FEDERAL UNIVERSITY OF PARANA

SANDRA REGINA BARROSO RUÍZ SELLA

DEVELOPMENT OF BIOPROCESSES FOR THE PRODUCTION OF A BIOLOGICAL INDICATOR FOR STERILIZATION PROCESSES FROM *Bacillus atrophaeus* SPORES

CURITIBA
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DEVELOPMENT OF BIOPROCESSES FOR THE PRODUCTION OF A BIOLOGICAL INDICATOR FOR STERILIZATION PROCESSES FROM *Bacillus atrophaeus* SPORES

Thesis presented as partial requirement for obtaining the Doctor degree in Bioprocess Engineering and Biotechnology, Animal and Human Health Area. Post-graduation program in Bioprocess Engineering and Biotechnology Department (Federal University of Parana)

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João Carlos Minozzo, Ph.D.

CURITIBA
2012

UNIVERSIDADE FEDERAL DO PARANÁ
Programa de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia
Setor de Tecnologia

UFPR

RELATÓRIO DE DEFESA DE TESE DE DOUTORADO

Aos vinte e dois dias do mês de novembro de 2012, no Salão Nobre do Setor de Tecnologia, Segundo Andar do Prédio da Administração do Centro Politécnico da Universidade Federal do Paraná, Jardim das Américas, foi instalada pela Profª Drª Luciana Porto de Souza Vandenberghe, Coordenadora do Curso de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia, a banca examinadora para a Septuagésima Nona Defesa de Tese de Doutorado, Área de Concentração: Saúde Humana e Animal. Estiveram presentes no Ato, além da Coordenadora do Curso de Pós–Graduação, professores, alunos e visitantes.

A Banca Examinadora, atendendo determinação do colegiado do Curso de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia, ficou constituída pelos Professores Doutores Maria Sueli Soares Felipe (UNB), Leon Rabinovitch (FIOCRUZ), João Carlos Minozzo (CPPI), Luciana Porto de Souza Vandenberghe (UFPR) e Carlos Ricardo Soccol (UFPR - orientador da tese).

Às 9h00, a banca iniciou os trabalhos, convidando a candidata **Sandra Regina Barroso Ruiz Sella** a fazer a apresentação da Tese intitulada: **"Development of a Bioprocess for the Production of a Biological Indicator for Sterilization from *Bacillus atrophaeus* Spores"**. Encerrada a apresentação, iniciou-se a fase de argüição pelos membros participantes.

Tendo em vista a tese e a argüição, a banca composta pelos professores Drª Maria Sueli Soares Felipe, Dr Leon Rabinovitch, Dr João Carlos Minozzo, Drª Luciana P S Vandenberghe e Dr Carlos Ricardo Soccol declarou a candidata APROVADA (de acordo com a determinação dos Artigos 59 a 68 da Resolução 65/09 de 30.10.09).

Curitiba, 22 de novembro de 2012

Profª Drª Maria Sueli Soares Felipe

Prof Dr Leon Rabinovitch

Prof Dr João Carlos Minozzo

Profª Drª Luciana P S Vandenberghe

Prof Dr Carlos Ricardo Soccol

Universidade Federal do Parana
Setor de Tecnologia
Curso de Doutorado em Proc s os
Biotecnológicos

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ABSTRACT

The genus *Bacillus* includes a great diversity of industrially important strains, including *Bacillus atrophaeus* (formerly *Bacillus subtilis* var. *niger*). This spore-forming bacterium has been established as industrial bacteria in the production of biological sterilization indicators, in studies of biodefense and astrobiology methods, and as potential adjuvants or vehicles for vaccines, among other applications. Two novels, cost-effective *B. atrophaeus* Sterilization Bioindicator Systems (BIS) with high quality and performance were developed from soybean molasses and glycerol and compared with commercial BIS. The BISs were composed of a recovery medium and dry-fermented. The production of biological indicators involving bacterial sporulation and multi-step downstream processes has been described. The first goal of the present work was to use fermented material as the final product in the BIS, thereby reducing processing steps and costs. The performance of three different inexpensive supports (vermiculite, sand and sugarcane bagasse) was assessed by determining *B. atrophaeus* sporulation during solid-state fermentation (SSF). Sand proved to be the best inert support, which enabled the direct use of the fermented product due to its easy homogenization, filling properties, and compatibility with the recovery medium. The BISs were developed and optimized using a sequential experimental design strategy. For soybean-based BIS, the optimum recovery medium contained soluble starch (1.0 g/L), soybean molasses (30.0 g/L), tryptone (40.0 g/L), and bromothymol blue (0.02 g/L) at pH 8.5. The SSF conditions of the bioreactor and environmental humidity had no significant impact on spore yield and dry-heat resistance. The only substrate mineral that showed a positive effect was Mn^{2+}, allowing Mg^{2+}, K^{+}, and Ca^{2+} to be eliminated from the formulation. Validation of optimized medium indicated a $D_{160°C}$ = 6.8 ± 1.0 min (3.6 min more than the minimum) and a spore yield = 2.3 ± 0.5 × 10^{9} CFU/g dry sand (10,000 × initial values). Cost reduction was of 23.9% and process cycle time was also reduced from 29 to 15 days. The study of the growth characteristics and the metabolic and enzymatic profiles confirmed that sporulation through SSF of *B. atrophaeus* occurs by biofilm formation and that this model of fermentation promotes important phenotypic changes in the spores. This study proposes a new concept regarding bacterial biofilm formation by SSF. For glycerol-based BIS, the proposed recovery medium enables the germination and outgrowth of heat-damaged spores, promoting a $D_{160°C}$ value of 6.6 ± 0.1 min. *B. atrophaeus* spore production by SSF reached 2.3 ± 1.2 x 10^{8} CFU/g dry matter. Sporulation kinetics results showed that only 5 days were sufficient for this process. Cost breakdowns were from 41.8% (quality control) up to 72.8% (feedstock). A performance evaluation of the proposed BIS against dry-heat and ethylene oxide sterilization showed compliance with regulatory requirements. Microwave disinfection tests demonstrated that the developed BISs were more resistant than the control BIS. Additional studies are necessary to determine if this is a positive factor for microwave medical waste treatment monitoring or if it may cause a false process failure indication. These processes may be utilized for spore production aimed at other applications or for the cost-effective production of spores from other *Bacillus* species.

Key words: *Bacillus atrophaeus*. Biological indicator. Sterilization. Spores. Solid-state fermentation. Soybean molasses. Glycerol. Biofilm.

FIGURE LIST

CHAPTER I ***Bacillus atrophaeus'*** **MAINS CHARACTERISTICHS AND BIOTECNOLOGICAL APLICATIONS – REVIEW**

TABLE LIST

SUMARY

INTRODUCTION

Microbial spores are usually considered to be much more resistant to sterilization processes than most other types of microorganisms. *Bacillus atrophaeus* is a suitable reference organism for testing the effectiveness of dry-heat, ethylene oxide, microwaves and electrothermal deactivation sterilization processes, among others applications, as described in **CHAPTER** I. Due to their resistance characteristics, *B. atrophaeus* spores are used for producing biological indicators (BIs) for sterilization. A BI for sterilization is a device intended for use by healthcare providers or quality control staff to accompany products through sterilization. The BI consists of a known number of microorganisms and a known resistance to the mode of sterilization, it is located on a carrier, and it is enclosed in a protective package. The subsequent growth or failure of the microorganisms to grow under suitable conditions indicates the adequacy of the sterilization. Bioindicators are considered as a system, with the spore characteristics, spore carrier, packaging, and recovery medium all contributing to the product effectiveness.

Several systems employed in the industrial production of *Bacillus* spores have been described. Although, a defined culture medium can provide a high-yield of spore production, its use makes the final product expensive, especially for extensive use in developing countries. Some advantages of solid-state fermentation (SSF) have been demonstrated for *Bacillus* spore production; however, downstream processes need to be applied after sporulation for spores to be utilized in BI production. These processes include spore detachment, harvesting and purification, followed by spore inoculation into a carrier. The use of an inert support to enable the direct use of fermented material in BI production is desirable. No reports were found in the literature describing the direct use of an SSF fermented substrate as a biological indicator for sterilization. **CHAPTER II** describes the study of *B. atrophaeus* spore production by SSF with different inert supports aiming the reduction of process steps.

The world's annual production of soybean is estimated at ~264 million tons, and Brazilian production represents approximately 30% of global production. Soybean molasses is a byproduct from the manufacture of protein-concentrate

soybean meal. Soybean molasses is highly available and is a low-cost raw material. The use of soybean molasses in a standardized culture medium for germination of spores and as a substrate for SSF, with sand as a support, has not been reported elsewhere. **CHAPTER III** describes the development and optimization of a production process for a cost-effective biological indicator system using soybean molasses.

In most studies and in industrial BI production, spores are obtained from laboratory standard strains cultivated in artificial media. However, in natural habitats, spores are predominantly formed from bacteria present in highly surface-associated communities of cells in extracellular polymeric matrices called biofilms. As SSF is the culture method that best mimics the natural environment of many microorganisms, it is a promising method to impact germinative cell phenotypes. No previous studies have compared spores of a standard strain using different sporulation methodologies and germination characteristics. The aim of the study reported in **CHAPTER IV** was to confirm that the sporulation of *B. atrophaeus* through SSF occurs by biofilm formation and that SSF promotes important phenotypic changes in germinated spores.

Glycerol is a byproduct of the biodiesel industry. World biodiesel production is increasing exponentially, and consequently, the excess glycerol that is generated is low-priced and may become an environmental problem. Thus, the development of new value-added applications for glycerol is of worldwide interest. The development of a new and economical bioprocess for the production of a *B. atrophaeus* sterilization BIS (spores on a carrier plus a recovery medium) using glycerol as the sole carbon source has not been described. **CHAPTER V** describes the development and optimization of another cost-effective biological indicator system using glycerol.

BISs are designed to provide a challenge to the disinfection or sterilization process, which exceeds the process resistance of the natural bioburden on the product. The developed BIS must meet specific standard requirements as set out by international standards or regulatory agencies to present a true challenge for sterilization process and to offer an alternative for commercial BIS. In **CHAPTER VI**, the performance of glycerol-based, soybean-based and commercial *B. atrophaeus* biological indicators were evaluated for dry-heat, ethylene oxide sterilization and microwave treatment.

OBJECTIVES

1. GENERAL OBJECTIVE

The purpose of this research was to develop new processes for the production of cost-effective *B. atrophaeus* biological indicators for sterilization using byproducts from the soybean agroindustry and from biodiesel production through solid-state fermentation.

2. SPECIFIC OBJECTIVES

a) Select an inert material for use as a support in solid-state fermentation process;
b) Study the ideal conditions for spore production by optimizing the physical and chemical conditions of cultivation;
c) Study the adhesion mechanism of microorganisms to inert supports;
d) Develop and optimize recovery medium formulations;
e) Evaluate the performance of the biological indicators produced.

CHAPTER I

Bacillus atrophaeus: MAIN CHARACTERISTICS AND BIOTECHNOLOGICAL APPLICATIONS – REVIEW

ABSTRACT

The genus *Bacillus* includes a great diversity of industrially important strains, including *Bacillus atrophaeus* (formerly *Bacillus subtilis* var. *niger*). This spore-forming bacterium has been established as industrial bacteria in the production of biological indicators for sterilization, in studies of biodefense and astrobiology methods as well as disinfection agents, in treatment evaluation, and as potential adjuvants or vehicles for vaccines, among other applications. This review covers an overview of the fundamental aspects of *B. atrophaeus* that have been studied to date. Although the emphasis is placed on recent findings, basic information such as morphology and growth characteristics, spore structure and life-cycle, as well as factors that influence spore resistance, is described. The wide biotechnological application of *B. atrophaeus* spores, including vegetative cells, is briefly demonstrated, highlighting their use as a biological indicator for sterilization.

Key words: *Bacillus atrophaeus*, spores, biological indicator, sterilization, disinfection

1. TAXONOMY AND MORPHOLOGY

The genus *Bacillus* includes a great diversity of industrially important strains that have a role in the production of a range of products, including fermented foods, industrial enzymes, heterologous proteins, bio insecticides, antibiotics, purine nucleotides, poly-ɣ-glutamic acid, D-ribose and other products with commercial applications (SCHALLMEY *et al.,* 2004). The metabolic diversity and lack of reported incidence of pathogenicity makes this bacterial genus the most suitable for the development of marketable products. However, the production of resistant spores is a major problem in the industries of sterile pharmaceutical and medical-hospital goods as well as the food industry. This feature is also used to produce biological

indicators for sterilization and to evaluate the capacity of biocide action (GORDON, 1977).

The *Bacillus* genus was established by Ferdinand Cohn in 1872 and later by Koch in the same year, although Christian Gottfried Ehrenberg is often credited with the first published description of *Bacillus subtilis* in 1835, when the bacterium was named *Vibrio subtilhe* (HARWOOD, 1989). Initially, the classification of the genus was based on the ability to sporulate and morphological, biochemical and physiological characterization. Currently, the 16 rRNA and the 16S-23S internally transcribed spacer are analyzed to phylogenetically group the *Bacillus* genus into sub clusters (XU; CÔTÉ, 2003). *B. atrophaeus* (also referred to in the literature as "*B. subtilis* var. *subtilis*," "*Bacillus globigii*," "*B. subtilis* var. *niger*," the "red strain," "*Bacillus niger*," or "*B. atrophaeus* subsp. *globigii)* is included in the *B. subtilis* group within *B. amyloliquefaciens, B. mojavensis, B. licheniformis, B. sonorensis* and *B. vallismortis* (VOS *et al.*, 2009).

Bacillus atrophaeus is a gram-positive, aerobic, spore-forming bacteria (FIGURE 1), phenotypically similar to *B. subtilis*, except for the production of a pigment when cultured in media containing an organic nitrogen source (NAKAMURA, 1989).

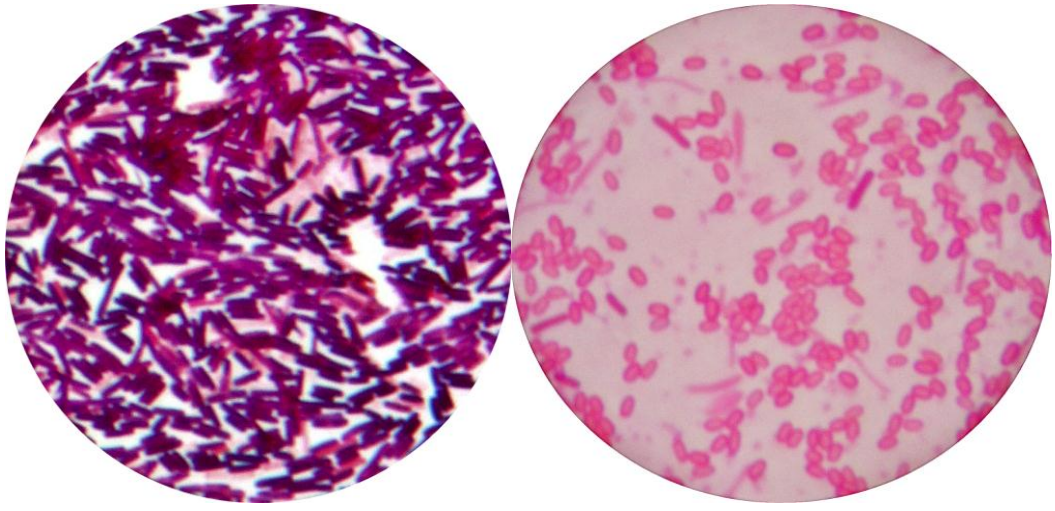


FIGURE 1- *Bacillus atrophaeus* VEGETATIVE CELLS (LEFT) FROM 12 HOURS CULTURE AND SPORES (RIGH) FROM 7 DAYS CULTURE. IT IS OBSERVATED THE GRAM LABILITY OF VEGETATIVE CELLS FROM OLD CULTURES (GRAM STAIN, MAGNIFICATION: X 1,000)

SOURCE: The author (2010)

The taxonomic position of *B. atrophaeus* has changed dramatically over the years. *B. atrophaeus* was first isolated by Migula in 1900 as *Bacillus globigii* (FRITZE; PUKAL, 2001). Smith *et al*. (1952) examined this strain and reclassified it as *B. subitilis* var *niger* after observing a pigment formation when cultured in a medium containing tyrosine. In 1989, Nakamura re-examined these dark pigment-producing *B. subtilis* strains. In comparative studies with a conventional strain of *B. subtilis* through pigment production (in two different media), DNA hybridization studies and multilocus enzyme electrophoresis, differences in the patterns were observed, which suggested that these species were distinct. After confirming the differences, the author described the following new specie: *Bacillus atrophaeus*, where "*ater*" refers to "black" and "*phaeus*" to brown. "*Atrophaeus"* means "dark brown" and refers to the formation of a dark brown pigment (NAKAMURA, 1989). Fritze and Pukall (2001) examined the strains ATCC 9372 and ATCC 51189 and, based on the results of ribotyping and DNA-DNA re-association, demonstrated the need to reclassify them as *B. atrophaeus* (FIGURE 2).

› Bacteria
› *Firmicutes*
› *Bacilli*
› *Bacillales*
› *Bacillaceae*
› *Bacillus*
› *Bacillus subtilis* group
› *Bacillus atrophaeus*
› *Bacillus atrophaeus* ATCC 6455, ATCC 6537 and ATCC 7972 = *Bacillus atrophaeus* Nakamura deposited as *Bacillus niger* Migula
› *Bacillus atrophaeus* ATCC 9372 and ATCC 49337 =*Bacillus atrophaeus* Nakamura deposited as *Bacillus subtilis* var. *niger* Smith et al.
› *Bacillus atrophaeus* ATCC 51189 =*Bacillus atrophaeus* Nakamura deposited as *Bacillus subtilis* (Ehrenberg) Cohn

FIGURE 2- *Bacillus atrophaeus* LINEAGE
SOURCE: Modified from Vos *et al.* (2009)

Bacillus atrophaeus is a soil-dwelling, non-pathogenic bacillus, with an optimal cultivation temperature between 20°C and 37°C. Catalase and Voges-Proskauer (acetoin) reactions are positive, glucose is fermented, and starch and casein are hydrolyzed (GORDON, 1977). Oxidase, hydrogen sulfide, indole, and di-hydroxyacetone are not produced. Nitrate is reduced to nitrite. Agar colonies are usually shiny, smooth, and circular, and the color varies from off-white up to orange. However, multiple colony morphotypes may be observed according to the strain origin and growth conditions (GIBBONS *et al*., 2011). Their vegetative cells have approximately 0.8 µm width and 2-3 µm length, but may vary according to cultivation conditions (MANDINGAN *et al*., 2002). *B. atrophaeus* spores are described with a length of 1.2-2.1 µm and a diameter of 0.6-0.8 µm, which vary based on the sporulation medium, the hydration state and the measurement technique (BUHR *et al*., 2008; YANG *et al*., 2010; MALKIN; PLOMP, 2011; SELLA *et al.,* 2012) (FIGURE 3).

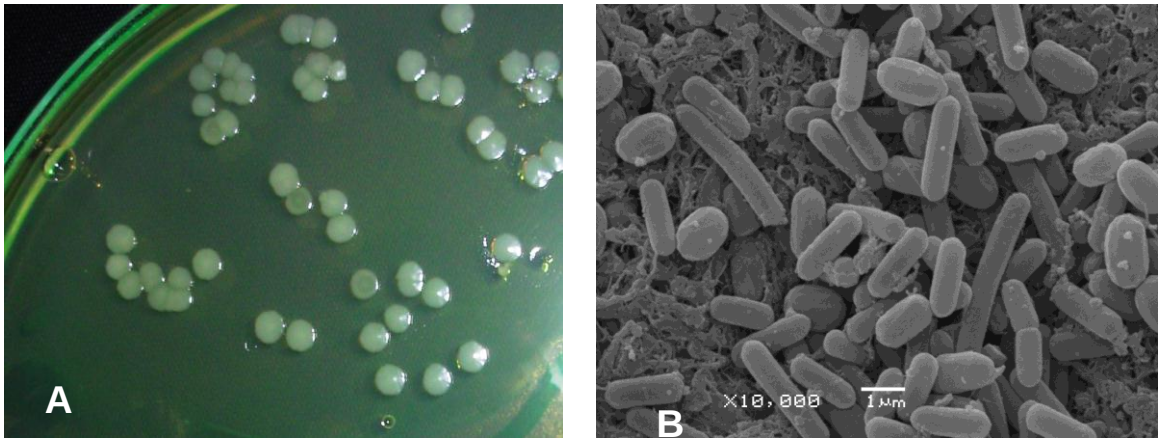


FIGURE 3 - *Bacillus atrophaeus:* (A) COLONIES GROWN ON AGAR PLATE (B) SCANNING ELECTRON MICROGRAPHS OF VEGETATIVE CELLS AND SPORES ON AGAR BASE
SOURCE: The author (2011)

2. MULTICELLULARITY

The growth of bacteria under laboratory conditions tends to select strains that lose many of their multicellular attributes. A phenomenon referred as ‘domestication’ causes populations of genetically identical bacteria to be viewed as if they were

homogeneous (BRANDA *et al.*, 2001; KEARNS; LOSICK, 2005). Based on observations of bacterial colony morphogenesis, Shapiro (1988) proposed multicellularity as a general bacterial trait. However, this idea did not persuade most microbiologists to embrace multicellularity as a basic tenet in bacteriology (AGUILAR *et al*., 2007). Koch, in 1877, identified and described *Bacillus anthracis* as the etiological agent of anthrax, and Ferdinand Cohn's description of *B. subtilis* cultures reveled the multicellular nature of these microorganism over a century ago (COHN, 1877; KOCH, 1877) (FIGURE 4).

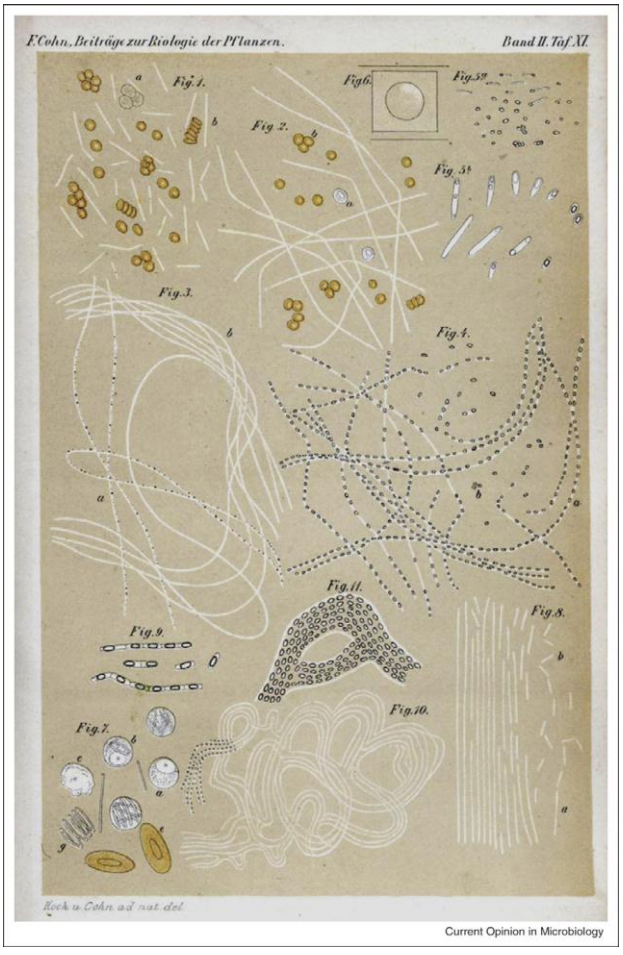


FIGURE 4 –

COLOR PLATE CONTAINING THE FIGURES FROM KOCH'S (FIG. 1–7) AND COHN'S (FIG. 8–11) PAPERS PUBLISHED IN 1877

SOURCE: Aguilar *et al*. (2007)

Bacillus sp. cells are capable of differentiating into subtypes with specialized attributes in response to different environmental cues (LOPEZ; KOLTER, 2010). This response to adverse environmental conditions occurs by inducing the expression of adaptive genes. Stochasticity allows bacteria to deploy specialized cells in

anticipation of possible adverse changes in the environment (LEWIS, 2007).

Under determined conditions, *Bacillus* populations are heterogeneous, consisting of mixtures of cells in distinct physiological or developmental states (FIGURE 5).

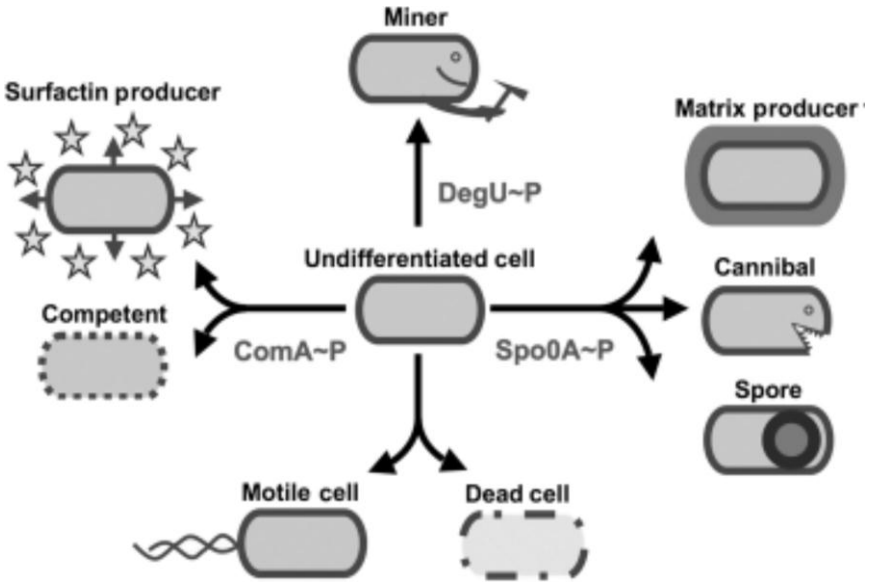


FIGURE 5 - SCHEMATIC REPRESENTATION OF THE DISTINCT CELL TYPES THAT DIFFERENTIATE IN THE COMMUNITIES OF *Bacillus sp*. EACH CELL TYPE HAS BEEN CARICATURIZED CONSIDERING ITS MOST REPRESENTATIVE ATTRIBUTE. THE CELLS WERE CLASSIFIED INTO SUBGROUPS, ACCORDING TO THE MASTER REGULATOR THAT TRIGGERS THEIR DIFFERENTIATION

SOURCE: Lopez and Kolter (2010)

Maugahan and Nicholson (2004) cited that a clonal population of *B. subtilis* has the potential to embark on different developmental pathways, including synthesis of degradative enzymes, competence for DNA uptake, motility, chemotaxis, biofilms fruiting body formation, adaptive mutagenesis and endospore formation. In nutrient-replete conditions, during the exponential phase of growth*, Bacillus sp.* may be found in one of two morphologically distinct forms: single, motile cells or long chains of sessile cells. The proportion of the two cell forms varies based upon the strain source or culture conditions (KEARNS; LOSICK, 2005). However, under conditions

of mild nutrient depletion, cell subpopulations may differentiate to produce the extracellular matrix required for biofilm formation (VLAMAKIS *et al.*, 2008; CHAI *et al.,* 2011). Nutrient depletion leads to the formation of dormant endospores that can remain dormant for many years (SONENSHEIN, 2000). Spores also arise in biofilms as they age, with matrix-producing cells differentiating into spore-forming cells at fruiting body-like aerial structures (BRANDA *et al*., 2001; VLAMAKIS *et al.*, 2008) (FIGURE 6).

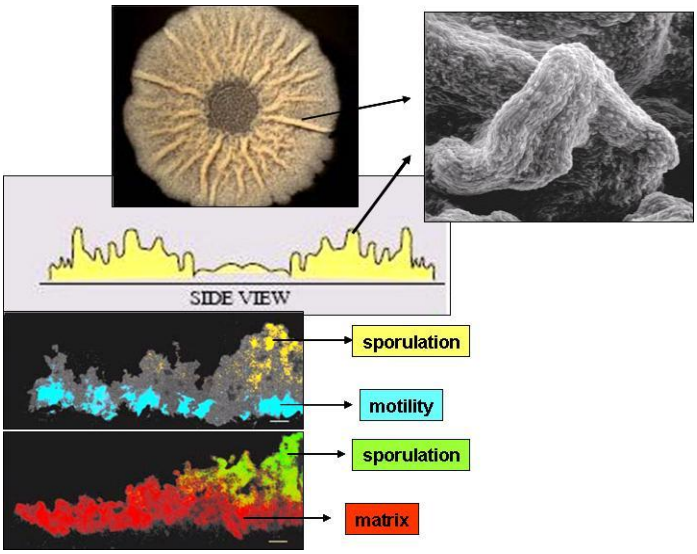


FIGURE 6 – MULTICELLULARITY REPRESENTED BY DISTINCT CELLS TYPES IN THE *Bacillus sp*. COLONY. SEM MICROPHOTOGRAPHY OF THE EDGE OF COLONY REVEALS TIGHTLY PACKED CHAINS OF CELLS WHERE SPORULATION OCCURS. FLUORESCENCE IMAGES SHOWS SPORULATION (GREEN AND YELOW), MOTILE (BLUE), AND MATRIX-PRODUCING (RED) CELLS IN THE SAME COLONY.

SOURCE: The author. Modified from Losick (2008)

To delay the sporulation process, cells that have entered the pathway to sporulate can differentiate into a subpopulation of specialized cells termed cannibals. Cannibal cells secrete two peptide toxins, *Skf* and *Sdp*, which kill their sensitive siblings. The dead cells can be used as nutrients to temporarily overcome the nutritional limitation and delay the onset of sporulation (GONZALEZ-PASTOR *et al.*,

2003; LÓPEZ *et al.*, 2009).

Competence is another state of *Bacillus* cells in which subpopulations become capable of up-taking external DNA, promoting genetic variability among the bacterial community (DUBNAU; LOVETT, 2002). The proportion of competent cells also depends on the strain and the environmental conditions.

Bacillus sp. also produces the lipopetide molecule surfactin, which functions to enhance the spreading of colonies on nutrient substrates. Surfactin is required for the morphogenesis of aerial projections that form along colony edges, called fruiting bodies (KINSINGER *et al*., 2003; ANGELINI *et al.,* 2009).

Some subpopulations of cells are termed ‘miner’ cells. They are responsible for exoprotease production, which degrades exogenous proteins and polysaccharides into smaller molecules that can be assimilated by the community (VEENING *et al*., 2008).

Prior studies have shown that the formation of these multicellular communities involves extensive intercellular communication and that it can be triggered in response to a variety of structurally unrelated natural products produced by *Bacillus* itself (LÓPEZ *et al.*, 2009). The study of the regulatory mechanisms that govern sporulation gene expression and novel pathways of intercellular signaling, the circuitry that governs multicellularity, and mechanisms that link spore formation to multicellularity are currently being investigated.

Because *B. subtilis* is a model microorganism for studies involving *Bacillus* species, there are no published reports regarding *B. atrophaeus* multicellularity. Sella *et al.* (2012) first described certain multicellular *B. atrophaeus* aspects of colonies from spores produced by solid-state fermentation, such as biofilm formation, motility, and variations in colony morphology and metabolic profile (see CHAPTER IV). However, the elucidation of these mechanisms and their potential applications in new *B. atrophaeus* biotechnological product development remain to be determined.

3. LIFE-CYCLE

The life-cycle of *B. atrophaeus*, similar to other endospore-forming bacteria, includes three different phases: vegetative growth, sporulation and germination

(FIGURE 7). Vegetative growth occurs when nutrients are available and is characterized by cells growing logarithmically by symmetric fission. When nutrients become limiting and following other environmental signals, these bacteria initiate the sporulation process. Spores can remain dormant for extended time periods and possess a remarkable resistance to environmental damages (i.e., heat, radiation, toxic chemicals, and pH extremes). Under favorable environmental conditions, the spore breaks its dormancy and restarts growth in a process called spore germination and outgrowth (MOIR, 2006).

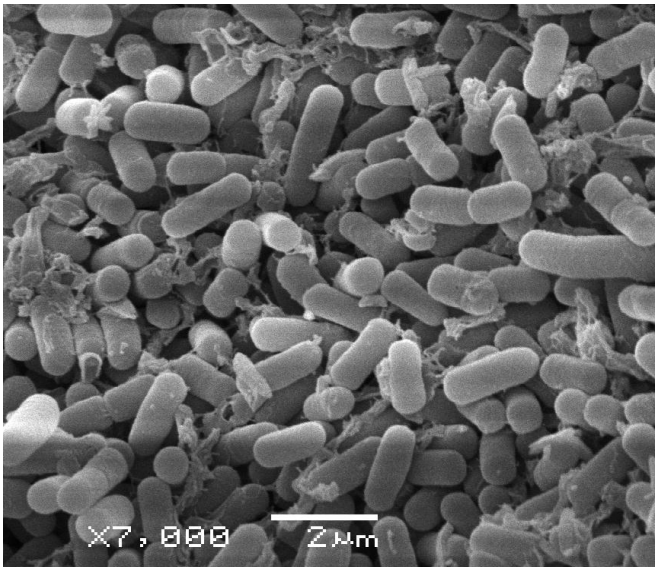
3.1 Sporulation

Most *B. atrophaeus* applications use its spore form. The process of sporulation in *Bacillus* and its initiation and regulatory pathways have been intensely studied by Sonenshein (2000), Errington (2003), Piggot and Hilbert (2004), Setlow (2007), De Hoon *et al.* (2010) and Higgins and Dworkin (2012).

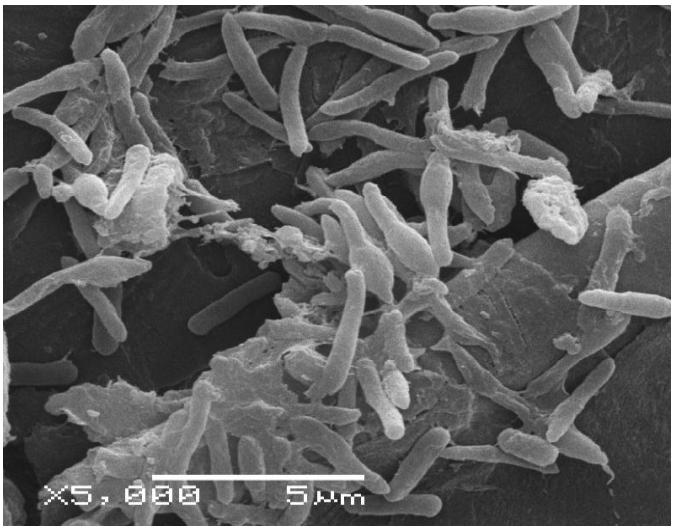
Sporulation can be triggered by multiple environmental signals, such as nutrient deprivation, high mineral composition, neutral pH, temperature, and high cell density. The cellular mass increase associated with the accumulation of secreted peptides, which are sensed by cell surface receptors. It initiates a sequential activation of the master regulator *SpoA*-denominated *phosphorelay* (transference of phosphate groups from ATP through histidine kinases and two intermediate proteins, *Spo0F* and *Spo0B*, to a transcription factor, *Spo0A*). Upon phosphorylation, *Spo0A-P* directly acts on more than 100 genes, setting off a chain of events that takes several hours to complete and culminates in the release of the mature spore from its mother cell compartment (MOLLE *et al.,* 2003; VEENING *et al.*, 2009).

The spore formation process may be briefly described by seven stages, as demonstrated in FIGURE 8:

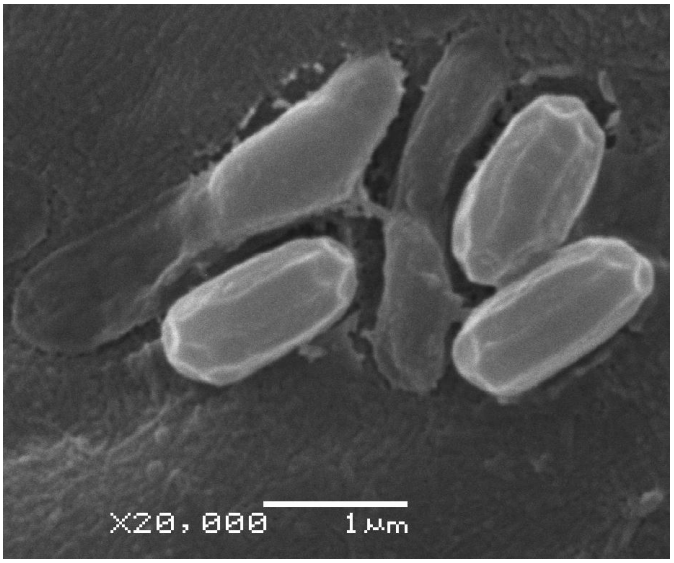
Stage I: The nuclear material is disposed axially into filaments;



A



B



C

FIGURE 7 –

SCANNING ELECTRON MICROGRAPHS OF *B. atrophaeus* SPORES. (A) VEGETATIVE CELLS, (B) VEGETATIVE CELLS AND SPORANGIA, (C) SPORES AND RELEASE OF THE MATURE SPORE FROM ITS MOTHER CELL COMPARTMENT

SOURCE: The author (2010)

Stage II: Completion of DNA segregation occurs concurrently with the invagination of the plasmatic membrane in an asymmetric position, near one pole of the cell, forming a septum;

Stage III: The septum begins to curve, and the immature spore is surrounded by a double membrane of the mother cell in an engulfment process (similar to phagocytosis). The smaller forespore becomes wholly contained within the mother cell;

Stage IV: The mother cell mediates the development of the forespore into the spore. The inner and outer proteinaceous layers of the spore are assembled, and the spore cortex, consisting of a thick layer of peptidoglycan contained between the inner and outer spore membranes, is synthesized. Furthermore, calcium dipicolinate is accumulated in the nucleus;

Stage V: The spore coat is synthesized, consisting of ~80 proteins deposited by the mother cell, arranged in inner and outer layers;

Stage VI: Spore maturation. In this stage, the spores become resistant to heat and organic solvents;

Stage VII: Lytic enzymes disrupt the mother cell, releasing the mature spore (FOSTER, 1994; ERRINGTON, 2003; SETLOW, 2007; HIGGINS; DWORKIN, 2012).

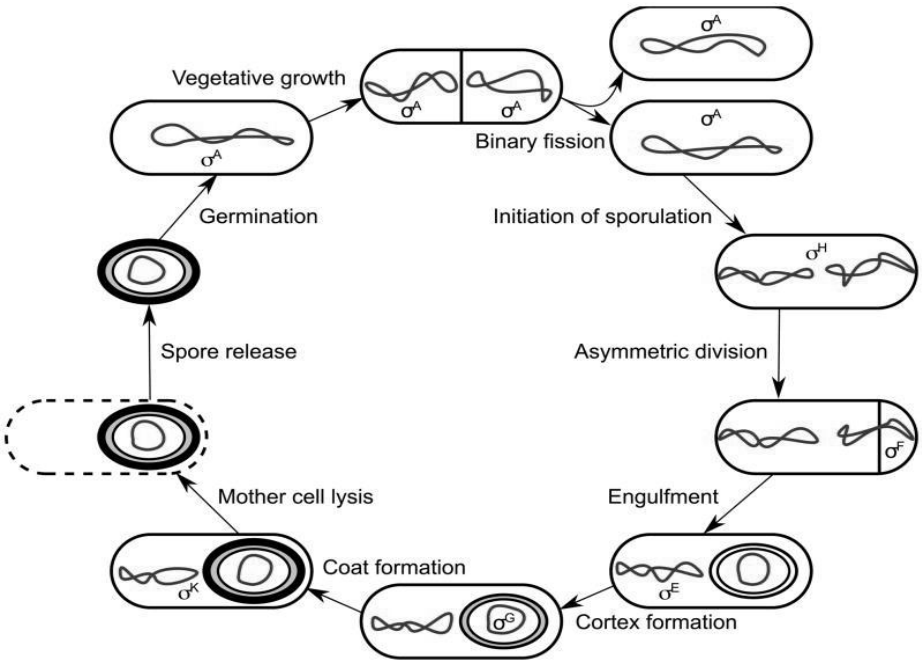


FIGURE 8 – CELL- CYCLE OF *Bacillus*: SPORULATION AND GERMINATION

SOURCE: de Hoon *et al.* (2010)

3.1.1 Spore structure

The structure and chemical composition of *Bacillus* spores differ considerably from those of vegetative cells. These differences largely account for the unique resistance properties of the spore to environmental stresses, including heat, radiation, disinfectants and sterilants. The dormant spore can survive for long periods of time, even in a hydrated state (SETLOW, 2007; LEGGETT *et al.,* 2012). There are many reports specifically describing spore structure, which are cited in this text, where the main points of spore structure are summarized (FIGURE 9):

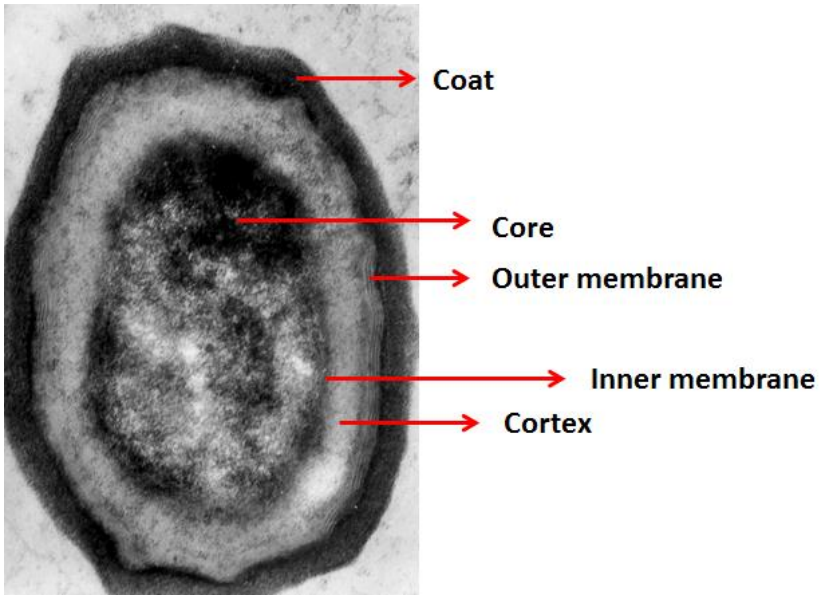


FIGURE 9 – CROSS-SECTION OF A SPORE OF *Bacillus atrophaeus*
SOURCE: The author (2012)

Coat: B. atrophaeus spores do not possess exosporia; the outer spore coat is the outermost surface. The spores are composed of more than 80 spore-specific proteins, which protect them from reactive chemicals and lytic enzymes. They include a series of thin, concentric layers, including the inner and outer spore coats, as well as a basement layer between the inner coat and the cortex and an outermost crust. The coat is predominantly made up of protein but also contains minor (6%)

carbohydrate components. The protein fraction of the coat represents 50%-80% of the total spore protein. The soluble fraction accounts for approximately 70% of the total coat protein. The 30% of insoluble coat proteins are characterized by high cysteine content; the formation of disulfide cross-links likely contributes to their insoluble nature. Cross-linked material in the spore coat is likely associated with the spore's chemical and mechanical resistance (DRIKS, 1999; PANDEY; ARONSON, 1979; ROSE *et al.* 2007*).* Functionally, the spore coat serves as an initial barrier to large molecules, such as the peptidoglycans (PG)-lytic enzyme lysozyme, which would otherwise have access to the spore cortex. The spore coat has also been identified as a resistance mechanism against many chemicals, especially oxidizing agents such as hydrogen peroxide, ozone, chlorine dioxide and hypochlorite (NICHOLSON *et al.,* 2000; YOUNG and SETLOW 2006). The coat surface may present peculiar structures (ridges) that seem to be formed when the spore volume decreases (during dehydration) and to disappear when the spores swell (during germination and/or hydration) (CHADA *et al*., 2003; SELLA *et al*., 2012). These findings suggest that the coat of *Bacillus* spores is a dynamic structure that might sense the external environment through active enzymes present on their surface and adapt to changes in the spore volume (DRIKS, 2003). Plomp *et al.* (2005), through the application of high-resolution imaging techniques, demonstrated that the outer spore coat of *B. atrophaeus* was composed of a crystalline array of ~11 nm thick rodlets, having a periodicity of ~8 nm (FIGURE 10). In the rodlet layer, planar and point defects, as well as domain boundaries, similar to those described for inorganic and macromolecular crystals, were identified by the authors. They suggested that rodlet structure assembly and architectural variation appear to be a consequence of species-specific nucleation and crystallization mechanisms that regulate the formation of the outer spore coat.

Outer membrane: The outer membrane surrounds the cortex, although this membrane might not act as a permeability barrier in the dormant spore. It is essential for spore formation, but its precise function remains unclear, reportedly having no great effect on resistance to radiation, heat or some chemicals (NICHOLSON *et al.,* 2000).

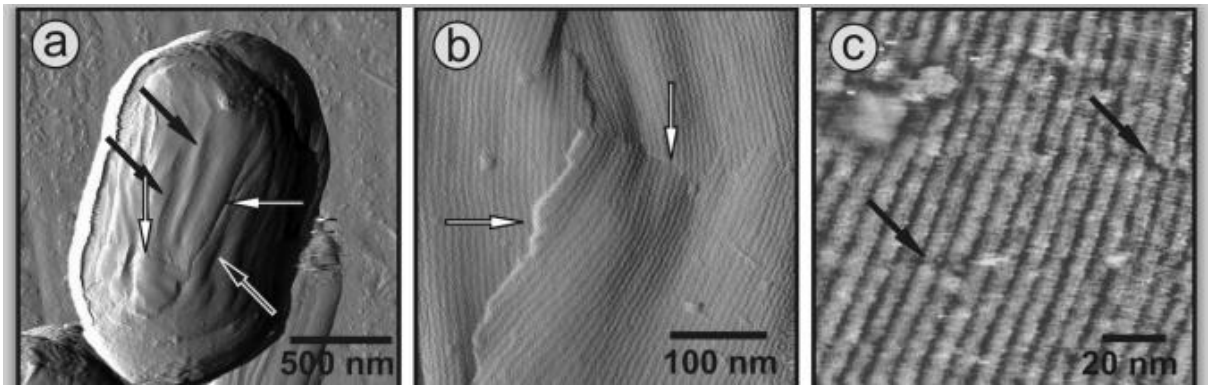


FIGURE 10 – (a) IMAGE SHOWING THE SPORE COAT RODLET STRUCTURE COVERED WITH STRAY RODLETS. (b) TAPPING AMPLITUDE IMAGE OF THE SAME AREA, (c) VISUALIZATION OF THE RODLET LAYER UNDERLYING INTEGUMENT

SOURCE: Plomp *et al*. (2005)

Cortex: The spore cortex is composed of peptidoglycans (PGs) that are broadly similar to PG in vegetative cells but with three major novel structural modifications: (a) only approximately one-quarter of cortex N-acetylmuramic acid (NAM) residues are substituted with short peptides, giving the cortex a lower degree of cross linking than the germ cell wall; (b) approximately 25% of the NAM residues carry a single L-alanine residue, a modification not found in the germ cell wall; and (c) approximately every second, muramic acid residues in the cortex PG are converted to muramic-d-lactam (MAL), a modification not found in the germ cell wall. The MAL residues appear to be the recognition element for cortex lytic enzymes (CLEs), which hydrolyze the cortex but not the germ cell wall during spore germination (PAREDES-SABJA *et al.,* 2011).

Germ cell wall: Surrounding the inner membrane is the germ cell wall, composed of PG, with a structure most likely identical to that of PG in a growing cell wall; this structure becomes the cell wall during spore outgrowth. There is currently no indication that it plays any major role in spore-resistance properties (LEGGETT *et al.,* 2012).

*Inner membrane***:** Surrounding the core is a significantly compressed inner membrane composed of lipids that are largely immobile. The low permeability of the inner membrane to small molecules, even water, most likely protects the spore core from DNA-damaging chemicals (PAREDES-SABJA *et al.,* 2011).

Core: The core is situated at the center of the spore and contains the spore's DNA, RNA, ribosomes and most of its enzymes. The core is relatively dehydrated (water content = 25-50% of wet weight), a factor that contributes to both the spore's enzymatic dormancy and its resistance to heat and certain chemicals. The core also contains high levels of the small molecule calcium dipicolinate (Ca–DPA). The conditions within the core are strongly linked to the resistance properties of the spore, many of which protect spore DNA from damage (PAREDES-SABJA *et al.,* 2011, LEGGETT *et al.,* 2012).

3.2 Germination

When the dormant spore encounters an appropriate environmental stimulus, it initiates the process of germination, which can result in the re-initiation of vegetative growth. The germination process was described in detail by deVries (2004), Moir (2006), Keijser *et al.* (2007), Plomp *et al.* (2007) and Zhang *et al.* (2010) and may be briefly explained in three stages:

Stage I: Activation, defined as the initiation or triggering process in response to nutritional replenishment, occurs when the germinating molecules (low-molecular-weight amino acids, sugars, and purine nucleosides) are sensed by germination receptors (GRs) located in the spore's inner membrane (such as *gerA*, *ger B*, or *ger K*) and binds them. The germinating spore initially releases H^+, K^+, Na^+ and Ca^{+2} raising the pH of the spore core from pH 6.5 to 7.7. Cortex lytic enzymes are activated, and the protective spore peptidoglycan cortex is degraded. Activation is a reversible process that does not necessarily commit the spore to germination and outgrowth (LOSICK *et al.*, 1986; SETLOW, 2003b).

Stage II: DPA (pyridine-2, 6- dicarboxylic acid) is degraded and released, followed by the rehydration of the spore core. Rehydration allows the initiation of protein mobility and reactivation of biochemical processes during outgrowth (ZHANG *et al.*, 2010).

Stage III: Spore coat hydrolysis allows emergence of the incipient vegetative cell.

Outgrowth is the transition of the germinated spore to a growing cell, during

which cell division occurs. In the first stage of outgrowth, ATP is generated through the conversion of 3-phosphoglycerate stored in the spore core. In a later stage, the outgrowing spore switches to the use of extracellular nutrients (ZHANG *et al.,* 2010).

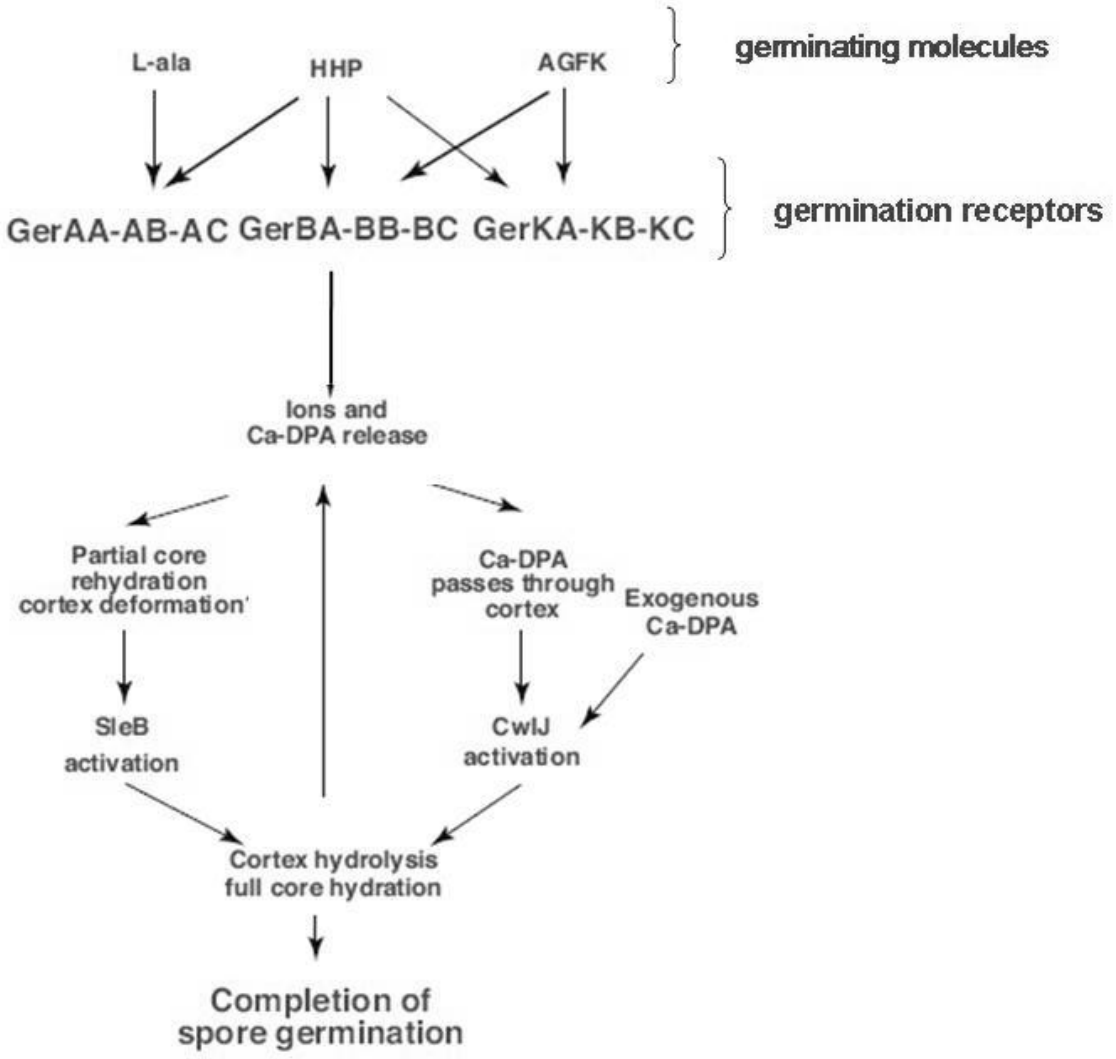


FIGURE 11 – MODEL OF THE INFORMATION FLOW DURING *Bacillus* SPORE GERMINATION
SOURCE: The author. Adapted from Paredes-Sabja *et al.* (2011)

Shah *et al*. (2008) found that germination of *Bacillus* spores could also be triggered by extremely low concentrations of muropeptides in germinant absences. Muropeptides are produced by the degradation of peptidoglycans that comprise the cell wall of most bacteria and spore coats. Muropeptides do not trigger germination

through nutrient germinant receptor sits; germination is triggered by binding to an inner membrane-bound protein kinase. Based on this study, Setlow (2008) suggested that muropeptides released from germinating spores may trigger spore germination, which might explain why, in some cases, spore germination efficiency increases at higher spore concentrations. In this case, even in the absence of nutrients, germination in a few spores may lead to germination in the total spore population.

The timing of germination and outgrowth varies stochastically among individual spores and germination medium characteristics (CHEN *et al.,* 2006). The germination of *B. atrophaeus* spores was investigated by Plomp *et al.* (2007) using atomic force microscopy, and the results indicated that swelling occurred within 0.5 h of germinant contact. Initiation of etching of the coat layers and outgrowth typically occurred within 1 h - 2 h and 3 h - 7 h, respectively.

4. FACTORS THAT CONTRIBUTE TO SPORE RESISTANCE

Spore resistance is multifactorial and cannot be accounted for by any single parameter (TABLE 1). The main causes of bacterial spore resistance can be attributed to a number of factors that have been identified, including the following.

4.1 Spore coat

The spore coat is an initial barrier to large molecules that would otherwise have access to the spore cortex (NICHOLSON *et al.,* 2000). The spore coat plays a role in spore resistance to predation by bacteriovorus (LAABERKI; DWORKIN, 2008) and to certain chemical biocide action, especially oxidizing agents (YOUNG; SETLOW 2004). It was evidenced that the coat protects a dormant spore from enzymes such as lysozyme and from mechanical disruption (GOULD, 1993). Riesenman and Nicholson (2000) cited that the spore coat, particularly the inner coat layer, influences spore resistance to environmentally relevant UV wavelengths. Spore coat proteins may also react with and detoxify chemical agents but have little

or no role in spore resistance to heat, radiation and certain other chemicals (SETLOW, 2006)

4.2 Cortex peptidoglycan

Atrih and Foster (1999) cited that the level of peptidoglycan cross-linking in the cortex was an important factor in maintaining spore heat resistance by attaining protoplast dehydration. However, other studies have demonstrated that the level of cross-linking in spore peptidoglycan does not alter spore dehydration (POPHAM, 2002).

4.3 Alpha/beta type small acid-soluble spore proteins (SASP)

The DNA in dormant spores of *Bacillus* is saturated with a group of nonspecific DNA-binding proteins termed alpha/beta-type small acid-soluble spore proteins (SASP). These proteins alter DNA structure *in vivo* and *in vitro*, providing spore resistance. These proteins are synthesized only during sporulation in the developing spore and are degraded early in spore germination, having a protective role that prevents DNA damage. The saturation of spore DNA with SASP provides protection against DNA base loss due to the wet heat and DNA single-strand breakage caused by desiccation and hydrogen peroxide (SETLOW; SETLOW, 1996). Protection of spore DNA from 254-nm UV radiation and free radical damage is associated with the binding of spore DNA by small, acid-soluble spore proteins in the spore core (SETLOW, 1995). The level of alpha/beta-type SASP is the major determinant of spore dry heat resistance and UV resistance, and the resistance to formaldehyde and hydrogen peroxide is determined, in part, by the level of alpha/beta-type SASP (PAIDHUNGAT; SETLOW 2001; SETLOW, 2000).

4.4 DNA repair

As dormant spores are metabolically inactive, the repair of DNA damage occurs during the process of germination when spores reactivate and prepare to return to vegetative growth (Moeller *et al*., 2007). DNA repair proteins may be

extremely important in spore outgrowth not only to repair damage that accumulated during spore dormancy but also to repair damage generated during spore germination and outgrowth, ensuring the return to vegetative life (FAIRHEAD *et al.* 1994; SETLOW, 1995). Spores contain enzymes found in growing cells that repair DNA damage, and DNA damage accumulated in the dormant spore will also often induce synthesis of DNA repair proteins upon subsequent spore germination (NICHOLSON *et al.,* 2000).

4.5 Core mineralization

Mineralization is associated with a high level of divalent ions in the core, mainly Ca^{2+}, Mg^{2+}, and Mn^{2+}. Spore core mineralization increases the resistance of spores, in part, by dehydrating the protoplast and by other mechanisms. In species such as *Bacillus atrophaeus*, heat resistance is strongly affected by the mineralization and demineralization of its spores. Different mineral ions have been observed to confer differing levels of wet heat protection, with Ca^{2+} providing greater protection than other divalent (Mg^{2+} and Mn^{2+}) or monovalent (K^{+} and Na^{+}) cations (BENDER; MARQUIS, 1985; BEAMAN; GERHARDT, 1986; YAMAZAKI *et al*., 1997).

4.6 Dipicolinic acid

Dipicolinic acid (DPA) or pyridine-2-6-dicarboxylic acid comprises approximately 10% of the dry weight of *Bacillus* spores. It is chelated with divalent cations, predominantly Ca^{2+}, in a 1:1 ratio. DPA is also extremely important in spore resistance and stability by protecting spore DNA from damage (SETLOW *et al.*; 2006).

A number of killing treatments, particularly desiccation, gamma irradiation, and UV radiation, exert their effects to a significant degree through the generation of reactive oxygen species (ROS), which cause cell killing through damage to proteins, including perhaps enzymes that repair oxidative DNA damage. Protein protection in spores against these treatments may be due, in part, to the high levels of DPA conjugated to divalent metal ions, predominantly Ca^{2+}, in spores (GRANGER *et al.,*

2011).

4.7 Spore maturation

Spores released first from their mother cells during sporulation are significantly less resistant than the total spores formed in the entire sporulation process. The reaction that may contribute to spore maturation is modification of spore coat structure, which is catalyzed by spore-associated enzymes (SANCHEZ-SALAS *et al*., 2011).

4.8 Core water content

Core dehydration might improve wet heat resistance by stabilizing proteins against thermal denaturation (RUPLEY; CARECI, 1991), presumably by entropically (by excluding extended polypeptide conformations) and energetically disfavoring the unfolded state (by restricting availability of water molecules for replacing intraprotein hydrogen bonds). Core dehydration might also act indirectly by immobilizing proteins, thereby preventing irreversible protein aggregation (SUNDE *et al.* 2009). Core water content is designed as the major determining factor of a spore's wet heat and hydrogen peroxide heat resistance. Core proteins in a more highly dehydrated spore core have greater resistance to wet heat as a result of reduced molecular motion (SETLOW, 2006).

4.9 Inner membrane permeability

Permeability of the inner membrane is an important factor in dormant spore resistance to chemicals; it acts as a barrier restricting the passage of small molecules into the spore core (PAIDHUNGAT; SETLOW, 2001). Higher permeability of the inner membrane is also associated with lower acid resistance of spores (ROSE *et al.,* 2007)

TABLE 1 – FACTORS INFLUENCING THE RESISTANCE OF *Bacillus* SPORES TO DIFFERENT KILLING TREATMENTS

Treatment	Major factors in *Bacillus* spore resistance	Mechanism of spores killing
Wet heat	Core water content, alpha/beta-type SASP,core mineralization, DPA	Damage of one or more essential spore proteins
Dry heat	Alpha/beta-type SASP, DNA repair	DNA damage
UV radiation	Dipicolinic acid, Alpha/beta-type SASP, Core water content, DNA repair	DNA damage
Microwave radiation	Core water content	Damage of one or more essential spore proteins Inner membrane damage DNA damage Coat disruption
Chemicals	Coat, inner membrane permeability, alpha/beta-type SASP, DNA repair	Depends on the chemical: DNA damage (nitrous acid, formaldehyde, alkylating agents, such as ethylene oxide); Inner membrane damage (ozone, hypochlorite, chlorine dioxide, ethanol and strong acid); inactivation of spore cortex lytic enzymes (strong alkali)

SOURCES: Young and Setlow (2003), Celandroni *et al.* (2004), Setlow (2006), Coleman *et al.* (2007), Kim *et al.* (2009) and Setlow *et al.* (2002)

4.10 Chemical state

Bacterial spores have a reversible cation exchange system loaded with calcium ions that give spores high wet and dry-heat resistance and, when acidified, allows them to be less resistant (sensitive state). The system can be manipulated by chemical pretreatments of the dormant mature spore (ALDERTON; SNELL, 1970). Spores subjected to acidic solutions (low pH) and slightly heated suffer mineral loss (calcium, manganese, magnesium, potassium or/and sodium), and their heat resistance declines. When heated at an alkaline pH (approximately 8.0), the spores are able to absorb mineral ions and become more heat resistant (BENDER; MARQUIS, 1985; BEAMAN; GERHARDT, 1986).

4.11 Sporulation conditions

Several studies described the influence of sporulation conditions on spore resistance, each owing its specificity to different culture characteristics and different components of the sporulation medium. Interactions among all nutrimental and environmental parameters must always be considered when studying the influence on spore resistance. The main variables are described below.

4.11.1 Sporulation medium

Spore properties vary with sporulation media. Spore resistance profile can be altered considerably according to spore preparation conditions (MELLY *et al.* 2002). Molin and Svensson (1976) demonstrated a 4 x the reduction decimal time at 160 °C ($D_{160°C}$) value variation for *B. atrophaeus* spores produced in 20 different types of media.

Divalent ions were found to induce sporulation and increase the thermal resistance; calcium was the key element for the development of resistance by inducing specific genes involved in the synthesis of the spore coat, and potassium and manganese stimulated the uptake of potassium (CAZEMIER *et al.,* 2001; HORNSTRA *et al.,* 2009). The addition of Mn^{2+} to the sporulation medium can promote sporulation efficiency, avoid spore expontanius germination, and increase wet heat resistance by affecting the peptidoglycan composition and core dehydration (ATRIH; FOESTER, 1999). Removing calcium ions from sporulation medium might inhibit the formation of the Ca^{2+}- dipicolinic acid, reducing spore resistance (MINH *et al.,* 2011).

Supplementation of the sporulation medium with cysteine, cystine or thioproline increases spore resistance to solar UV radiation and H_2O_2 (MOELLER *et al.,* 2011). Rose *et al.* (2007) demonstrated higher wet-heat and some chemical resistance of *B. subtilis* spores prepared on plates compared to spores prepared on liquid.

Craven *et al.* (1990) and Mazas *et al.* (1997) studied the effect of sporulation pH on the heat resistance of *Clostridium perfringens* and *B. cereus* and observed that increased pH resulted in the formation of spores with greater resistance to

inactivation at elevated temperatures. Guizelini *et al.* (2012) demonstrated that pH is the most significant variable affecting *G. stearothermophilus* spore wet-heat resistance and that alkaline pH (~ 8.5) results in more heat resistant spores.

Nguyen Thi Minh *et al.* (2008) observed that *B. subtilis* spores produced at lower water activity are smaller and more sensitive to heat but that their resistance to high hydrostatic pressure was not altered. The authors concluded that the water activity of the sporulation medium significantly affects the spores' properties, mainly heat resistance.

4.11.2 Sporulation temperature

Lindsay *et al.* (1990) demonstrated that spores of *B. subtilis* produced at different temperatures (23°C- 49°C) had increased heat resistance as sporulation temperature increased up to 45°C. However, spores grown at 49°C showed a dramatic reduction in resistance. This may be related to the spore coat composition, which varies significantly with sporulation temperature. Spores produced at lower temperatures were also more sensitive to DNA damage by chemicals such as formaldehyde and nitrous acid than were spores produced at higher temperatures (CORTEZZO; SETLOW, 2005). Higher sporulation temperatures were also correlated with higher levels of spore mineralization (PALOP *et al.,* 1999).

Melly *et al.* (2002) cited that *B. subtilis* spores prepared at 22 to 48°C had identical amounts of dipicolinic acid and small acid-soluble proteins (SASP); however, the core water content was lower in spores prepared at higher temperatures. These spores were more resistant to wet-heat than the spores prepared at lower temperatures. Spores prepared at higher temperatures were also more resistant to oxidizing agents, formaldehyde, and glutaraldehyde and exhibited nearly identical resistance to UV radiation and dry heat.

4.12 Other factors

Spore resistance is significantly induced by the spores but also by the supporting material, recovery culture medium composition, osmolarity, pH and

presence of germinating substances (such as alanine, Ca-DPA and dodecylamin) (PFLUG *et al.*, 1981;CAZEMIER *et al.,* 2001;SETLOW *et al.,* 2003b;PIZÓN-ARANGO *et al.,* 2009). The influence of the recovery medium on measured recovery was discussed by Pflug *et al.* (1981), who reported the effect of different lots on the number of recovered spores, and Shintani *et al.* (2000), who observed differences in recovery capacity with differing calcium concentrations as well as the presence and the absence of K_2HPO_4 in different lots and/or manufacturers of soybean casein digest (SCD) culture medium.

The presence of organic substances, such as proteins and carbohydrates, is known to increase heat resistance, while glucose may reduce resistance (MOLIN, 1997). Molin and Snygg (1967) demonstrated the effect of lipids on the heat resistance of bacterial spores and observed increased variability according to the type of lipid used. The interactions that frequently occur between environmental factors must be considered when studying spore resistance.

5.0 BIOTECHNOLOGICAL APPLICATIONS

Because of their complex structure and high resistance to physical and chemical factors, *Bacillus atrophaeus* spores have been the subject of investigation for a number of important applications, including sterilization monitoring, vaccine and drug delivery, astrobiology biodefense, antimicrobial compound production, and water treatment system evaluation.

5.1 Biomolecule producer

B. atrophaeus is a known producer of antimicrobial compounds. Stein *et al.* (2004) described the production of the bacteriocin subtilosin A, a macrocyclic bacteriocin, at the end of exponential growth, particularly under stress conditions. *B. atrophaeus* bacteriocin production with antimicrobial properties and prominent lipolytic activity, similar to a bacteriocin produced by others *Bacillus* species, was described by Shelar *et al.* (2012). Liu *et al.* (2012) studied the *B. atrophaeus* C89,

isolated from a marine sponge, as a potential producer of bioactive compounds, such as neobacillamide A and bacillamide C.

The capability of the *B. atrophaeus* strain to produce biosurfactant proteins with detergents, emulsifiers, and antimicrobial actions was shown by Neves *et al.* (2007). Thermotolerant neutral lipase, which hydrolyses castor oil, was also reportedly found in *B. atrophaeus* SB-2. This lipase activity was markedly stimulated by the addition of diethyl ether to the medium (BRADOO *et al.,* 1999). Youssef and Knoblett (1998) demonstrated the antifungal activity of the broth filtrate of *Bacillus atrophaeus* on *Ascosphaera apis*, a pathogen of honeybee larvae. Significant growth inhibition and hyphal lysis were observed, but the biomolecule was not isolated.

This organism also plays an important role in the biotechnology industry as a source of restriction endonucleases and the human intestinal sucrase inhibitor 1-deoxynojirimycin, also known as the glycosylation inhibitor nojirimycin (GIBBONS *et al.,* 2011). Stein *et al.* (1894) first described the synthesis of this compound, which was detected concomitantly with heat-resistant spores. The amount of 1-deoxynojirimycin produced was highly dependent on the carbon source.

Chandrapati and Woodson (2003) reported the synthesis of β-glucosidase during germination and outgrowth of *B. atrophaeus* spores in the presence of a germinant such as L-alanine and the inducer 4-methylumbelliferryl-β-D-glucoside. This research provided the first biological basis for the development of a rapid readout biological indicator to monitor the efficacy of ethylene oxide sterilization. This study was complemented by Setlow *et al.* (2004), who determined the mechanism of the hydrolysis of 4-methylumbelliferyl-beta-D-glucopyranoside (beta-MUG) by germinating and outgrowing spores of *Bacillus*.

As a biomolecule producer, *B. atrophaeus* is not as well cited as other *Bacillus* species partially because it only became a new species in 1989 (NAKAMURA, 1989).

5.2 Vehicle and adjuvant for vaccine

Barnes *et al.* (2007) reported that inactivated *B. subtilis* spores were as an effective microparticle adjuvant for the induction of higher IgG titers against tetanus

toxoid as titers induced by the toxoid alone. Huang *et al*. (2010b) described that live and inactivated spores were both capable of inducing an effective cellular and humoral immune response against a number of tested antigens, including tetanus toxin, *Clostridium perfringens* alpha toxin and anthrax toxin. Based on these studies, Oliveira-Nascimento *et al.* (2012) demonstrated the use of *B. atrophaeus* inactivated spores (BAIS) as an alternative method to boost the inactivated rabies virus response. The results showed that BAIS was effective in augmenting antibody titers, but in combination with saponin, it doubled the titers. BAIS was regarded as a viable alternative to commercial adjuvants as it had high vaccine potency with good stability (21 months when stored at 4°C - 8 °C).

Ricca and Cutting (2003) reported that the protective coat of the bacterial spore may allow its use in nanobiotechnology research as a substrate for the delivery of biomolecules. Spore coat has been shown to provide a suitable surface for the display of heterologous antigens using the *CotB* and *CotC* proteins. Vaccine vehicle spores have a number of advantages, as described by Barák *et al.* (2005), including (*a*) heat stability, ensured by the well-documented resistance of the bacterial spore; (*b*) safety record, established through the common use of spores of several species as probiotics; and (c) simple and economic production of large amounts of spores, based on the commonly used procedures for industrial-scale production. These same carrier systems (*CotB* and *CotC*) can also be used for drug delivery or for proteins important for industry (e.g. xylanases). It is likely that other spore coat proteins can also be used for surface expression and delivery (RICCA; CUTTING, 2003).

5.3 Planetary Protection Assays

Planetary protection is the term given to the practice of protecting the solar system from contamination by Earth life and protecting Earth from possible life forms that may be returned from other solar system bodies (*http://planetaryprotection.nasa.gov/about*). National Aeronautics and Space Administration (NASA) uses a variety of methods to measure, control and reduce spacecraft microbial contamination for planetary protection purposes. *B. atrophaeus*

endospores are the reference microorganism used as a model for assay procedures that apply to all spacecraft hardware and pertinent assembly, test, and launch facilities required to meet planetary protection standards and/or requirements established by the NASA Planetary Protection Officer (NASA, 2010). Some of the applications described address biological challenges in development and validation of microbiological sample methods (PROBST *et al*., 2011). Furthermore, these applications can analyze the efficacy of different cleaning approaches to remove bacterial spores from a series of surrogate and/or spacecraft materials (CHEN *et al.,* 2008). They can also determine the efficacy of disinfection methods (KEMPF *et al.,* 2008; POTTAGE *et al.* 2012), develop biological sensing and novel methods for spore detection or/and enumeration (JONSSON *et al*., 2005; YUNG *et al*., 2006; PROBBT *et al*., 2012) and lead to model systems for studying biological responses to extraterrestrial conditions (MOSHAVA *et al*., 2011).

5.4 Control for DNA extraction during nucleic acid testing

Polymerase Chain Reaction (PCR) is used to detect pathogens from various samples. Prior to performing PCR, DNA must be extracted efficiently from samples. An optimal extraction procedure will efficiently extract DNA from any microorganism present in the samples (Rose *et al.*, 2011). Picard *et al.* (2009), Geissler *et al.* (2011) and Rose *et al.* (2011) described the use of *B. atrophaeus* spores as controls to investigate the efficiency of nucleic acid extraction. Because its structure is difficult to lyse, *B. atrophaeus* is added to test samples prior to cell lyses and DNA extraction and after the extraction process. The genomic DNAs from *B. atrophaeus* and sample microorganisms can be detected and quantified using a PCR assay. *B. atrophaeus* spores are considered a universal cell lysis control.

5.5 Water treatment system evaluation

Water supplies are critical resources that are vulnerable to accidental or intentional contamination by potential human pathogens (WHO, 2004). Szabo *et al*.

(2007) studied the persistence of *B. atrophaeus* subsp. *globigii* spores on corroded iron coupons in drinking water using a biofilm annular reactor. *B. atrophaeus* was pulse-injected into the reactor in spore form, and the spore density on the coupons was monitored over time under dechlorinated and chlorinated bulk conditions. The results indicated that these spores are capable of persisting for an extended time in the presence of high levels of free chlorine, indicating that decontamination with alternative disinfectants and physical removal of corrosion are important subjects for future research. Shane *et al*. (2011) complemented this study by adding free chlorine at a concentration of 5 mg/L and observed a decrease in the adhered spore density by 2 logs within 4 hours. Furthermore, spores were not detected after 67 h and 49 h in the presence of 1 mg/L and 5 mg/L free chlorine, respectively.

B. atrophaeus spores were also utilized by Szalbo *et al.* (2012) to demonstrate that germinating spores before chlorinating cement-mortar or flushing corroded iron was more effective than chlorinating or flushing alone.

Kearns *et al.* (2008) utilized *B. atrophaeus* spores as a challenger microorganism to evaluate an automated concentration system placed online in drinking water distribution systems, projected to facilitate the detection of microorganisms and mitigate the risk to public health. This study represented an initial step toward automated monitoring of critical water resources for potential pathogens.

B. atrophaeus has also been applied to several aerated stabilization basins and settling ponds in tracer studies due to its resistance and non-reproductive capacity in this environment. This organism requires a sugar to grow, which is not present in wastewaters. The collected samples were plated onto an agar that allowed the development of spore colonies, which were enumerated (CHRISTIANSEN *et al.,* 2003).

The commercial product BI-CHEM® MicroTrace (Novozymes Biologicals Inc., Salem, VA, USA) is composed of *B. atrophaeus* spores and is indicated for determining hydraulic retention times of once-through unit processes, estimating basin loss due to sludge accumulation, estimating basin recovery from sludge removal, analyzing mixing efficiencies (short circuiting) to make decisions on aerator and curtain placement, and measuring improvements in efficiency following changes (NOVOZYMES, 2006).

5.6 Simulant for biological warfare (WF)

For almost seven decades, *B. atrophaeus* spores have played an integral role in biodefense activities as a stimulant for biological warfare and terrorism events and as a *B. anthracis* surrogate (GIBBONS *et al.,* 2011). Several recent reports regarding this use are described in the following sections. The microorganism has been mainly used to develop or assess methods for the following:

a) *Investigation of the effectiveness of decontamination methods by surface sampling*

Krauter *et al.* (2012) affirmed that the recovery of spores from environmental surfaces varies due to sampling and analysis methods, spore size and characteristics, surface materials, and environmental conditions. The authors used *B. atrophaeus* spores to evaluate the effects of spore concentration and surface material on the efficiency of sponge wipe sampling method recovery. Using *B. atrophaeus* spores, Calfee *et al*. (2012) evaluated the effectiveness of two spray-based decontamination methods for surfaces aimed at reducing contamination and determined the potential of spreading contamination by these methods. The authors concluded that achieving conditions that effectively inactivate surface biological contamination are critical for preventing the spread of contamination. Lewandowski *et al.* (2010) also used *B. atrophaeus* spores as a *B. anthracis* surrogate to evaluate the use of a foam spatula for sampling surfaces after bioaerosol spore deposition.

b) *Study of spore inactivation methods and products*

A number of studies have aimed to determine the susceptibility of *B anthracis* to current antiseptics in order for healthcare workers and patients to avoid contamination. Weber *et al.* (2003) evaluated the efficacy of several hand disinfectants against *B. atrophaeus* and concluded that hand washing with soap and water, 2% chlorhexidine gluconate, or chlorine-containing towels reduced the amount of spore contamination, whereas the use of a waterless rub containing ethyl alcohol was not effective in removing spores. Oie *et al.* (2011) evaluated the

disinfection methods for environments contaminated with bioterrorism-associated microorganisms by studying the sporicidal effects of sodium hypochlorite on spores of *B. atrophaeus* and other related species. Their results suggest that hypochlorite-containing vinegar is effective in disinfecting vinyl chloride, tile, and cement plates, and peracetic acid is effective in disinfecting plywood plates contaminated with *B. atrophaeus*. Tufts and Rosati (2012) verified the thermal inactivation of viable *B. anthracis* simulant spores (*G. stearothermophilus* and *B. atrophaeus*) for municipal waste landfill flare applications, and their results indicated that all spores were inactivated in the flare.

c) *Validation of current mathematical fate predictions and transport models for predicting the distribution of pathogenic particles after their release into air or water*

Greenberg *et al.* (2010) reported that in 1949, the US Army experimentally sprayed *B. atrophaeus* and *Serratia marcescens* over the coastal population centers of Hampton, Virginia, and San Francisco, California. Furthermore, during the mid-1960s, *B. atrophaeus* was disseminated in Greyhound bus and New York subway terminals via covert spray generators hidden in briefcases in order to evaluate spore dissemination.

Kournikakis *et al.* (2011) showed that *B. atrophaeus* spores (in dry powder form) were released by opening a letter to characterize airflow and unmitigated spore aerosol dissemination within an office test site using a tracer gas, smoke tubes (containing stannic chloride) and aerosol spectrometry. *B. atrophaeus* spores were also utilized by Raber and Burklund (2010) to determine effective decontamination alternatives for use in a contaminated drinking water supply.

d) *Development and evaluation of methods to detect and identify Bacillus spores*

Detection and identification of microorganisms in environmental samples for biosecurity applications are challenging due to the strict requirements for specificity, sensitivity and time (GÖRANSSON *et al.,* 2012).

Czerwieniec *et al.* (2005) utilized *B. atrophaeus* spores and vegetative cells to identify key biomarkers from microorganisms grown in C^{13} and N^{15} isotopically enriched media by bioarerosol mass spectrometry. Létant *et al.* (2011) developed and tested a novel RT-PCR method for the detection of live, virulent *Bacillus anthracis*, in which the incubation time was reduced from 14 h to 9 h, using surrogate organisms including *B. atrophaeus* and the non-virulent *B. anthracis* Sterne strain. Göransson *et al.* (2012) developed a quick, specific and sensitive method of microorganism identification in environmental samples. Target identification was realized by padlock and proximity probing, and reacted probes were amplified by RCA (rolling-circle amplification). The individual RCA products were labeled by fluorescence and enumerated by an instrument, developed for sensitive and rapid digital analysis. This method was demonstrated by the identification of similar biowarfare agents for bacteria (*Escherichia coli* and *Pantoea agglomerans*) and spores (*B. atrophaeus*) released in the field.

5.7 Standard microorganisms

The International Standard Organization (ISO) indicates the employment of *B. atrophaeus* spores in some biological tests as described in the following standards: ISO 14698-1 (2003), Cleanrooms and associated controlled environments--Biocontamination control; ISO 3826-1 (2003), Plastics collapsible containers for human blood and blood components - Part 1: Conventional containers; ISO 15747 (2003) Plastic containers for intravenous injection; and ISO 14160 (1998) Sterilization of single-use medical devices incorporating materials of animal origin--Validation and routine control of sterilization by liquid chemical sterilants. ISO also recommends the use of *B. atrophaeus* spores as standard microorganisms for the production of biological indicators for sterilization, as described in the following standards: ISO 11.138 (2006) Sterilization of health care products-Biological indicators, Part 2: Biological indicators for ethylene oxide sterilization and ISO 11.138-4 (2006) Sterilization of health care products-Biological indicators: Biological indicators for dry-heat sterilization.

This strain is recommended for use in the tests described in the military

specification MIL S-36586A (1976) substituted by A-A-50879 (US Department of Defense, 1991) - Sterilization Test Strip Set, Bacterial Spore and US Pharmacopeia <1211> "Sterilization and Sterility. Assurance of Compendial Articles" (USP 23, 1995).

The AOAC 997.17 Official method “Microbial Ranking of Porous Packaging Materials (Exposure Chamber Method)”, used to measure the microbial barrier effectiveness of porous packaging designates the use of *B. atrophaeus* spores as a challenge microorganism (AOAC, 1997).

5.8 Study and evaluation of sterilization systems, products and processes

5.8.1 Plasma sterilization

Sharma *et al.* (2002) exposed *B. atrophaeus* spores to a downstream plasma afterglow plume emitted from a slotted plasma device operating in open air at atmospheric pressure to study bacterial inactivation on surfaces exposed to the plasma afterglow at different exposure times. The results suggested that the mechanistic action of the plasma appeared to affect both nucleic acids and the cell wall structure, indicating that it was a promising method of microorganism inactivation. Halfmann *et al.* (2007) utilized *B. atrophaeus* spores to identify the role of sterilization agents in argon plasma (with the addition of nitrogen and oxygen) sterilization, an alternative to traditional sterilization processes. Another example of *B. atrophaeus* endospore inactivation was studied by Opretzka *et al.* (2007) using plasma discharges, which offers the benefits of short treatment times, minimal damage to the objects being sterilized and minimal use of hazardous chemicals. The simultaneous impact of H atoms and low energy ions was shown to cause a perforation of the endospore shell (FIGURE 12), explaining the efficient inactivation of bacteria.

Hauser *et al.* (2011) sprayed *B*. *atrophaeus* spores (10^6 colony-forming units) on the surfaces of 12 silicone implant material samples as biological indicators aimed at evaluating the effectiveness of the low-pressure gas plasma sterilization technique

on a silicone implant material.

Cold atmospheric Surface Micro-Discharge (SMD) plasma sterilization was also tested against *B. atrophaeus* spores by Klämpfl *et al.* (2012). The experimental $D_{23°C}$-values obtained at 0.6 min were significantly lower compared to *D*-values obtained from other reference methods, demonstrating the strong biocidal effect of SMD air plasma.

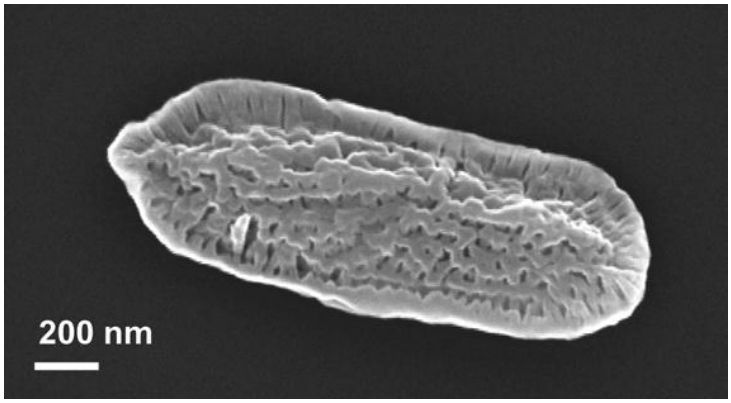


FIGURE 12 – SCANNING ELECTRON MICROSCOPY PICTURE OF *B. atrophaeus* SPORE TAKEN AFTER A 60 MIN EXPOSURE TO H ATOMS AND Ar^+ IONS (115 EV). THE SPORE APPEARANCE IS TYPICAL OF THIS PARTICULAR SAMPLE

SOURCE: Opretzka *et al.* (2007)

5.8.2 Chemical sterilization and disinfection

Chemical sterilization is typically used for devices that would be sensitive to the heat used in steam or dry-heat sterilization and for devices that may be damaged by irradiation. For highly spore-contaminated environments or surfaces such as laminar flow biological safety cabinets, gaseous sterilization or decontamination is recommended. The use of *Bacillus* spores is advised, the sporicidal capacity of the chemical agent should be evaluated, and their "*in use*" action should be validated.

Mazzola *et al.* (2006) used *B. atrophaeus* ATCC 9372 and *E. coli* ATCC 25922, among others microorganisms, as 'standard' bacteria to evaluate resistance at 25°C against either 0.5% citric acid, 0.5% hydrochloric acid, 70% ethanol, 0.5% sodium bisulfite, 0.4% sodium hydroxide, 0.5% sodium hypochlorite, or a mixture of 2.2%

hydrogen peroxide (H_2O_2) and 0.45% peracetic acid. *B. atrophaeus* spores were used by Landa-Solis *et al.* (2005) to assess the sporicidal activity of super-oxidized water (commercial product). Under these test conditions, the product demonstrated complete inactivation of the spores within 2–3 min, confirming its sporicidal activity. Grand *et al*. (2010) studied the influence of the properties of solid substrates in peracetic acid bactericidal activity with *B. atrophaeus* and compared it with material characteristics associated with inoculation.

Luftman and Regits (2008) studied a validation program that used chlorine dioxide (CD) gas in the decontamination of laminar flow biological safety cabinets (BSCs) using *B. atrophaeus* and *G. stearothermophilus* endospores as biological indicators (BIs) of CD gas decontaminations. The authors concluded that *B. atrophaeus* spores on paper substrates should be used as standards for the validation of chlorine dioxide gas in the decontamination of BSCs. Andersen *et al.* (2012) reported the regular use of commercial spore control biological indicators with spores of *B. atrophaeus*, placed each time into defined equipment to monitor the effect of decontamination with hydrogen peroxide gas.

5.9 Biological indicators for sterilization and disinfection validation and monitoring

Biological indicators (BIs) are a test system containing viable microorganisms providing a defined resistance to a specific sterilization process (ISO 11139, 2006). BIs contain high numbers, generally 10^3 to 10^6, of bacterial endospores that are highly resistant to the sterilization process for which they are designed. The spores are placed on or in a carrier material such as paper, glass or liquid and packaged to protect the BI. After being subjected to sterilization or other similar treatment, the indicator is cultivated to determine the effectiveness of the sterilization, disinfection, and so on. Biological indicator systems allow for qualitative evaluation, e.g., by visual identification of a color change or turbidity of the substrate media containing a pH indicator. The visual color identification method detects acid metabolites produced after germination and growth of the spores. The acid metabolites are the result of a series of enzyme-catalyzed reactions that occur during the growth, producing a pH change in the medium. This pH change causes the medium to visually change color

(GILLIS *et al*., 2010).

Sterilization monitoring initially emerged with the use of small quantities of garden soil cultured after exposure to the sterilization process. In the early 1890s, Robert Koch refined the BI concept by using flannel rolls contaminated with spores. The exposition of dry heat demonstrated the survival and free germination of spores, while complete destruction occurred when the contaminated flannel was exposed to moist heat (PERKINS, 1978; PFLUG, 2009).

Currently, BIs have been useful for validation and routine monitoring of ethylene oxide, steam, dry heat, low temperature steam formaldehyde, vapor hydrogen peroxide, microwave and related plasma sterilization systems (GILLIS *et al.,* 2010). While physical monitors and chemical indicators provide valuable information regarding the sterility of a processed load, BIs are recognized by most authorities as being closest to the ideal monitor of the sterilization process because they are the only type of indicator that can measure the direct lethality, i.e., killing power, of that process (RUTALA; WEBER, 2008; RUTALA *et al.,* 1993; SCHNEIDER 2011*).*

It is important to note that BIs are defined as a system consisting not only of the sensing element, the microorganisms, but also the carrier material onto or into which the spores are placed and the packaging characteristics. When evaluating BI as a system, stressed microorganisms do not always have the same responses to methods as laboratory stock cultures. It is not unusual for stressed microorganisms (post sterilization process) to show different growth characteristics in recovery media than cultures that have not been stressed (used as a control) (SHINTANI; AKERS, 2000; SASAKI *et al*., 2000).

5.9.1 Validation and monitoring of dry-heat and ethylene oxide sterilization

Bacillus atrophaeus have been established as industrial bacteria for the production of biological indicators for sterilization (FDA, 2007; FRITZE; PUKALL, 2001; GIBBONS *et al*., 2011; WEBER *et al*., 2003). *B. atrophaeus* BIs are routinely used by medical-device manufacturers and healthcare providers to monitor the efficacy of various sterilization processes, such as dry heat and ethylene oxide

(HALFMANN *et al*., 2007; ISO 11138-4, 2006).

B. atrophaeus BIS are commercially available in the following types:

a) Spores on an inert carrier

Inert carriers are used as spore supports. These carriers are usually paper strips but may be metal discs, plastic discs, glass, steel wire, aluminum strips, quartz sand, or cotton yarn (FIGURE13). The BIs are packaged in different varieties, e.g., protective glassine envelope, to maintain the integrity and viability of the inoculated carrier. After processing through the sterilizer, the strip is aseptically removed from the envelope and placed in an appropriate recovery medium that is incubated between 48 h and seven days. If live bacterial spores are still present, they will grow and produce cloudiness and/or change the color of the recovery medium, indicating sterilization failure.

Spore suspensions should also be inoculated into or on parts that are representative units of the products to be sterilized (USP 29, 2006).

FIGURE 13 – *Bacillus atrophaeus* BIOLOGICAL INDICATORS MADE WITH DIFERENT CARRIERS

SOURCE: The author, based on http://www.cruinn.ie/home/Default.aspx?id=11320, http://www.catalyststerilityproducts.com/eo.html and http://www.cherwell-labs.co.uk/products/biological-indicators/industrial-use-bis.html.

b) Self-contained vial

Spore strips or disks and an ampule filled with recovery medium are contained in a plastic vial with a vented cap to permit the entrance of the sterilizing agent into the vial (FIGURE 14). After processing, the vial is squeezed or the cap is pushed down to break the internal ampule, which mixes the growth medium with the spores.

The vial is then incubated; if live bacterial spores are still present, they will grow and change the color of the recovery medium, indicating sterilization failure.

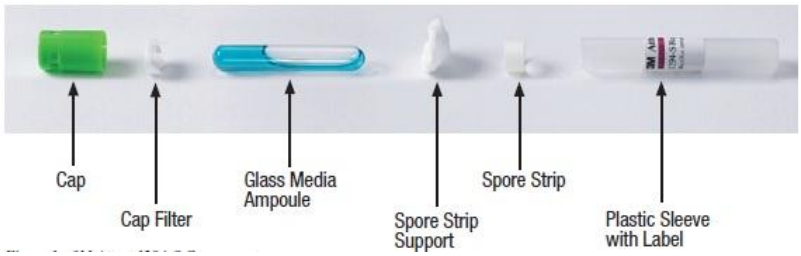


FIGURE 14 – SELF-CONTAINED BIOLOGICAL INDICATOR

SOURCE: 3M (2012)

c) Rapid readout

This system detects the activity of the enzyme β-glucosidase produced during bacterial spore germination and outgrowth. β-glucosidase catalyzes the hydrolysis of β-glucosidic linkages between glucose and alkyl, aryl or saccharide groups of the fluorogenic substrate β-MUG (4-methyllumbelliferyl-β-D-glucoside) to release its moieties, glucose and the fluorescent compound 4-MUG (4-methyllumbelliferone) (FIGURE 15).

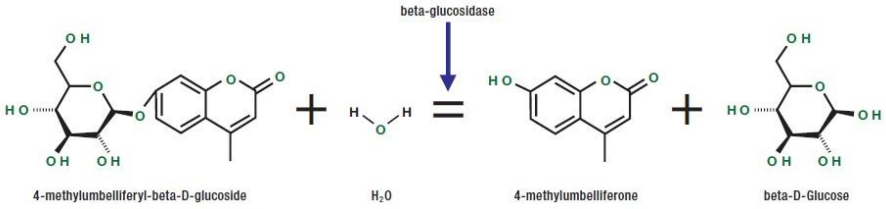


FIGURE 15 - A RAPID READOUT BIOLOGICAL INDICATOR: FLUOROGENIC SUBSTRATE REACTION

SOURCE: 3M (2012)

4-MUG is a fluorescent compound that is detected thought a fluorescence reader. Due to its high sensitivity, the method provides results in one to three hours (CHANDAPRATI; WOODSON, 2003) (FIGURE 16). The *B. atrophaeus* rapid readout BI has been employed to monitor ethylene oxide sterilization.

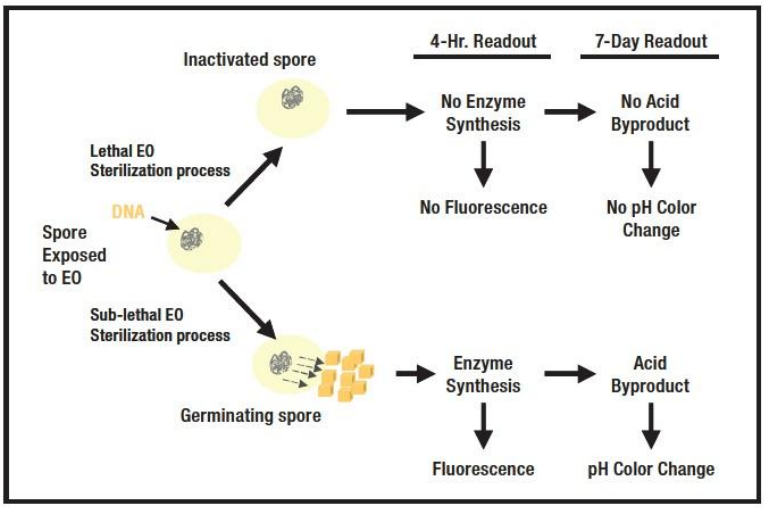


FIGURE 16 - SCHEMATIC OF THE RAPID READOUT BIOLOGICAL INDICATOR

SOURCE: 3M (2012)

5.9.1.1 Biological Indicators production and performance requirements

The production and performance requirements of *B. atrophaeus* BIS are normalized by the U.S. Pharmacopeia (USP 34, 2011a, b) and by the International Standard Organization-ISO ISO11138 - Sterilization of health care products – Biological indicators and ISO 11138, part 1 (general requirements), part 2 (requirements for biological indicators for ethylene oxide sterilization processes) and part 4 (requirements for biological indicators for dry-heat sterilization). These rudiments are also included in the Association for the Advancement of Medical Instrumentation (AAMI, 2006 a,b,c) sterilization standards collection, and the FDA regulates biological indicators (BI) intended to monitor sterilizers used in healthcare facilities as class II medical devices, which require premarket notification (FDA, 2007).

a) Conventional production

Around 1950, a sporulation medium containing Liver Fraction "B" buffered with phosphate was used to obtain high yields of *Bacillus* spores. In 1964, Donnellan *et*

al. (1964) described a chemically defined media for sporulation with glucose and glutamic acid with the addition of Ca^{+2} and Mn^{+2}. Schaeffer *et al.* (1965) described a complex medium using nutrient broth, KCl, $MgSO_4$, $MnCl_2$, $CaCl_2$ and $FeSO_4$ solidified with agar. This medium and its modifications were utilized until recently. *B. atrophaeus* spores for use as BIs are usually produced in a commercial medium such as Nutrient Sporulation Media (NSM), a solid growth medium, Casein Acid Digest (CAD), a liquid medium adapted from US Military Specification MIL-S-50003A (US DEPARTMENT OF DEFENSE, 2001) and Chemically Defined Sporulation Medium (CDSM), as described by Hageman *et al. (*1984).

b) Production by solid-state fermentation

Solid-state fermentation (SSF) is defined as a process in which the microorganisms grow over and inside a solid matrix in the absence of free water (PANDEY *et al.,* 2000). In SSF, the moisture necessary for microbial growth exists in a complexed form within the solid matrix or 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). The most commonly SSF system involves cultivation on a natural material or on an inert support impregnated with liquid medium (BHARGAV *et al.,* 2008). The utilization of agro-industrial residues as substrates or supports in SSF processes provides an alternative use and value-addition to these otherwise under- or non-utilized residues. The establishment of the relationships between the physiology of the used microorganisms and the physical-chemical factors of the SSF process is the key for the development of proper models. These factors include temperature, pH, aeration, water activity and moisture, bed properties, nature of solid substrate employed, substrate composition, etc (SINGHANIA *et al.*, 2009). The main advantages of SSF are its low technology, high productivity, low energy and low water requirements, low wastewater generation and the low risk of bacterial contamination due to the low water activity used (PANDEY, 2003).

Sella *et al.* (2009) first described solid-state fermentation using sugarcane bagasse as a support for *B. atrophaeus* spore production, with the intent to produce a biological indicator. The authors concluded that the thermal resistance of the spores was not affected by the SSF sporulation process conditions, indicating that

the SSF technique may be a highly attractive alternative for spore production and use as a bioindicator.

The performance evaluation of the *B. atrophaeus* BI includes a determination of the viable spores' population, *D*-value, survival time and kill time:

a) Viable spore population

Biological indicators typically have between 10^5 and 10^6 spores per unit. Thus, the typical biological indicator dies halfway through the time required to achieve the 10^{-6} sterility assurance level (SAL). The survival of the BI is a consequence of its resistance and population size. Therefore, BIs with a lower population number but higher resistance can be used to validate a sterilization process. It is up to the user to define the best BI according to the specifics of the process or product (Farmacopeia Brasileira, 2010).

BI standards for the population should be replicated within 50% to 300% of the manufacturer's label claims (ISO 11138-1, 2006). The potential sources of variability that may adversely affect a population determination are: 1) equipment, 2) materials, and 3) methods. Only the stated microorganisms should be present.

.

b) D-value determination

The *D*-value for a biological indicator (BI) is the time (or dose) required at a specified set of exposure conditions to reduce the viable spore population by one log or 90%. It important to note that the *D*-value is for the BI, not the spore, because the *D*-value is a measure of resistance performance that refers to the entire BI system (spore, packaging and recovery medium) and is not a value for the spore itself (GILLIS, 2008). BI standards resistance (*D*-value) should be within 20% of manufacturers label claims (ISO 11138-1, 2006). The *D*-value should be determined by:

b.1) Fraction negative analysis, using the Limited Spearman–Kaber Method (LSKM)

A sufficient number of groups of specimens of biological indicators in their original and individual containers, consisting of not less than 5 units, are exposed to the evaluated sterilizing condition at different exposition times. These differences in sterilizing times over the series must be constant, and the difference between adjacent times should be no greater than 75% of the estimated *D*-value. After completion of the sterilizing procedure, the tested BIs are cultivated in the proper recovery medium (USP 29, 2006). The *D*-value determined by the Limited Spearman–Kaber Method is calculated by the following formulas (equation *1 and 2*):

$$D_{160^{o}C} = \frac{U_{sk}}{\log N_o + 0.2507} \qquad (1)$$

where:
N_o = initial number of organisms on BI
U_{sk} = Spearman–Kaber heating time/dosage estimate

$$U_{sk} = U_k - \frac{d}{2} - \left(\frac{d}{n} \cdot \sum_{i=1}^{k-1} \frac{r_i}{n} \right) \qquad (2)$$

where:
U_k = first heating time with all units negative
d = time interval (minutes)
n = BIS per exposure time
r_i = number of units negative

b.2) Survivor curve

A survivor curve or death rate curve is a graphic representation of microbial inactivation over increased exposure to sterilizing conditions. This curve is based on

the direct enumeration of the spores after BI submission at two or more sterilization times. For *D*-value determination, the Stumbo-Murphy Cochran formula (equation 3) is utilized:

$$D = \frac{t}{\log No - logN} \qquad (3)$$

where:
N_o = initial number of organisms on BI
N = final number of organisms on BI
t = exposure time

b.3) Survival/Kill Window

Biological indicators must be designed to meet certain specifications, including a time point at which all spores need to survive (referred to as survival) and a time point at which all spores need to be inactivated (referred to as kill) (CHANDRAPATI; YONG, 2008). USP 32 (2008) and ISO 11138-1(2006) provides an acceptable test method to calculate and verify the survival/kill window. The minimum expected survival time and maximum expected kill time can be calculated from the following equations (USP 32, 2008; ISO 11138-1, 2006; FDA, 2007):

Survival Time = (log_{10} population – 2) x D-value (4)

Kill Time = (log_{10} population + 4) x D-value (5)

For verification or determination of BI resistance performance, the recommendation includes performing at least four cycles: one cycle resulting in total survival, two cycles resulting in fractional growth (some positive BIs, some negative BIs) and one cycle resulting in total kill (all BIs negative for growth). Minimum populations and resistance characteristics are demonstrated in TABLE 2.

TABLE 2 - MINIMUM BIOLOGICAL INDICATOR POPULATIONS AND RESISTANCE CHARACTERISTICS FOR DRY-HEAT AND ETHYLENE OXIDE STERILIZATION

Characteristics	Dry-heat (160°C)	Ethylene Oxide
Population	10^{5} (a) - 10^{6}(b,c)	10^{5} (a) - ≥10^{6}(b,c)
D-value	1-3 min (b) 3 min (c)	≥ 2.5 min (b) 3 min (c)
Survival time	12 min (a,b,c)	≥10 min (b) 15 min(a,c)
Kill time		60 min (a) 40 min (b)

(a) European Pharmacopoeia (2005); (b) ISO 11138 (2006); (c) FDA (2007)

5.9.2 Validation and monitoring of biomedical waste disinfection

Biomedical waste or healthcare waste is any solid and/or liquid waste, including its container and any intermediate products, that is generated during research or in the diagnosis, treatment or immunization of human beings or animals. Inappropriate treatment and final disposal of this waste can result in negative impacts to public health and the environment. Infectious healthcare waste may be a source of infection and may pose serious occupational risks to those who participate in the management cycle (DIAZ *et al.,* 2005).

Among the more common treatment and disposal methods utilized in the management of infectious healthcare waste are those employing autoclaving, incineration, shredding associated with chemical disinfection, thermal treatment (injected steam into waste), plasma pyrolysis, microwave disinfection and electro-thermal deactivation (ETD). The chosen method should consider environmental protection, waste reduction, work safety and treatment costs (EDLICH *et al*., 2006).

Entities performing the treatment of biomedical waste must submit proof that the waste is being properly treated. The monitoring procedures should ensure that the treatment processes will provide an acceptable level of microbial inactivation and will continue to do so during the regular operation of the facility. Based on recommendations by the State and Territorial Association of the USA (STAATT), all treatment technologies are required to attain Level III inactivation as a minimum. Level III inactivation requires a reduction of ≥ 6 $\log_{10}$ of vegetative bacteria, fungi,

lipophilic/hydrophilic viruses, parasites and mycobacteria, and a ≥ 4 Log_{10} reduction of *G. stearothermophilus* and *B. atrophaeus* spores. The biological indicators should be included in the surrogate test loads for initial efficacy tests of the treatment system, with a minimum of three surrogate test loads that differ in the concentrations of organic to non-organic compounds and fluid to solid components (STAATT, 1998). At least monthly, the biological indicator should be placed at the center of a load processed under standard operating conditions to confirm the attainment of adequate sterilization conditions (CDPH 2007). Goodfrey *et al.* (2003) recommends the use of bacterial spores once per week, or once every 40 h of machine operation.

B. atrophaeus ATCC 9372 is the organism of choice for the control of nonionizing radiation biomedical waste treatment, including microwave and electro-thermal deactivation (ETD). Commercial spore suspensions may be employed to proper discs or strips; however, spores on strips are available commercially. The use of spore suspensions in sealed vials is not recommended, as this method does not reflect actual waste treatment conditions (MESON *et al.,* 1993). The Brazilian Company of Environmental Technology and Sanitization (CETESB, 2007), however, does recommend its use.

The use of *B. atrophaeus* to evaluate waste treatment was initially described by Goldblith and Wang (1967) when *E. coli* and *B. atrophaeus* spores were exposed to conventional thermal and microwave energy at 2,450 MHz and the degrees of inactivation by the different energy sources were compared quantitatively. This was also tested by Barbeito *et al.* (1968) to determine the operating conditions to sterilize contaminated air by a semi-portable air incinerator and by Barbeito and Shapiro (1997) to determine the minimum operating temperature of a gas-fired waste incinerator needed to prevent the release of spores into the atmosphere.

Jeng *et al.* (1987) utilized dry spores of *Bacillus atrophaeus* simultaneously treated with heat in a convection dry-heat oven and a microwave oven to demonstrate that in dry-state, sporicidal action of the microwaves was caused solely by thermal effects. Huang *et al.* (2010) compared the thermal effect of microwave sterilization and the lethal effects of microwave and water-bath heating treatments on B. *atrophaeus* at various heating temperatures and time. The authors observed that at the identical conditions of heating temperature (85°C) and time, the microwave treatment was more lethal than the water-bath heating treatment, which was due to

an obvious non-thermal effect. However, there were no significant differences in the lethality rates of microwave and water-bath heating treatments at 92°C for 20 min.

Oliveira *et al.* (2010) inoculated pre-sterilized healthcare waste with spores of *Bacillus atrophaeus* to study their inactivation by microwave processing at different conditions in a pilot scale. The most favorable conditions for waste heating included 50% moisture with surfactant addition. The kinetic parameters obtained in this study allowed predicting the degree of inactivation of *B. atrophaeus* spores in typical healthcare waste, based on radiation exposure time and temperature. The authors estimated that the waste disinfection time required for the inactivation level to reach 4 $\log_{10}$ ranged from 13.0 h to 4.3 h for wastes processed at 100 W/kg and 200 W/kg, respectively, at a temperature of 100°C. They concluded that the microwave process of spore inactivation in real-scale equipment is most likely ineffective for spore inactivation, as exposure time is usually only 30 min at an average power of approximately 80 W/kg. Their findings suggest that it is necessary to use another method of disinfection concomitant to this one or that the current process must be optimized.

6. CONCLUDING REMARKS

The literature analysis demonstrates that *B. atrophaeus,* mainly in this sporulate form, has wide and diverse biotechnological applications.

B. atrophaeus spores are widely recommended as a bacteriological control for dry-heat and ethylene oxide sterilization. Although the regular use of biological indicators to evaluate the efficiency of the sterilization processes is a legal requirement for health services, studies for the development of new sporulation processes, the reduction of processing steps, the enhancement of spore yields and the reduction of biological indicator costs are still lacking.

Due to their non-pathogenicity, facility of culture and resistance characteristics, *B. atrophaeus* spores are known to have a fundamental role in the validation and monitoring of a series of new sterilization and disinfection products and process performance testing, with applications in the pharmaceutical, food and biodefense industries.

Studies of *B. atrophaeus* biomolecule production are rare and should be conducted more frequently. As *B. atrophaeus* is closely related to *B. subtilis*, which has a range of industrial applications, the study of biomolecule production by *B. atrophaeus* remains an exciting field.

The development of new generations of vaccines and drugs employing genetically engineered bacterial spores to express vaccine antigens or others products in the coat are promising. Because of their high resistance as well as their safety, cost effectiveness and high production yield, *B. atrophaeus* spores are a highly attractive microorganism for surface display studies that aim to develop new vaccines and drugs.

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CHAPTER II

DEVELOPMENT OF A LOW-COST STERILIZATION BIOLOGICAL INDICATOR USING *Bacillus atrophaeus* BY SOLID-STATE FERMENTATION

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ABSTRACT

The production of biological indicators involving bacterial sporulation and multi-step downstream processes has been described. The goal of the present work was to use fermented material as the final product in a biological indicator, thereby reducing processing steps and costs. The performance of three different inexpensive supports (vermiculite, sand, and sugarcane bagasse) was assessed by determining *Bacillus atrophaeus* sporulation during solid-state fermentation and by assessing the direct use of the fermentation products in the subsequent steps of the process. All three supports allowed spore production of between 10^7 and 10^9 CFU/g. Sand proved to be the best inert support enabling the direct use of the fermented product due to its easy homogenization, filling properties, and compatibility with recovery medium. Bacterial adhesion to the sand surface was supported by biofilm formation. The resistance to sterilization of the dried fermentation product was evaluated. For dry-heat resistance (160°C), the *D*-value was 6.6 min and, for ethylene oxide resistance (650 mg/L), the *D*-value was 6.5 min. The cost reduction of this process was at least 48%. No previous studies have been published on the application of sand as a support in solid-state fermentation for the production of biological indicators.

Key words *Bacillus atrophaeus*; spores; biological indicator; solid-state fermentation; sand; biofilms

1. INTRODUCTION

Microbial spores are usually accepted as being much more resistant to sterilization processes than most other types of microorganisms, so a sterilization process that will kill microbial spores is also assumed to eliminate any other contaminating microorganisms (TAUTVYDAS, 2000). *Bacillus atrophaeus* (formerly *Bacillus subtilis var. niger*) is a suitable reference organism for testing the effectiveness of dry-heat, ethylene oxide, microwaves, low-temperature gas plasma,

and electrothermal deactivation sterilization processes (ISO 11138-4, 2006; OLIVEIRA *et al.*, 2010; ROTH *et al*., 2010; TURNBERG, 1996). Because of their resistance characteristics, spores are used for producing biological indicators (BIs). A non-self-contained BI consists of glass ampoules containing paper strips (or silica sand) inoculated with 10^3-10^6 spores, and culture tubes (or vials) containing sterile recovery culture medium.

Several systems employed in the industrial production of *Bacillus* spores have been described, such as sporulation in industrialized or chemically defined media, such as agar (SCHAEFFER *et al*., 1965; CAZEMIER *et al*., 2001) or broth in batch bioreactors (HAGEMAN *et al.,* 1984; MONTEIRO *et al.,* 2005). Although a defined culture medium can provide a high-yield of spore production, its use makes the final product expensive, especially for extensive use in developing countries. Some sporulation processes using different complex media, aimed at lowering production costs, have been described, such as submerged fermentation (Smb) using distillery effluents, corn starch, corn flour, and wheat bran (SHI; ZHU, 2006; CHEN *et al.,* 2010). Some advantages of solid-state fermentation (SSF), relative to Smb, have been demonstrated for *Bacillus* spore production (PRYOR *et al.,* 2007; ZHAO *et al.*, 2008; SELLA *et al.*, 2009) however, downstream processes need to be applied after sporulation for spores to be utilized in BI production. These include spore detachment, harvesting and purification, followed by spore inoculation into a carrier (FIGURE 1). The present study investigated solid-state fermentation supports that would allow sporulation and the maintenance of spore attachment, thus enabling the support to also be used as the carrier in the BI. These features should make it possible for fermented substrate to be used directly in a follow-up process, thereby reducing processing steps. For this purpose, three relatively inexpensive by-products were evaluated and compared: vermiculite, a hydrated magnesium aluminum iron silicate; sand, usually in quartz form and composed of silica (SiO_2); and sugarcane bagasse, composed of cellulose, hemicellulose and lignin. We found no reports in the literature concerning the direct use of SSF fermented substrate as a biological indicator for sterilization.

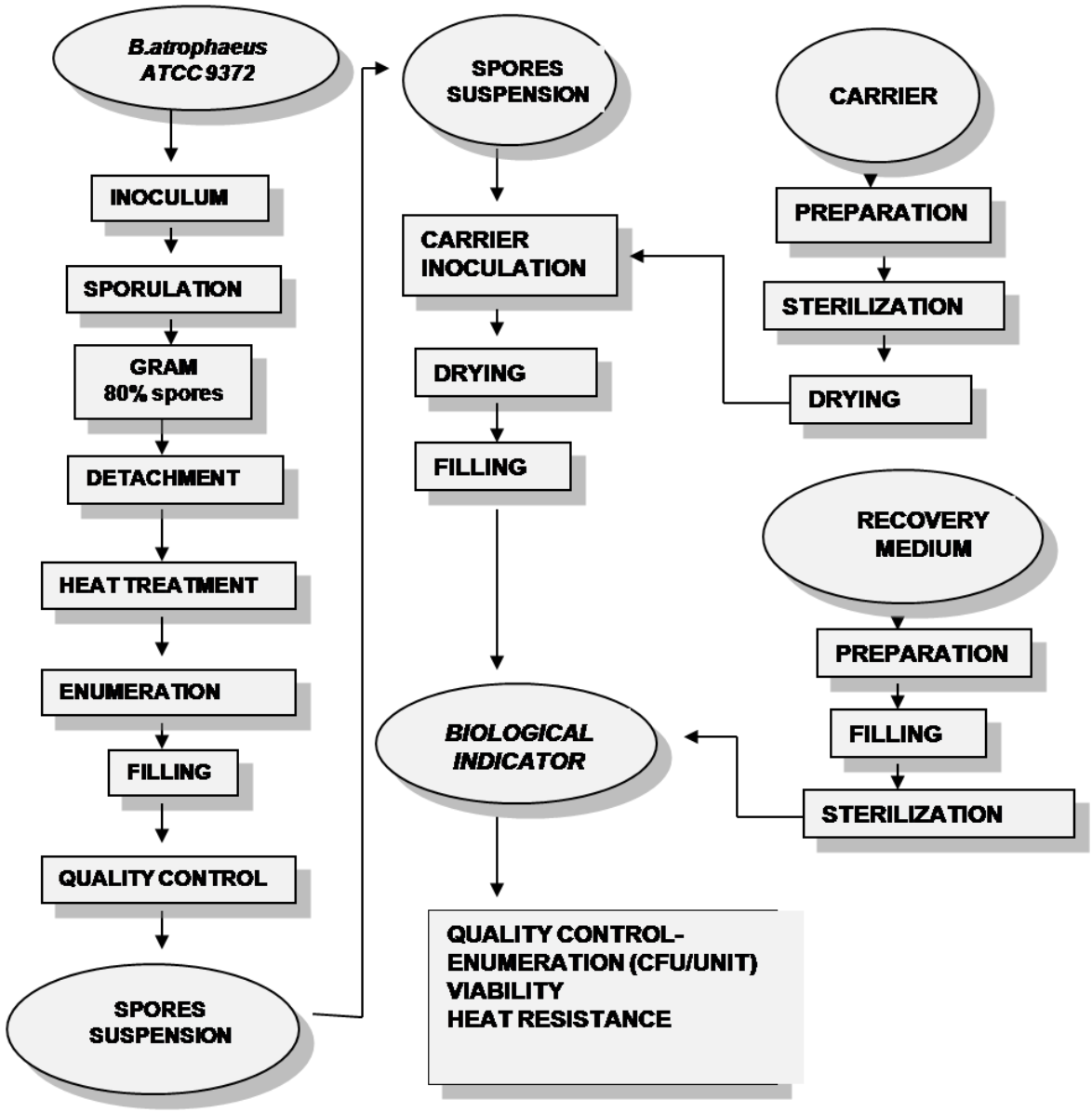

FIGURE 1 - FLOWCHART FOR BIOLOGICAL INDICATOR PRODUCTION

SOURCE: The author (2011)

2. MATERIAL AND METHODS

2.1 Bacterial strain

B. atrophaeus ATCC 9372, Bach-1403349, was obtained from the *National*

Institute of Health Quality Control (INCQS/MS, Brazil).

2.2 Preparation and characterization of support

Supports were washed once in tap water, twice in distilled water, and dried in trays for 24 h at 90°C in an air oven. Sugarcane bagasse was supplied by Cocamar (Cianorte, Brazil). The silica sand-quartz used was commercial grade and classified as medium size particle (0.4 - 2.0 mm). The washing process was done to ensure that the sand has an exceptionally low turbidity, free from clay and fines, and low in unwanted salts. Both bagasse and sand were sieved to obtain a ~1.0 mm particle size (MESH 14-20). The vermiculite (~4.0 mm particles) was not sieved because this damages its laminar characteristics. For pH determination, before and after sterilization, 1.0 g sample was diluted with 9.0 ml distilled water, blended for 2 min, and then centrifuged. The supernatant was used for the assay. The pH was determined with a pHmeter (PG-1800-Gehaka, SãoPaulo, Brazil). Moisture content was determined gravimetrically by drying the sample in an oven at 105ºC approximately for 24 h. Water activity (a_w) measurements were determined at 19-20ºC, using an AquaLab CX-2 water activity meter (Decagon Devices, Pullman, WA, USA).The water absorption capacity of the supports was determined using 5.0 g of tested support in 100 mL of water after a 15-min exposure to 121ºC.

2.3 Inoculum

For SSF utilizing commercial industrialized media as substrate, 100.0 µL of spore suspension (batch: 01/08-CPPI) was inoculated in 30.0 mL of commercial medium (tryptone soy broth, TSB). For SSF with complex substrates, inoculums were produced in soybean molasses based medium [4% (*w/v*) tryptone and 4% (*w/v*) soybean molasses (80ºBrix)]. These cultures were incubated for 18 h at 36°C.

2.4 Media composition

Media consisted of: (1) *complex substrate:* 2% soybean molasses, supplemented with: 0.005% $K_2HPO_4.H_2O$, 0.004% $MnSO_4.4H_2O$, 0.004% $CaCl_2.6H_2O$, 0.005% $MgSO_4.7H_2O$ and (2) *commercial or synthetic substrate*: 0.8% yeast extract, 0.4% nutrient broth, 0.005% $MnSO_4.4H_2O$, 0.005% $CaCl_2.6H_2O$. The pH was adjusted before sterilization with 0.1 N NaOH to 8.0 $\pm$ 0.1.

2.5 Sporulation

SSF was carried out in 250 mL Erlenmeyer flasks each containing 26.0 mL of substrate. The support impregnated with medium was weighed and added to the flask based on water retention capacity: 4.0 g for sugarcane bagasse, 5.0 g for vermiculite, and 100.0 g for sand. Flasks were autoclaved at 121ºC for 15min. The inoculum size was 4% (*v/v,* substrate). Sporulation SSF was carried out at 36ºC for 7 days. Static submerged fermentation was carried out to evaluate substrate sporulation induction using 150 mL of broth medium with the same culture conditions. The fermented material was dried in trays for 24 h at 65°C in an air oven. Spore detachments for counting were performed as previously described (SELLA *et al*., 2009). All spores' suspensions were filtered through cotton and gauze tissue and, subsequently, washed three times with cold, sterile 0.02 M calcium acetate solution adjusted to pH 9.7, by centrifugation at 2,500 rpm (1,048 × g) for 20 min at 4°C. Spores' suspensions kept in flasks were subjected to a heat shock (80°C, 10 min) and were stored at 4°C. 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°C. SSF yield based on substrate consumption was available by CFU/ g dry matter (CFU / g dried medium - support + substrate) and by CFU/ mL substrate (number of cells produced / substrate volume).

2.6 Recovery medium compatibility

This analysis was conducted to verify the influence of the recovery medium on the fermentation products. Ten milligrams of each dried fermented material (~ 10^6 CFU spores) was loaded into 7.0 mL borosilicate glass vials, closed with silicone rubber stoppers, and sealed with aluminum seals. Forty vials for each support were dry-heat sterilized for 2 h at 160ºC. After cooling, the vials were filled with 3.0 ml of the recovery medium (3.0% TSB, 0.018% $CaCl_2.6H_2O$, 0.1%soluble starch and 0.002% bromothymol blue). Compatibility was determined by a no color change in the medium or by a no detection of turbidity after exposure to 4ºC for up to 30 days, or exposure to 36ºC for up to 7 days. Bromothymol blue is a pH indicator that gives a green color to the recovery medium in a neutral pH environment. Chemical changes were differentiated from spore germination or contamination by inoculation of recovery medium samples in TSB medium, followed by Gram staining. Viability was determined before and after sterilization.

2.7 Microtiter plate biofilm assay

Biofilm formation was evaluated using the assay described by Merritt *et al.* (2005). The inoculum was obtained by growing *B. atrophaeus* spores [unattached to sand, vermiculite, sugarcane bagasse or agar (control)] in TSB medium until the stationary phase (18 h) and then diluting the cells (1:100) in the respective media. Samples of 100 µL were grown in four wells in sterile PVC 96-well microtiter plates. The negative control wells contained media only. The plate was covered by a lid and incubated at 36ºC for 24 h. Growth medium and non-adherent cells were removed by shaking out the plate over a waste tray, and plates were then rinsed in tap water. Adherent cells were stained with a 0.1% crystal violet solution (CV) for 10 min at room temperature. Excess CV was then removed and the wells were rinsed in water. After drying, 200 µL of 33% (*v/v*) glacial acetic acid was added to the wells to dissolve the attached and colored cells. Biofilm formation was measured by determining the optical density of each well at 570 nm.

2.8 Scanning electron microscopy

For scanning electron microscopy (SEM), air-dried (65ºC - 24 h) sand fermented samples were fixed with 3% glutaraldehyde in cacodilate buffer (0.1 M, pH 7.0) for a minimum of 24 h, rinsed in the same buffer, and then dehydrated in an ethanol series (70%, 90% and 100%) (Merck) and propylene oxide. Samples were then mounted in stubs and metalized with gold in an argon atmosphere and under vacuum system before being analyzed on a JEOL JSM-6360LV electron microscope. Spore lengths and widths (micrometers) were measured, and the data presented are the average of over 100 spores.

2.9 Sterilization resistance

Dry-heat sterilization was carried out at 160ºC for 25 min as a partial cycle time. Ethylene oxide sterilization was performed using 650 mg/L at 55ºC, 55% humidity, with a partial cycle time of 15 min. *D*-values (decimal reduction time) were calculated based on logarithmic curve inactivation, after determining the number of surviving spores.

2.10 General conditions and statistical analysis

Experiments were carried out in a good manufacturing practices certified laboratory, in triplicate and performed in aseptic conditions in an ISO 5 clean room (ISO 14644-1, 1999). Experimental designs and analyses were carried out using Statistic 8.0 software (Stat Soft) and the SGWIN program (Stat Graphic Plus for Windows version 5.0, Statistical Graphics, 2000). The results were subjected to analysis of variance (ANOVA), and differences were considered significant when $p<0.05$. Cost production analysis was performed based on the production of one lot of 50,000 units, each BI having 5.0×10^6 CFU spores by comparison with the usual method of BI production (sporulation on agar). Costs took also into account each usual media component compared to the costs of media formulated with alternative

supports and carbon sources.

3. RESULTS

3.1 Evaluation of substrates and supports for sporulation production

The properties of the inert supports, sugarcane bagasse, sand and vermiculite were compared with respect to the production of bacterial spores by SSF. Vermiculite had an approximately neutral pH, while sand and sugarcane bagasse had a slightly acidic pH (TABLE1). The mineral compounds did not produce acid products upon sterilization, a highly desirable factor to avoid final pH adjustments before inoculation, or to avoid addition of a buffering component to the basal medium.

TABLE 1 - COMPARISON OF SUGARCANE BAGASSE, SAND, AND VERMICULITE REGARDING PROPERTIES RELATED TO BACTERIAL SPORE PRODUCTION BY SSF

Support/ Assay	Sugarcane bagasse	Sand	Vermiculite
pH before sterilization	5.2± 0.1	5.1± 0.1	6.1± 0.1
pH after sterilization	4.4 ± 0.1	5.1± 0.1	6.1± 0.1
Moisture (%)	4.7± 0.2	0.2 ± 0.1	5.5± 1.4
a_w	0.314	0.716	0.708
Water absorption (mL/g)	6.6	0.26	5.5

All three supports and both substrate culture media allowed *B. atrophaeus* spore production (TABLE 2). The spore production results were analyzed according to substrate and support productivity. The ANOVA of the substrate spore productivity (CFU per milliliter of substrate in different supports) demonstrated an *F*-ratio of 7.0343 and the *p*-value of the *F* test was 0.007 (<0.05) indicating that there is a statistically significant difference between the averages of the tested formulations. To determine which means are significantly different from each other, the multiple range test was applied, and it was demonstrated that the complex substrate with sugarcane bagasse as support (assay 4) and industrialized and complex substrates

with sand as support (assays 5 and 6) showed the best results and that there were no statistically significant differences among these assays.

TABLE 2 - COMPARISON OF THE SOLID STATE FERMENTATION PRODUCTION OF *B. atrophaeus* SPORES USING SUGARCANE BAGASSE, SAND AND VERMICULITE AS INERT SUPPORTS AND INDUSTRIALIZED OR COMPLEX SUBSTRATES

Assay	Support	Substrate Medium (type)	Spores (CFU/mL substrate)	Standard Deviation	Spores (CFU/g dry matter)	Standard Deviation
1	(1)	Industrialized	2.0×10^8	2.8×10^8	-	-
2	(1)	Complex	7.4×10^7	3.7×10^7	-	-
3	Sugarcane bagasse	Industrialized	8.1×10^6	4.9×10^5	5.2×10^7	3.5×10^6
4	Sugarcane bagasse	Complex	4.9×10^8	1.4×10^7	3.2×10^9	7.1×10^7
5	Sand	Industrialized	4.1×10^8	2.1×10^7	1.1×10^8	7.1×10^6
6	Sand	Complex	3.9×10^8	7.1×10^6	9.9×10^7	1.4×10^6
7	Vermiculite	Industrialized	7.6×10^7	1.6×10^7	4.0×10^8	7.8×10^7
8	Vermiculite	Complex	4.0×10^7	2.8×10^6	2.1×10^8	1.4×10^7

(1) submerged fermentation

ANOVA testing of spore productivity (CFU per gram of dry matter) using the different supports demonstrated an *F* ratio of 1573.54 and the *p* value of the *F* test was 0.000 (<0.05) indicating that there was a statistically significant difference between the averages of the tested formulations. The multiple range test was applied, and it was demonstrated that sugarcane bagasse as support with complex medium as substrate showed the best results. There were no statistically significant differences among the others assays. Based on these results, a complex medium was chosen to continue the studies.

3.2 Evaluation of supports for compatibility with recovery medium and BI production

The results of the viability test showed that all the dried fermented materials studied promoted spore germination and outgrowth when cultivated with the recovery medium (TABLE 3). Dry-fermented material of sugarcane bagasse

presented the disadvantage of absorbing part of the recovery medium and promoting its color change to yellow due its acidification.

TABLE 3 - COMPARISON OF DRIED SSF PRODUCT VIABILITY AND INCOMPATIBILITY WITH RECOVERY MEDIUM

Test/ Support	Viability		Incompatibility	
	Before sterilization	After fermented sterilization	4ºC	36ºC
Sugarcane bagasse	100% growth	No growth	pos	pos
Sand	100% growth	No growth	neg	neg
Vermiculite	100% growth	No growth	neg	neg

neg = no medium color change or turbidity; pos = medium color change or turbidity

3.3 Bacterial adhesion on supports and biofilm formation

In this study, bacterial adhesion and surface colonization on the support surface was demonstrated by the adherence of SSF germinated spores throughout the microtiter plate. The results of this assay confirmed the microorganism's ability to form biofilm. Sand proved to be the best biofilm inductor among the tested supports, facilitating support colonization and achieving a high cell density before sporulation (FIGURE 2).

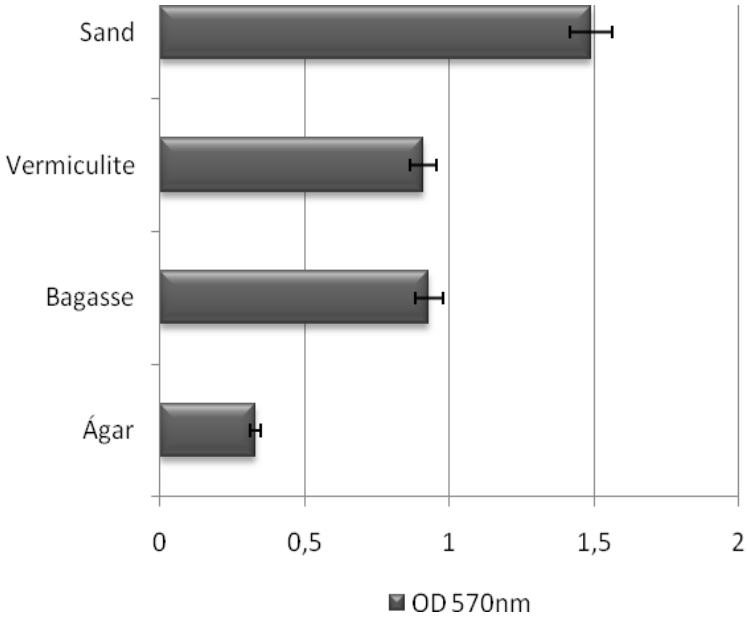

FIGURE 2 - BIOFILM ASSAY OF *B. atrophaeus* SPORES PRODUCED BY SSF ON DIFFERENT SUPPORTS AND GERMINATED IN TSB MEDIUM

SOURCE: The author (2011)

SEM was used to examine spore morphology and distribution on each support. The presence of spores on all exposed surfaces of sand was confirmed by SEM analysis (FIGURE 3). Size distributions from several large populations of spores (~100) were determined to investigate how SSF conditions influence spore size. Both spore width and length were measured and compared with data in the literature. Measurements of 1.6 ± 0.2 µm for length and 0.8 ± 0.1 µm for width are close to those found by Yang *et al.* (2010) - 1.5-1.8 µm for length and 0.8 µm for diameter.

3.4 Spore quality

The quality of produced spores was evaluated according to sterilization resistance. Aiming the production of a multi-use BI, the produced spores were

submitted to both challenges: dry-heat and ethylene oxide. Microbial inactivation by ethylene oxide and dry-heat sterilization has been considered to follow first-order kinetics, and the *D* value refers to the time required, under certain sterilization conditions, to kill 90% of the organisms. The dry heat resistance $D_{160°C}$ value and ethylene oxide D_{EO} values were 6.6 ± 0.1 min, and 6.5 ± 0.1 min, respectively. All results were in excess of the established characteristics of typical commercially supplied bioindicator systems: $D_{160°C}$ from 1.0 to 3.0 min and D_{EO} from 2.5 up to 5.8 min for 1.0 x 10^6 to 5.0 x 10^7 spores/unit (USP 31, 2008). This indicates that to produce a BI within these suggested *D*-values it is necessary to dilute the fermented material, thus improving productivity.

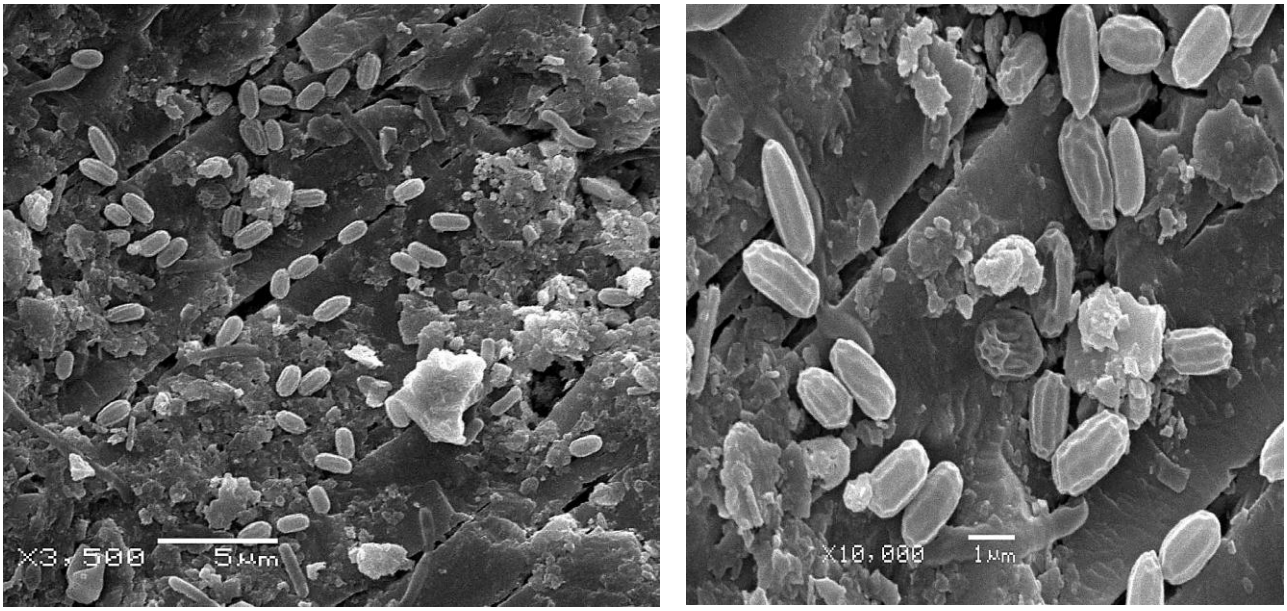

FIGURE 3 - SCANNING ELECTRON MICROGRAPHS OF *B. atrophaeus* SPORES ON SAND SURFACES AFTER SSF PRODUCTION USING SAND AS SUPPORT AND COMPLEX SOYBEAN MOLASSES MEDIUM AS SUBSTRATE

SOURCE: The author (2010)

3.5 Comparative production cost analysis

The labor cost, which is the greatest contributor to the cost of BI production, was reduced by approximately 49% with the new proposed process. Quality control

tests, the second highest cost, were reduced by approximately 48%, while raw materials costs were reduced by 78%. The process cycle time was reduced from 25 days to 18 days.

4. DISCUSSION

The comparison of the physical properties among the three studied supports showed that sugarcane bagasse has the highest water retention capacity, followed by vermiculite and sand. High-water retention could have a positive impact on bacterial biomass production; however, low water retention may reduce the drying process time.

High-yield spore production was expected for sugarcane bagasse, which has been well explored by others (SOCCOL; VANDENBERGHE, 2003; SELLA *et al.*, 2009). Vermiculite also allowed microbial growth due to its plate-like multi-laminar structure that holds large quantities of water and positively charged nutrients and allows air circulation to enable microbial growth (MEISINGER, 1984). The dry sand water adsorption should be described as a film formation around the grains. This film is as thin as the empty spaces between grains, producing what is called ‘swelling’, where microorganisms can grow.

A good production was observed using submerged fermentation, which used the same SSF substrates as growth media, indicating the capacity of the substrates to induce sporulation. Although the objective of this work was not the comparison between SSF and submerged fermentation processes, some aspects deserve discussion. Initially, submerged fermentation production was thought to be more advantageous. However, this is not true because in standing cultures spores are formed at the air-liquid interface, a floating biofilm. Therefore, spore production is associated with surface area and not the volume of the nutrient medium. Raman and Ano (2009) described the same observation for a lipopeptide antibiotic production by *Bacillus subtilis.* In this case, a suitable reactor design allowing a large surface fermentation area was necessary to achieve cost-effective spore production. Even though some studies cite that good sporulation can be obtained for different *Bacillus* species in agitated and aerated media, this method of production would not be

applicable to the production of BIs because of the introduction of new costs to the process. Furthermore, spores produced in liquid media have less resistance to sterilizing agents (ROSE *et al.*, 2007). The advantages of the use of soybean based medium as substrate for BI production have been previously explained by SELLA *et al.* (2009) and it use was possible due *B. atrophaeus* ability by using complex sugars as carbon source.

The first problems observed in the direct use of the fermented material in BI production began during the pre-filling homogenization: (a) Due to its non-homogeneous shape and low relative density, a large volume of sugar cane bagasse per vial was needed to achieve the desired 10^6 CFU/unit; (b) vermiculite manipulation destroyed part of its laminar physical property; (c) expanded vermiculite should not be pressed or compacted because this could lead to a heterogeneous spore distribution in the vials. Vermiculite proved not to be a suitable option for BI production, because its manipulation requires some care to avoid its disintegration, which means higher costs. Sand was the best inert support to enable the direct use of fermented material in BI production due to its easy homogenization, filling properties and recovery medium compatibility (FIGURE 4). No previous studies have been published on the application of sand as an SSF support for *B. atrophaeus* sporulation.

The knowledge of how and where spores are located on a support is of fundamental importance to predict the access of the sterilizing agent, especially if the agent is in gaseous form. *Bacillus* spore adhesion to inert surfaces has been described by Rönner *et al.* (1990) who observed that spores generally adhered to a greater extent to hydrophobic and hydrophilic surfaces than did vegetative cells. Gutiérrez-Correa and Villena (2003) showed that the one of the advantages of solid state fermentation is related to the adhesion of microorganisms to solid particles. Bacterial adhesion to sand was described by Zhu and Chen (2005) who concluded that surface colonization of abiotic surfaces was correlated to physicochemical properties of bacterial surfaces and was determined by their interaction free energies. Although it is known that biofilm cultures have advantages over suspension cultures, such as high cell concentrations achieved without washout leading to high productivities, *B. atrophaeus* biofilm formation on SSF supports related to high spore production has not yet been described.

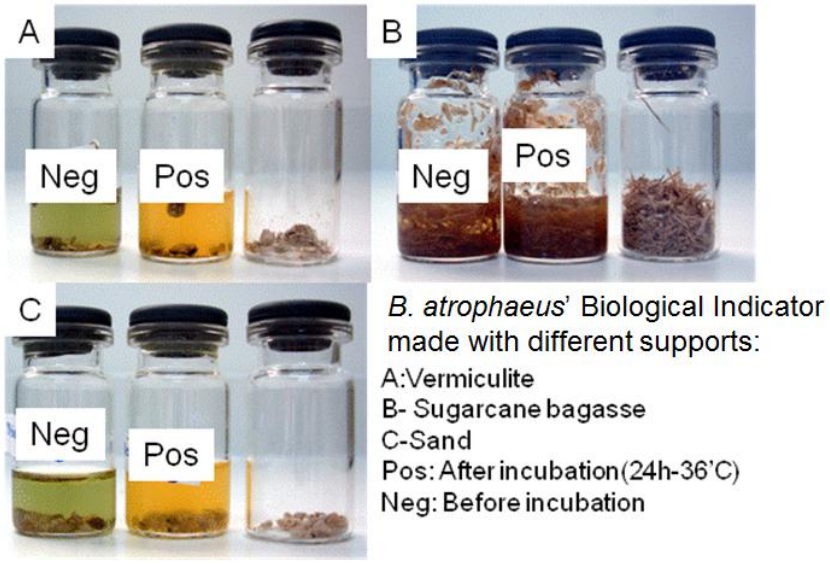

FIGURE 4 - *B. atrophaeus* BIOLOGICAL INDICATOR MADE WITH DIFFERENT SUPPORTS: (A) VERMICULITE, (B) SUGAR CANE BAGASSE, AND (C) SAND

SOURCE: The author (2011)

Environmental and physiological factors during sporulation can have significant effects on spore dimensions and structure. Scanning electron microscopy demonstrated that the culture conditions didn't alter spores size (width and length) and that all visible cells were located on exposed sites of the mineral grain and not entrapped in holes or dents, theoretically permitting access for the sterilizing agent.

The spores quality and process costs analysis confirmed that the production of *Bacillus atrophaeus* spores by SSF using sand as a low cost support is viable and successful. Sand is a very inexpensive, widely available, inert and non-toxic dry material. This approach was successful because of the ability of spores to attach to sand and also because of bacterial adhesion and surface colonization through biofilm formation before sporulation. The present work focused on reducing down-stream processing steps, which was achieved by the direct use of the fermented material (FIGURE 5), reducing cost and process cycle time. The dry-heat and ethylene oxide resistance of the BIs produced with these spores were not affected by the SSF sporulation conditions. Further research is needed to complete evaluation of spore resistance in different sterilization processes. An optimization of the process is suggested.

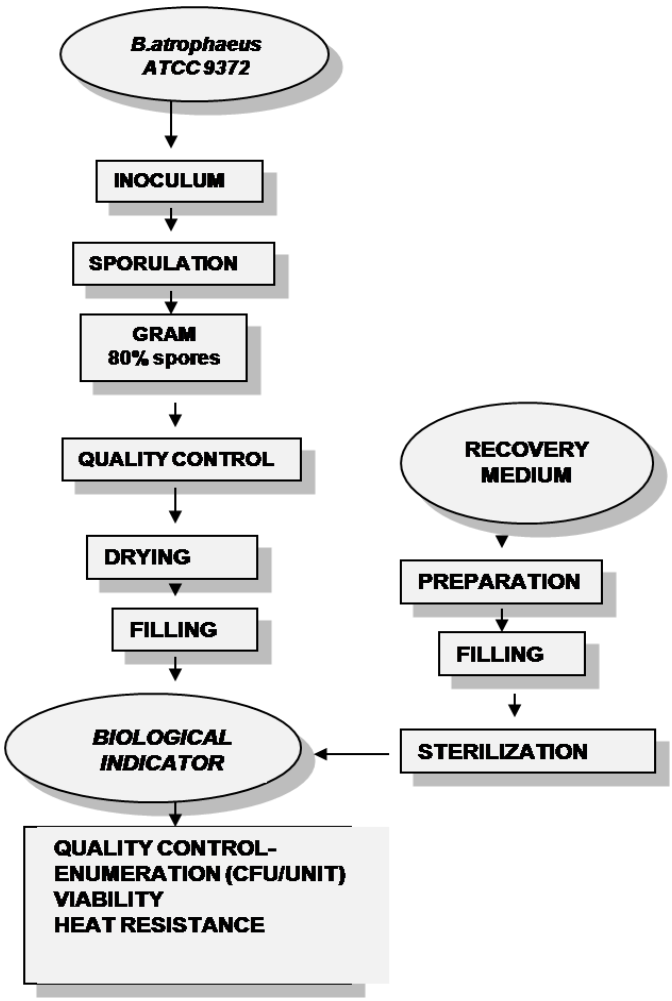

FIGURE 5 - FLOWCHART FOR THE PROPOSED PROCESS OF BIOLOGICAL INDICATOR PRODUCTION USING DRY-FERMENTED SAND SPORES

SOURCE: The author (2011)

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CHAPTER III

DESIGNING AND PERFORMANCE EVALUATION OF A SOYBEAN MOLASSES-BASED BIOINDICATOR SYSTEM FOR MONITORING STERILIZATION PROCESS

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ABSTRACT

A novel cost-effective *Bacillus atrophaeus* Sterilization Bioindicator System (BIS) with high quality and performance was developed from a soybean by-product and compared with commercial BIS. It was composed of recovery medium and dry-fermented spores with sand as support. The BIS was developed and optimized using a sequential experimental design strategy. The recovery medium contained soluble starch (1.0 g/L), soybean molasses (30.0 g/L), tryptone (40.0 g/L), and bromothymol blue (0.02 g/L) at pH 8.5. The solid-state fermentation conditions of bioreactor and environmental humidity had no significant effects on spore yield and dry-heat resistance. The only substrate mineral that showed a positive effect was Mn^{2+}, allowing Mg^{2+}, K^{+}, and Ca^{2+} to be eliminated from the formulation. Validation of optimized medium indicated $D_{160°C}$ = 6.8 ± 1.0 min (3.6 min more than the minimum) and spore yield = 2.3 ± 0.5 × 10^9 CFU/g dry sand (10,000 × initial values). BIS performance was evaluated and had $D_{160°C}$ = 6.6 ± 0.1 min. Sporulation and germination kinetics allowed the sporulation process to be reduced to three days, and the growth of heat-damaged spores was sufficient to achieve visual identification of a non-sterile BIS within 21 hours. Process economics was a minimum of 23.9% and process cycle time was reduced from 29 days to 15 days. The new BIS parameters demonstrated compliance to all regulatory requirements. No studies have yet described a BIS production from soybean molasses.

Keywords *Bacillus atrophaeus*; solid-state fermentation; bioindicator system; soybean molasses; recovery medium, sterilization

1. INTRODUCTION

A bioindicator (BI) for sterilization is a device intended for use by health care providers or quality control staff to accompany products through sterilization process.

It consists of a known number of microorganisms, of a known resistance to the mode of sterilization, on a carrier and enclosed in a protective packaging. Subsequent growth or failure of the microorganisms to grow under suitable conditions indicates the adequacy of the sterilization (FDA, 2007). Bioindicators must be considered as a system, with the spore characteristics, spore carrier, packaging, and recovery medium all contributing to its effectiveness.

Bacillus atrophaeus (formerly called *Bacillus subtilis* var. *niger*) spores are resistant to sterilization using ethylene oxide, low-temperature steam, dry heat, plasma, U.V. radiation, electrothermal deactivation (ETD), and microwaves (HALFMANN *et al.,* 2007; ISO- 11138-4, 2006; OLIVEIRA *et al.,* 2010; ROTH *et al.,* 2010; TURNBERG *et al.,* 1996). Thus, *B. atrophaeus* is a suitable BI to test the effectiveness of these sterilization and disinfection processes. These spores are also used as indicators to assess the efficiency of medical waste, water, and wastewater treatment systems (OLIVEIRA *et al.,* 2010; SZABO *et al.,* 2007) and as a surrogate for *Bacillus anthracis* in biodefense assay (WEBER *et al.,* 2003).

The world annual production of soybean was estimated at ~264 million tons in 2010/2011, and Brazilian production represents around 30% of global production (HIRAKURI; LAZZAROTTO, 2011). Soybean molasses is a co-product from the manufacture of protein-concentrate soybean meal. Its main constituents are sugars (~57% carbohydrates, consisting of sucrose ~28%, stachyose ~19%, and raffinose ~10%) and other components, such as proteins (~9%) and lipids (~21%), wich are extracted from the de-oiled biomass (SIQUEIRA *et al*., 2008). Solid-state fermentation (SSF) using soybean molasses as the substrate was first described by Sella *et al.* (2009a) as process with a high-yield of *B. atrophaeus* spores. SSF makes the spores at lower cost than other processes, especially in developing countries. Sand was used as an inert support to enable the direct use of fermented material in BI production due to its easy homogenization, filling properties, and recovery medium compatibility. BI growth on sand particles occurs by bacterial adhesion and biofilm formation (SELLA *et al*. 2012)**;** because biofilms are very difficult to eradicate, they are advantageous for BI production. No studies have yet described the effects of the SSF process variables on *B. atrophaeus* spore yield and heat resistance. This knowledge is fundamental to improve and standardize the spore-production process.

The recovery medium significantly influences germination and growth of the spores, and thus, it can have profound effects on evaluation of the resistance of the spores to the sterilization process. Several research groups have reported that heated spores showed different levels of viability when cultured on different formulations of recovery media (PFLUG *et al.,* 1981; SHINTANI; AKERS, 2000). Others have reported that the *D*-value (time taken for inactivation of 90% of spores) was dependent on the recovery medium (LÓPEZ *et al.,* 1997; SASAKI *et al*., 2000). The nutritional composition of the culture medium should allow the organism to repair sub-lethal defects caused by the sterilization treatment. It should enable spore germination and a sufficient level of multiplication for the identification of the non-sterile BI. The use of soybean molasses as for *B. atrophaeus* inoculum medium has been reported previously (SELLA *et al*., 2008). However, its use in a standardized culture medium for germination of spores has not been reported elsewhere.

The objective of this study was to develop a cost-effective biological indicator system (BIS) and to compare the performance of the developed products (spores plus recovery medium) with that of a reference BIS. Ultimately, the new BIS is intended to replace the currently-used commercial BI.

2. MATERIALS AND METHODS

This study was executed in three steps: a) development and optimization of the recovery medium; b) development and optimization of spore production by SSF; and c) performance evaluation of the developed IBS by the comparing sporulation and germination kinetics, as well as resistance characteristics and the process costs, with a commercial BI.

2.1 Bacterial strain and soybean molasses characteristics

Bacillus atrophaeus ATCC 9372 (batch: 1403349) was obtained from the standard strain supplied by Instituto Nacional de Controle de Qualidade em Saúde (INCQS/MS, Rio de Janeiro, Brazil). Soybean molasses (80° Brix) was obtained

from the IMCOPA Company (Araucaria-Pananá, Brazil). It was composed of 508.0 ± 18.0 g/L total sugars, 51.0 ± 2.0 g/L protein, 3.0 ± 0.06 g/L lipids, and 123.0 ± 1.0 water, pH 6.1 ± 0.1.

2.2 Development and optimization of the BI recovery medium

The recovery medium was developed and optimized as described below.

2.2.1 Screening of significant variables for recovery medium development

A Plackett-Burman design was used to evaluate the effects of medium components on the main BIS property: heat-resistance. The variable factors (concentrations of soybean molasses, tryptone, casein peptone, yeast extract, soluble starch, $CaCl_2$, bromothymol blue, and pH) were examined at two coefficient levels, low (-) and high (+), using a set of orthogonal contrasts. Central points were investigated in triplicate to identify any experimental anomalies. For the viability assay, germination and growth of the spores (10 units) were observed over 24h – 48 h incubation time, and positive results were indicated by changes in the color (green to yellow) and turbidity of the recovery medium. The medium stability was determined by observing changes in color or turbidity during incubation at 36°C for 7 days; in this case, positive results were indicated by a no change in the color (green). The design and results are shown in TABLE 1.

2.2.2 Recovery medium optimization

The medium was optimized using two central composite designs (CCD): a) 2^3 full-factorial CCD was used to optimize the three main components of the media: yeast extract, tryptone, and soybean molasses. Independent factors were studied at five different levels with two replications at the center points (16 experiments in total). The amounts of the main components were as follows: 20 g/L – 50 g/L

soybean molasses, 15.0 g/L - 45.0 g/L tryptone, and 15.0 g/L - 45.0 g/L yeast extract. The concentrations of soluble starch (1.0 g/L) and bromothymol blue (0.02 g/L) were fixed, and the pH was adjusted after sterilization with 0.1N sterile NaOH, until the color of the medium changed to green. The design and results are shown in TABLE 2.

TABLE 1 - PLACKETT–BURMAN DESIGN MATRIX WITH UNCODED VALUES AND EXPERIMENTAL RESULTS TO INVESTIGATE THE EFFECTS OF THE RECOVERY MEDIUM COMPOSITION ON *Bacillus atrophaeus* SPORE HEAT RESISTANCE

	Variables								Results		
Assay	Soybean molasses (g/L)	Casein peptone (g/L)	Tryptone (g/L)	Yeast extract (g/L)	Calcium chloride (g/L)	Soluble starch (g/L)	BB (g/L)	pH	Stability	Viability (%)	$D_{160°C}$ (min)
1	45.0	0	16.0	0	0	0	0.04	9.1	+	100	6.6 ± 0.2
2	45.0	50.0	0	50.0	0	0	0.02	9.1	+	0	0
3	15.0	50.0	16.0	0	0.04	0	0.02	8.3	-	-	0
4	45.0	0	16.0	50.0	0	2.0	0.02	8.3	+	100	6.6 ± 0.2
5	45.0	50.0	0	50.0	0.04	0	0.04	8.3	+	100	5.6 ± 0.3
6	45.0	50.0	16.0	0	0.04	2.0	0.02	9.1	+	0	0
7	15.0	50.0	16.0	50.0	0	2.0	0.04	8.3	+	0	6.7 ± 0.1
8	15.0	0	16.0	50.0	0.04	0	0.04	9.1	+	10	0
9	15.0	0	0	50.0	0.04	2.0	0.02	9.1	+	0	0
10	45.0	0	0	0	0.04	2.0	0.04	8.3	-	-	0
11	15.0	50.0	0	0	0	2.0	0.04	9.1	+	0	0
12	15.0	0	0	0	0	0	0.02	8.3	-	-	0
13(CP)	30.0	25.0	8.0	25.0	0.02	1.0	0.03	8.7	+	100	6.6 ± 0.2
14(CP)	30.0	25.0	16.0	25.0	0.02	1.0	0.03	8.7	+	100	6.6 ± 0.1
15(CP)	30.0	25.0	16.0	25.0	0.02	1.0	0.03	8.7	+	100	6.6 ± 0.2
CM	-	-	-		-	-			+	100	6.7 ± 0.1
Effect	3.150	-0.920	3.150	-1.290	-0.920	0.950	-0.950	1.280			
F-ratio	2.520	0.210	2.520	0.420	0.210	0.230	0.230	0.420			
p-value	0.163	0.660	0.163	0.541	0.660	0.649	0.649	0.541			

R^2=72.4%

For media with negative stability (-) or viability (0) results, $D_{160°C}$ was considered = 0.
BB= Bromothymol blue; CM= control medium; CP=Center Point.

b) A 2^2 full-factorial CCD was used to determine the optimal pH conditions (before medium sterilization) and soluble starch concentration. Each variable was tested at five levels with four axial points and two replications at the center points (10 experiments in total). The concentrations of soybean molasses and tryptone were fixed based on the results of the first CCD (TABLE 3)

TABLE 2 - A 2^3 FULL-FACTORIAL CENTRAL COMPOSITE DESIGN WITH UNCODED VALUES AND EXPERIMENTAL RESULTS FOR OPTIMIZATION OF *Bacillus atrophaeus* BIOLOGICAL INDICATOR RECOVERY MEDIUM

Assays	Variables			Results		
	Soybean molasses (g/L)	Tryptone (g/L)	Yeast extract (g/L)	Stability	Viability (%)	$D_{160°C}$ (min)
1	20.0	15.0	15.0	+	100	6.7 ± 0.1
2	20.0	15.0	45.0	+	100	5.6 ± 0.1
3	20.0	45.0	15.0	+	100	6.7 ± 0.1
4	20.0	45.0	45.0	+	100	3.7 ± 0.2
5	50.0	15.0	15.0	+	100	5.1 ± 0.2
6	50.0	15.0	45.0	+	100	3.8 ± 0.2
7	50.0	45.0	15.0	+	100	6.7 ± 0.1
8	50.0	45.0	45.0	+	0	0
9	10.0	30.0	30.0	+	100	5.1 ± 0.2
10	60.0	30.0	30.0	+	0	0
11	35.0	5.0	30.0	+	0	0
12	35.0	55.0	30.0	+	100	5.6 ± 0.2
13	35.0	30.0	5.0	+	100	6.7 ± 02
14	35.0	30.0	55.0	+	100	5.1 ± 0.3
15(CP)	35.0	30.0	30.0	+	100	6.6 ± 0.1
16 (CP)	35.0	30.0	30.0	+	100	6.6 ± 0.1
CM	-	-		+	100	6.6 ± 0.2
Effect	-3.021	0.779	-1.434			
F-ratio	8.360	0.550	1.870			
p-value	0.028	0.485	0.220			
R^2= 75.93%						

For media with negative stability (-) or viability (0) results, $D_{160°C}$ was considered = 0.

CM= control medium; CP=Center Point

2.3 Development and optimization of spore production

The fermentation process for spore production was developed and optimized as described below.

2.3.1 Fermentation process

Solid-state fermentation was carried out in 18 × 8 × 0.5 cm (length × width × height) trays, with the initial moisture 21.0 ± 1.0% and water activity (a_w) 0.0997 ± 0.0002. Sand washed once in tap water, twice in distilled water, and dried in trays for

24 h at 90°C in an air oven was utilized as inert support. The silica sand-quartz used was commercial grade and classified as medium size particles (0.4 mm – 2.0 mm). The substrate consisted of 20.0 g/L soybean molasses, supplemented with: 0.05 g/L $K_2HPO_4.H_2O$, 0.04 g/L $MnSO_4.4H_2O$, 0.04 g/L $CaCl_2.6H_2O$, and 0.05 g/L $MgSO_4.7H_2O$. Inoculums were produced in soybean molasses based medium (40.0 g/L tryptone and 40.0 g/L soybean molasses inoculated with 100 µL of 10^9 CFU/mL spore suspension) and incubated for 18 h at 36°C. Fermentation was carried out at 36°C in a climate-controlled chamber under circulating air (which maintained a uniform temperature) and after 7 days of incubation the fermented material was dried in trays for 24 h at 65°C in an air oven. Spore detachment for counting was performed as previously described (SELLA *et al*., 2009a). SSF yield was measured as CFU/g dry matter. For pH determination before and after sterilization, 1.0 g sample was diluted with 9.0 mL distilled water, blended for 2 min, and then centrifuged. The supernatant was used for the assay. Moisture content was determined gravimetrically by drying the sample in an oven at ~105°C for 24 h. Water activity measurements were determined at 19°C - 20°C using an Aqua Lab CX-2 water activity meter (Decagon Devices, Pullman, WA, USA).

TABLE 3 - VARIABLES AND RESULTS OF THE COMPLEMENTARY 2^2 FULL-FACTORIAL CENTRAL COMPOSITE DESIGN FOR DEVELOPMENT OF *Bacillus atrophaeus* RECOVERY MEDIUM

Assays	Variables		Results		
	Soluble starch (g/L)	pH	Stability	Viability (%)	$D_{160°C}$ (min)
1	1.7	8.0	-	100	0
2	1.7	9.0	+	100	4.7 ± 0.3
3	1.0	7.8	+	100	4.7 ± 0.2
4	2.0	8.5	-	100	0
5	1.0	9.2	+	100	4.7 ± 0.1
6	0	8.5	+	100	4.7 ± 0.3
7	0.3	8.0	-	100	0
8	0.3	9.0	+	100	0
9(CP)	1.0	8.5	+	100	6.6 ± 0.2
10 (CP)	1.0	8.5	+	100	6.5 ± 0.1
CM	-	-	+	100	6.6 ± 0.1
Effect	-0.487	1.175			
F-ratio	0.060	0.370			
p-value	0.814	0.576			

R^2= 59.09%. For the medium that results negative (-) stability or viability=0, $D_{160°C}$ was considered =0; CM= control medium; CP=Center Point

2.3.2 SSF optimization

A series of experimental designs studies were conducted to elucidate the effects of particle size, bed height, environmental humidity, pH, inoculum size, and bioreactor on spores yield, as follow:

(a) *Particle size and bed height.* A 3^2 fractional factorial experimental design, with two factors and three levels, was used to study the effects of particle size (0.4mm – 1.6 mm) and bed height (0.5 cm – 1.5 cm), generating nine experiments. The design and results are shown in TABLE 4.

(b) *Substrate pH, bed height, incubation time, and environmental humidity.* A mixed two- and three-level experimental design was used to study the effects of substrate pH before sterilization (7.2 – 8.0), bed height (0.5 cm – 3.5 cm), incubation time (7 d and 10 d), and environmental humidity (100% and uncontrolled), generating 24 experiments. The design and results are shown in TABLE 5.

(c) *Bed height, inoculum size, and bioreactor type.* A 2^2 full-factorial CCD was used to determine the optimal bed height (2.0 cm – 5.0 cm) and inoculum size (4% – 12%). Each variable was tested at five levels with four axial points and two replications at the center points, generating 10 experiments in total (TABLE 6). To verify the influence of the bioreactor type, the optimized conditions also were tested in 20 L plastic-bag bioreactors and in 250 mL Erlenmeyer flasks.

TABLE 4 - A 3^2 FULL-FACTORIAL EXPERIMENTAL DESIGN AND RESULTS FOR MEDIUM PARTICLE SIZE AND BED HEIGHT EFFECTS ON *Bacillus atrophaeus* SPORE PRODUCTION BY SOLID-STATE FERMENTATION ON SAND

Assays	Variables		Results
	Particle size (mm)	Bed height (cm)	Spores (CFU/g dry matter)
1	1.6	1.0	6.4×10^4
2	1.6	0.5	6.3×10^4
3	0.4	1.0	50
4	0.4	0.5	50
5	1.0	0.5	1.3×10^5
6	1.0	1.5	1.6×10^5
7	0.4	1.5	50
8	1.6	1.5	6.7×10^4
9	1.0	1.0	1.9×10^5
Effect	6.5×10^4	1.1×10^4	
F-ratio	0.460	0.000	
p-value	0.026	0.270	

R-squared = 97.2%

(d) *Mineral composition*. A Plackett-Burman design was used to evaluate the effects of medium mineral components on spore yield and dry-heat resistance: $K_2HPO_4.H_2O$ (0 g/L – 0.1 g/L), $MnSO_4.4H_2O$ (0 g/L – 0.08 g/L), $CaCl_2.6H_2O$ (0 g/L – 0.08 g/L), and MgSO4.7H_2O (0 g/L – 0.1 g/L). The variable factors were examined at two coefficient levels, low (-) and high (+), using a set of orthogonal contrasts. Central points were added to the design in duplicate (TABLE 7).

(e) *Determination of the optimum concentration of the medium components*

A 2^2 CCD was used to optimize the concentration of the two main components of the media: soybean molasses (5.0 g/L – 35.0 g/L) and manganese sulfate (0.04 g/L – 0.12 g/L). Each variable was tested at five levels with four axial points and two replications at the center points.

TABLE 5 - MIXED TWO - AND THREE- LEVEL EXPERIMENTAL DESIGNS AND RESULTS FOR *Bacillus atrophaeus* SOLID-STATE FERMENTATION SPORE PRODUCTION

Assays	Variables				Results		
	Substrate pH	Bed height (cm)	Incubation time (days)	Environmental humidity (%)	Spore yield (CFU/g dry matter)	Water activity (a_w)	Moisture content (%)
1	7.2	2.0	7	100	1.8×10^8	0.998	13.7 ± 0.5
2	7.2	3.5	7	100	6.4×10^7	0.994	8.4 ± 0.9
3	8.0	2.0	7	100	9.5×10^7	0.996	10.9 ± 0.8
4	8.0	3.5	7	100	3.1×10^7	0.996	9.7 ± 0.4
5	7.2	0.5	7	100	7.4×10^7	0.996	8.3 ± 0.3
6	8.0	0.5	7	100	4.4×10^7	0.997	12.5 ± 0.7
7	7.2	2.0	10	100	2.2×10^8	0.994	12.9 ± 0.1
8	7.2	3.5	10	100	3.4×10^8	0.997	11.1 ± 1.1
9	8.0	2.0	10	100	1.2×10^8	0.997	13.1 ± 0.1
10	8.0	3.5	10	100	5.9×10^7	0.996	8.4 ± 1.3
11	7.2	0.5	10	100	7.3×10^7	0.996	9.8 ± 0.4
12	8.0	0.5	10	100	1.2×10^8	0.995	9.6 ± 0.7
13	7.2	2.0	7	*	1.6×10^8	0.765	0.5 ± 0.3
14	7.2	3.5	7	*	1.2×10^8	0.740	1.7 ± 0.1
15	8.0	2.0	7	*	1.1×10^8	0.730	0.8 ± 0.1
16	8.0	3.5	7	*	1.5×10^8	0.791	1.9 ± 0.2
17	7.2	0.5	7	*	2.8×10^4	0.747	0.9 ± 0.1
18	8.0	0.5	7	*	3.6×10^6	0.665	0.3 ± 0.1
19	7.2	2.0	10	*	3.4×10^8	0.602	0.3 ± 0.1
20	7.2	3.5	10	*	1.4×10^8	0.575	0.6 ± 0.1
21	8.0	2.0	10	*	1.4×10^8	0.556	0.3 ± 0.3
22	8.0	3.5	10	*	4.5×10^8	0.545	0.3 ± 0.1
23	7.2	0.5	10	*	2.6×10^3	0.547	0.2 ± 0.1
24	8.0	0.5	10	*	2.4×10^7	0.556	0.4 ± 0.3

*Uncontrolled environmental humidity

TABLE 6 - VARIABLES AND RESULTS OF THE 2^2 FULL-FACTORIAL CENTRAL COMPOSITE DESIGN ASSAYS TO DETERMINE THE BED HEIGHT AND INOCULUM EFFECTS ON *Bacillus atrophaeus* SOLID-STATE FERMENTATION SPORE PRODUCTION

Assays	Variables		Results
	Bed height (cm)	Inoculum size (%)	Spores (CFU/g dry matter)
1 (CP)	3.5	8.0	2.5×10^8
2	2.0	12.0	1.6×10^8
3	2.0	4.0	4.5×10^8
4	5.0	12.0	4.2×10^8
5	5.0	4.0	6.6×10^7
6 (CP)	3.5	8.0	2.6×10^8
7	3.5	2.3	8.8×10^7
8	3.5	13.7	5.6×10^8
9	1.4	8.0	2.2×10^8
10	5.6	8.0	3.2×10^8

CP = center point

TABLE 7 - EXPERIMENTAL ASSAYS AND RESULTS OF THE PLACKETT-BURMAN DESIGN FOR THE EFFECTS OF MINERAL COMPOSITION ON *Bacillus atrophaeus* SOLID-STATE FERMENTATION SPORULATION MEDIUM

Assay	Potassium phosphate (g/L)	Manganese sulfate (g/L)	Calcium chloride (g/L)	Magnesium sulfate (g/L)	Spores (CFU/g dry matter)	$D_{160°C}$
1	0	0	0	0	9.5×10^7	3.4
2	0. 1	0	0	0. 1	1.3×10^8	4.5
3	0	0.08	0	0. 1	1.5×10^8	6.9
4	0. 1	0.08	0	0	1.5×10^8	6.9
5	0	0	0.08	0. 1	1.3×10^8	4.8
6	0. 1	0	0.08	0	9.9×10^7	4.6
7	0	0.08	0.08	0	1.4×10^8	3.3
8	0. 1	0.08	0.08	0. 1	9.8×10^7	5.9
9(CP)	0.05	0.04	0.04	0.05	1.2×10^8	5.4
10(CP)	0.05	0.04	0.04	0.05	1.3×10^8	5.5
p-value	0.078	0.033	0.099	0.066		

R^2=98.10%

2.4 BIS performance characteristics

To check the effectiveness of the developed formulation, three different BIS batches were evaluated using different lots of the raw material and of the commercial BIS.

Recovery medium: Performance was determined by spore growth viability and dry-heat resistance ($D_{160°C}$ value) after inoculation with *B. atrophaeus* commercial spore strips (CPPI, Piraquara, Brazil).

Fermented medium with spores: Biological indicators were prepared by diluting the dried fermented product with sterilized dry sand to obtain ~10^6 CFU spores/g and filling 7.0 mL borosilicate glass vials with 1.0 g of material. The loaded vials were closed with silicone rubber stoppers and sealed with aluminum seals. After the dry-heat resistance test, the vials were cultured with 3.0 mL of commercial recovery medium (CPPI, Piraquara, Brazil). Germination and growth of the spores were observed over 24 – 48 h incubation time.

Biological Indicator System: Dry-heat resistance was tested for our BIS, and a commercial BI system (CPPI, Piraquara, Brazil) was used as control.

2.4.1 Dry heat resistance test

The *D* value for a specified set of conditions is defined as the time taken for the spore population to decrease by 90%, or one order of magnitude. The *D* value was determined by fraction negative analysis, using the Limited Spearman–Kaber Method. Dry heat conditions were 160°C for 25, 30, 35, 40, 45, and 50 min in a tabletop air oven. The survival/kill times were determined by the formula suggested by the United States Pharmacopeia (USP 31, 2008).

2.4.2. Sporulation kinetics

Solid-state fermentation was carried out under the optimum conditions using plastic bags as bioreactors. For standard agar sporulation, the cultures were grown in Roux flasks containing 400.0 mL of sporulation medium: 8.0 g/L yeast extract, 4.0 g/L nutrient broth, 0.05 g/L $MnSO_4.4H_2O$, 0.05 g/L $CaCl_2.6H_2O$, and 30.0 g/L agar. Growth was monitored by total cell and spore counts. Time course results of spore formation were compared with sporulation on agar.

2.4.3 BI recovery kinetics

Unheated BI (spores in vials) and those that were subjected to sub-lethal heating (160°C for 25 min) were inoculated into the developed recovery medium and cultured for 24 h at 36°C. Growth was monitored by total cell and spore counts. The 160°C dry-heat exposure time of 25 minutes was calculated based on the optimized medium *D* value obtained for unheated spores as the time to kill ~50% of the initial population (10^6 spores).

2.4.4 Complementary analysis

Viable vegetative-cell and spore counts were done by serial decimal dilutions

in distilled sterile water. Fifty microliters of each dilution were inoculated onto a tryptone soy agar plate surface in duplicate. Plates were incubated overnight at 36°C. The pH was determined with a pH meter. The concentration of total sugars in the cell-free growth medium was determined using the Somogyi and Nelson method (NELSON, 1944). Specific sugar consumption profiles on SSF were observed by high performance liquid chromatography in a Varian HPLC (Varian, Palo Alto, USA) with a Shodex KS 801 column (Shodex, Munich, Germany), with a refraction index detector. The mobile phase was purified water at 70°C and a flow rate of 0.5 mL/min. The samples were centrifuged at 10,000 × *g* and filtered with 0.22 µm PVDF membranes, diameter 13 mm (Millipore, MA, USA). Oligosaccharides were identified by comparing retention times with known standards.

2.5 General conditions and statistical analysis

The assays were carried out in triplicate. Microbiological assays were conducted under aseptic conditions in a Good Manufacturing Practices (GMP) certified laboratory ISO 5 clean room (ISO 14644-1, 1999). Experimental designs and analyses were carried out using Statistic 8.0 software (Stat Soft, USA) and the SGWIN program (Stat Graphic Plus for Windows version 5.0, Statistical Graphics, USA). Analysis of variance was used to evaluate the model, and 95.0% was used as the significance level. The quality of the polynomial model equation was judged statistically using the coefficient of determination (R^2), and its statistical significance was determined by an *F* test. To validate the optimization of the medium composition, three tests were carried out under the optimized conditions and the results analyzed statistically. Commercial *B. atrophaeus* Biological Indicators (IB Calor Seco, CPPI, Piraquara, Brazil) were utilized as controls in all assays and for comparative results. The commercial BI samples (spore on strips plus recovery medium) had the following labeled characteristics*:* $D_{160°C}$ = 6.5 ± 0.2 min and initial number of spores $N_0 = 3.6 \pm 0.8 \times 10^6$ CFU/unit.

3. RESULTS AND DISCUSSION

3.1 Recovery medium development and optimization

Cell division and multiplication are required to identify a nonsterile BI, and visual detection of turbidity and/or metabolic pH alteration requires cellular metabolism and abundant growth of the microorganisms in the medium (GILLIS *et al.,* 2010). The recovery medium should provide all necessary conditions for nonsterile BI to germinate and grow. The effects of pH and the concentrations of soybean molasses, tryptone, casein peptone, yeast extract, soluble starch, $CaCl_2$, and bromothymol blue on spore growth (viability assay), stability, and heat resistance are shown in TABLE 1. As determined by analysis of variance (ANOVA), tryptone was the nitrogen source that contributed the most variation to spore heat resistance (*p*-value = 0.1632). The highest *p*-value for the independent variables was 0.6602 (calcium chloride and casein peptone). Due to their lack of statistical significance, these components were removed from the recovery medium. Calcium and soluble starch were reported to affect heat resistance and germination of *Bacillus* spores (LÓPEZ *et al.,* 1997; SASAKI *et al.,* 2000). Although appropriate levels of calcium are essential for germination of heat-damaged spores, the negative effects of calcium observed in the present study may have been due to the additional calcium present in the tested spore strips. High concentrations of calcium can result in the formation of calcium phosphate in the presence of phosphoric acid, which forms from potassium phosphate. Microorganisms cannot utilize calcium phosphate (DE VRIES, 2004). Phosphate ions are also usually available in the complex media made using agro-industrial by-products. Therefore, calcium phosphates are highly likely to form in such media.

Most commercial BI recovery media contain one or more sources of nitrogen, *e.g*., peptone from casein, soy peptone, or other extracts or digests. These compounds provide inorganic ions, purine and pyrimidine, essential vitamins, amino acids, and peptides that are necessary for growth. Nutritional analyses of soybean molasses showed that an additional organic nitrogen source was required to support the growth of microorganisms (SIQUEIRA *et al.,* 2008).

The optimum concentrations of soybean molasses (main carbon source) and nitrogen sources (tryptone and yeast extract) were determined. The results are shown in TABLE 2. Statistical analysis revealed that yeast extract had negative effects on the recovery of spores and that tryptone was in the optimal range. There

was no significant interaction between the two nitrogen sources (*p*-value = 0.9581). These results suggested that the yeast extract may be removed from the medium. The importance of optimized *Bacillus* growth media supplemented with an organic source of nitrogen were described by Puri *et al.* (2002) and Xiao *et al.* (2007). In this study, tryptone provided sufficient nitrogen for spore germination and growth.

The relationship between the experimental variables and the heat resistance of spores was plotted on a surface graph (FIGURE 1- UP) and fitted to a second-order polynomial equation describing the surface:

$$Z = -2.52 + 0.32\,X + 0.31\,Y - 0.0006\,X^2 - 0.0002\,XY - 0.005\,Y^2 \qquad (1)$$

Where $Z = D_{160°C}$, X = soybean molasses concentration, and Y= tryptone concentration.

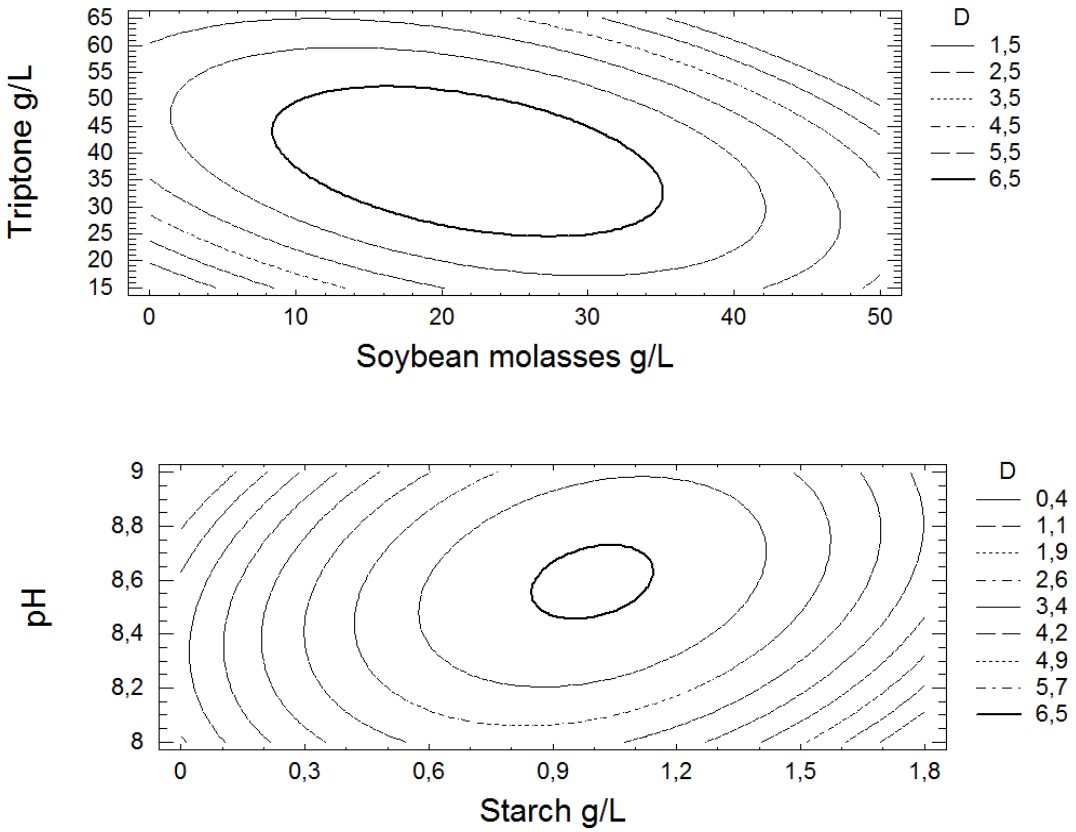

FIGURE 1 - CONTOURS OF ESTIMATED RESPONSE SURFACE PLOTS SHOWING THE EFFECTS OF SOLUBLE STARCH AND pH (DOWN), AND TRYPTONE AND SOYBEAN MOLASSES (UP) ON THE RECOVERY OF *Bacillus atrophaeus* SPORES, AS DETERMINED BY $D_{160°C}$

SOURCE: The author (2012)

Equation *(1)* was employed in the determination of the maximum predicted $D_{160°C}$-value. The result of $D_{160°C}$ = 6.9 ± 2.1 min was obtained for the medium containing 30.0 g/L soybean molasses, 35.0 g/L tryptone, and no yeast extract.

In the ANOVA of the pH and starch optimization, no significant effects were observed (*p*-value < 0.05) for the variables or their interactions. Starch in the growth medium has been reported to increase the value of the decimal reduction time. In such cases, the primary role of starch is not as a carbon source, but to bind toxic compounds that inhibit germination and cell development (MALLIDIS; SCHOLEFIELD, 1986; PENNA *et al*., 2000). The pH should be adjusted taking into account that the medium becomes more acidified after the soybean molasses are heated; determining the optimum pH of the medium before sterilization avoids contamination.

The response surface plots illustrate the behavior of the $D_{160°C}$ value (FIGURE 1-DOWN). Application of the RSM for the $D_{160°C}$ value gave the following regression equation, which is an empirical relationship between $D_{160°C}$ and the tested variables in a coded unit:

$$Z = -424.26 - 17.81\,X + 102.37\,Y - 5.54\,X^2 + 3.36\,XY - 6.15\,Y^2 \qquad (2)$$

Where $Z = D_{160°C}$, X = soluble starch concentration, and Y = pH.

The differential equation *(2)* was used to calculate the optimal parameters. The optimum values of the tested variables in coded units were X= 0.1 g/L and Y= 8.5 with the corresponding $D_{160°C}$ = 6.6 min. The predicted optimal concentrations of media components were as follows: 30.0 g/L soybean molasses (80° Brix), 40.0 g/L tryptone, 0.02 g/L bromothymol blue, 1.0 g/L soluble starch, and pH 8.5.

Finally, three tests were carried out under the optimized conditions to validate the model. The experimentally derived $D_{160°C}$ value was 6.6 ± 0.2 min. This was equal to the predicted $D_{160°C}$ value and to that obtained using the commercial recovery medium (control). The developed recovery medium was in compliance with the official standards for dry-heat sterilization BIS recommendation ($D_{160°C} \geq 3.0$ min) (FDA, 2007; ISO- 11138-4, 2006, USP 31, 2008).

3.2 Development and optimization of spore production

The BI industrial production also requires a process with high spore efficiency and good resistance to the specific sterilant. A clear relationship between sporulation conditions and spore yield and resistance is well described (PENNA *et al.*, 2003; ROSE et al., 2007; NGUYEN THI MINH *et al.*, 2011)

In the evaluation of sand particle size and bed height, the best initial spore yield was 1.9×10^5 CFU/g dry matter (TABLE 4) with 1.0 mm particle size and 1.0 cm bed height. The 0.4 mm particle size did not support sufficient sporulation for BI production. The ANOVA confirmed that the main effect of particle size was statistically significant (p-value = 0.0264). The results demonstrated that sand particles ≤ 0.4 mm in size negatively affected spore yield. Support properties are critical for the proper microbial growth in SSF. The negative effect of small particles (≤ 0.4 mm) on spore yield was explained by Pandey *et al.* (2001): smaller particles provide large surface area for microbial growth, but they may result in substrate agglomeration, interfering in microbial respiration and substrate diffusion and limiting microbial action.

The treatment means for the effects of substrate pH, bed height (up to 3.5 cm), incubation time, and environmental humidity on total spore formation, fermented final moisture content, and water activity are demonstrate in TABLE 5. Drastic a_w and moisture content reductions were observed from fermented cultivate with uncontrolled environmental humidity: from a_w = 0.9 to 0.7 with 7 days incubation time and from 0.9 to 0.6 with 10 days incubation time and initial moisture content = 21 ± 1% to 1.0 ± 0.8%. However, these effects were not observed for fermentation under 100% environmental humidity: a_w was maintained at 0.9 and the moisture content fell to only 11 ± 0.3%. The ANOVA results showed a statistically significant positive effect for bed height (p = 0.026) and no significant effects for the others variables or their interactions ($p \geq 0.050$). The role of water in an SSF system was extensively described by Gervais and Molin (2003). The low water-holding capacity of the sand (0.3 mL/g) associated with an uncontrolled environmental humidity reduced the moisture content of the medium and this limitation might decrease substrate diffusion and affect microbial growth. However, the water activity, which is related to solute concentration, capillary forces, and absorption properties of the insoluble substrate in

equilibrium with the air and the microorganisms, remained. This thermodynamic state during all processes avoided the negative influence on spores' properties, like germination capacity and heat resistance, as discussed by Nguyen Thi Minh *et al.* (2008). Thus, the environmental humidity need not be adjusted or controlled during the process studied here. The optimum pH was 8.0 for *B. atrophaeus* spores production by SSF (SELLA *et al.*, 2009a) when using sugar cane bagasse as a support medium, due its slight acidification after medium sterilization; in this case, the inert characteristics of sand resulted in an optimum pH of 7.2.

Under the different experimental conditions, the effect on spore yield of the combination of inoculum size and bed height (up to 5.0 cm) was studied (TABLE 6). The ANOVA *p*-value was greater than 0.05 for the variables studied and their interactions, indicating that there were no statistically significant differences between the means. The mean spore yields in different bioreactors were $7.6 \times 10^8 \pm 3.0 \times 10^8$ CFU/g dry matter, $1.0 \times 10^9 \pm 7.7 \times 10^8$ CFU/g dry matter, and $1.0 \times 10^9 \pm 9.5 \times 10^8$ CFU/g dry matter in trays, plastic bags, and Erlenmeyer flasks, respectively. ANOVA indicated that fermentation in trays gave total spores counts that were statistically equivalent to those in plastic bags and Erlenmeyer flasks under the studied conditions ($p \geq 0.05$) (FIGURE 2). Bed height (≥ 3.5 cm) and bioreactor type did not influence spore formation. These results show the advantage of growth by cell adherence and biofilm formation: almost all types of bioreactor can be used, including trays, bed columns, flasks, and disposable plastic bags, avoiding the costs of cleaning and cleaning-process validation. The microorganism affixes itself onto the support surface, forming cell communities inside the bioreactor, regardless its size or shape. This property makes scale-up of the process easier.

. Inoculum size generally plays an important role in SSF productivity; theoretically, a large inoculum allows more rapid substrate consumption, inducing sporulation. However, inoculum size was not a significant variable in this study, indicating that sporulation was triggered by other factors than high cell density and nutrient deprivation (GROSSMAN; LOSICK, 1988). The absence of interference of these studied parameters on SSF sporulation was demonstrated by Sella *et al.* (2009 a,b) when using sugarcane bagasse as support, indicating that sand, the support used here, did not affect the behavior of these variables.

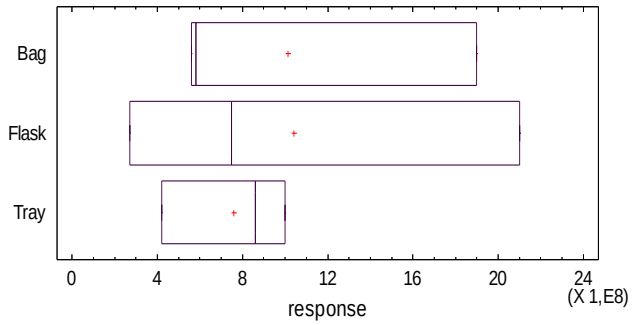

FIGURE 2 - BOX PLOT FOR THE EFFECTS OF THREE BIOREACTOR TYPES ON *Bacillus atrophaeus* SPORE YIELD: 20 L PLASTIC BAGS, 250 ML ERLENMEYER FLASKS, AND 18 × 8 × 5 CM (LENGTH × WIDTH × BED HEIGHT) TRAYS

SOURCE: The author (2012)

Mineral composition of the sporulation medium may significantly affect spore properties. The presence of certain mineral ions during sporulation, specifically calcium, potassium, and manganese, has been described as having a clear effect on spore heat-resistance properties (CAZEMIER *et al*., 2001; NGUYEN THI MINH *et al*., 2008; OOMES *et al.,* 2007). Mineral composition can affect the mineralization of the spore core and influence the protoplast water content, which appears to be inversely correlated to spore heat resistance (BEAMAN; GERHARDT, 1986, NICHOLSON *et al.*, 2000). Molin and Svensson (1976) reported an increase in dry-heat resistance by adding a mixture of $CaCl_2$, $MgSO_4$, and KH_2SO_4 to the sporulation medium, and Cazemier *et al.* (2001) concluded that *Bacillus* spores prepared on nutrient agar supplemented with Ca^{2+}, Mg^{2+}, Mn^{2+}, Fe^{2+}, and K^{+} were more heat resistant at 114°C than spores obtained from nutrient agar with Mn^{2+}. However, the Plackett-Burman assays results indicated that the mineral composition of the sporulation medium did not substantially affect spore yield, but the dry-heat resistance, demonstrated by the $D_{160°C}$ value, varied by 3.6 minutes among the treatments (TABLE 7). The ANOVA demonstrated the statistical significance of the ionic component of the sporulation medium by comparing the mean square against an estimate of the experimental error. In this case, three ions (potassium, calcium, and magnesium) had *p*-values greater than 0.05, indicating that they did not have significant effects on spore dry-heat resistance. Only manganese had a positive influence on spore dry-heat resistance (FIGURE 3). These results allowed us to

eliminate the other salts from the sporulation medium, simplifying its composition. It is in agreement with the results of Granger *et al.* (2011) in which sporulation in Mn^{2+}-deficient media was very poor because this ion is required by the enzyme phosphoglycerate mutase, an important enzyme for *Bacillus* growth. The other necessary ions were probably present in the soybean molasses substrate, an agro-industrial by-product with about 10% of mineral residue and 75% mineral solids (SANADA *et al.,* 2009), eliminating the need for mineral complementation.

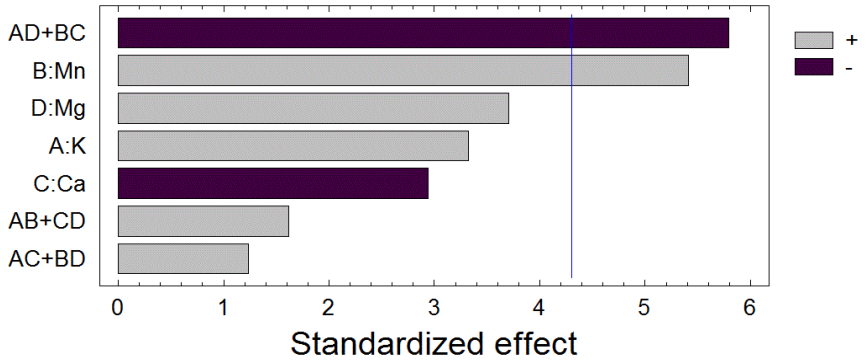

FIGURE 3 - PARETO CHART OF THE EFFECTS OF IONS IN THE SOLID-STATE FERMENTATION SPORULATION MEDIUM ON *Bacillus atrophaeus* SPORE DRY-HEAT RESISTANCE: K^+ (A), Mn^{2+} (B), Ca^{2+} (C) and Mg^{2+}(D)

SOURCE: The author (2012)

The optimum concentrations of the main components of the sporulation medium (soybean molasses and $MnSO_4$) were obtained from the CCD. The ANOVA of the $D_{160°C}$ and N (spores yield) results demonstrated that the effects of soybean molasses and manganese concentration had p-values less than 0.05, indicating that they were significantly different from zero. The R^2 statistic indicated that the fitted model explained 99.8% of the variability in $D_{160°C}$ and 97.6% in N. The application of the RSM gave the following regression equations and response surface plot (FIGURE 4) to the tested variables:

$$Z_1 = -2.74 + 3.3\,X + 1391.1\,Y - 0.62\,X^2 - 41.7\,XY - 78124.6\,Y^2 \quad (3)$$

$$Z_2 = -4.5 \times 10^9 + 1.7 \times 10^9\,X + 1.2 \times 10^{12}\,Y - 4.1 \times 10^8\,X^2 + 2.0 \times 10^{10}\,XY - 7.9 \times 10^{13}\,Y^2 \quad (4)$$

Where $Z_1 = D_{160°C}$ value, $Z_2 = N$, X = soybean molasses, and $Y = MnSO_4$.

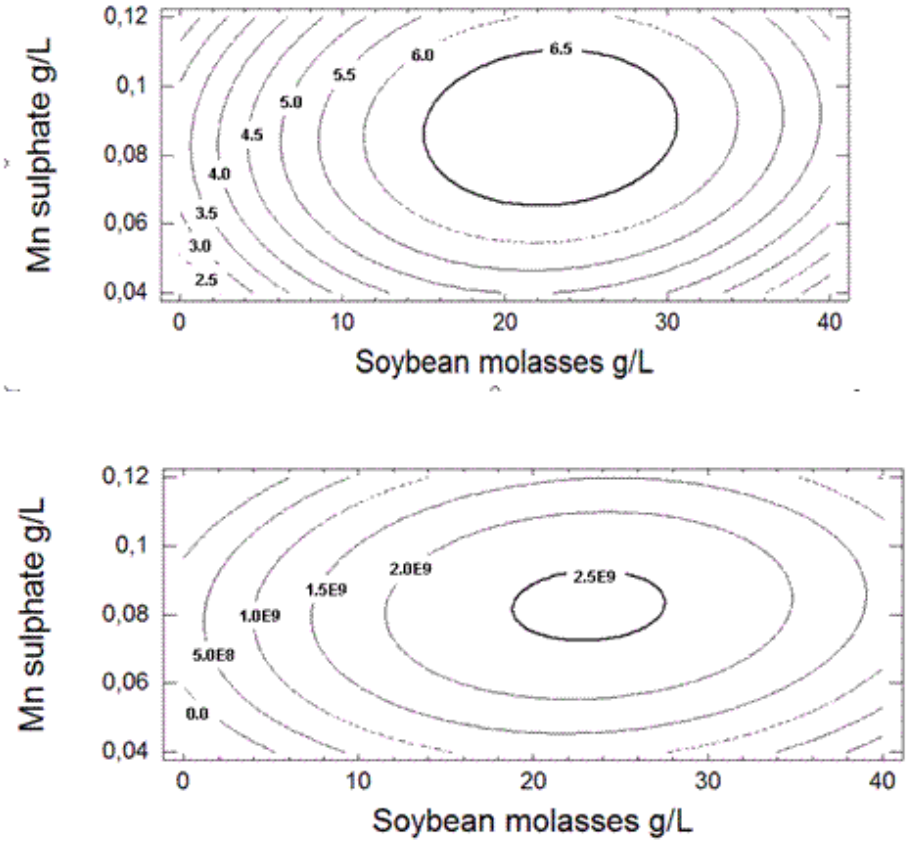

FIGURE 4 - CONTOURS OF ESTIMATED RESPONSE SURFACE PLOTS SHOWING THE OPTIMUM CONCENTRATIONS OF SOYBEAN MOLASSES AND $MnSO_4$ ON *Bacillus atrophaeus* SPORE YIELD – *N* (CFU/g DRY MATTER) AND DRY-HEAT RESISTANCE - $D_{160°C}$ (min)

SOURCE: The author (2012)

The differential equations *(3)* and *(4)* were used to calculate optimal parameters. The optimum values of the tested variables were: soybean molasses = 24.0 g/L and $MnSO_4$ = 0.08 g/L, with the corresponding $D_{160°C}$ = 6.9 min and $N = 2.5 \times 10^9$ CFU/g dry matter. Finally, three tests were carried out under the optimized conditions to validate the model. The experimentally derived $D_{160°C}$ value was 6.7 ± 0.1 min and $N = 2.3 \pm 0.5 \times 10^9$ CFU/g dry matter, equal to the calculated values. Validation of the RSM regression equations demonstrated non-significant variation

among the three batches. The design of experiments (DoE) methodology resulted in a significant improvement in spore yield (~ 4 orders of magnitude over initial values) and in $D_{160°C}$ value (3.6 min higher than the lowest results). Thus, this methodology was a useful model to study and optimize the major variables of the SSF process and has the advantage limiting the number of experiments.

3.3 Performance evaluation of the new BI system

A time course of spore production using the optimized medium was performed and compared with sporulation on standard agar medium (FIGURE 5). Maximal spore yield of 2.4×10^9 CFU/g dry matter was achieved at 72 h for SSF, and 2.5×10^{10} CFU/g dry matter was achieved at 144 h for standard agar medium. At 72 h incubation the difference in number of spores formed between SSF and agar were less than one order of magnitude, indicating that the process biomass were equivalent at that time. The pH increased for both sporulation methods.

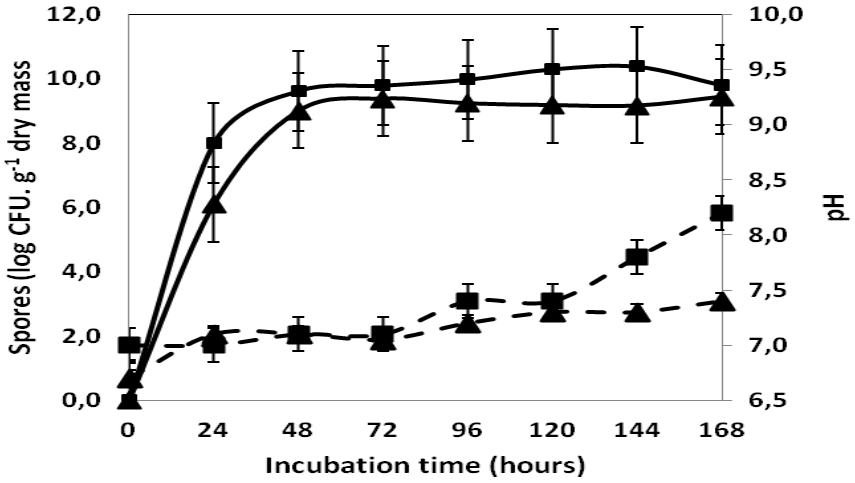

FIGURE 5 - SPORULATION KINETICS (SOLID LINES) AND pH MEASUREMENT (DASHED LINES) OF *Bacillus atrophaeus* DURING SOLID-STATE FERMENTATION ON OPTIMIZED SOYBEAN MOLASSES-BASED SPORULATION MEDIUM (▲) AND ON STANDARD AGAR MEDIUM (■)

SOURCE: The author (2012)

The consumption kinetics of total and specific sugars was evaluated to confirm the suitability of the soybean molasses sugars as the only carbon source for *B. atrophaeus* (FIGURE 6). These results allowed the sporulation process to be reduced from 7 days to 3 days. An increase in pH during sporulation was correlated with the consumption of metabolic acids by the remaining vegetative cells; such pH changes are typical of *Bacillus* spp. cultured in complex media (YAZDANY; LASHKARI, 1975).

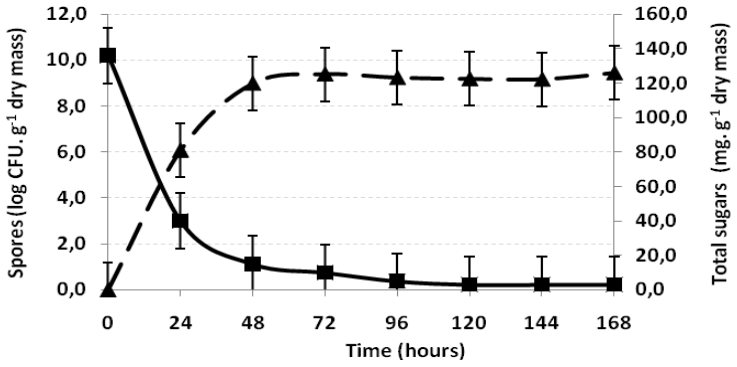

FIGURE 6 - *Bacillus atrophaeus* TOTAL SUGARS AND SPORULATION KINETICS IN THE OPTIMIZED SOLID-STATE FERMENTATION PROCESS

SOURCE: The author (2012)

The HPLC analysis of specific sugars demonstrated that the strain was able to cleave sucrose, raffinose, and stachyose (a tetrasaccharide), which were preferentially consumed in this order. Sucrose decreased completely within the first 24 h, and by 72 h only stachyose remained (FIGURE 7). Stülk and Hillen (2000) reported that *Bacillus* can use different saccharides as single-carbon and energy sources; catabolic enzymes were synthesized only when their substrates were present in the growth medium and when preferred carbon and energy sources were absent. The pathway for the use of sucrose involves cleaving sucrose-phosphate to glucose-6-phosphate and fructose and the production of levansucrase, an extracellular enzyme that cleaves sucrose to glucose and fructose.

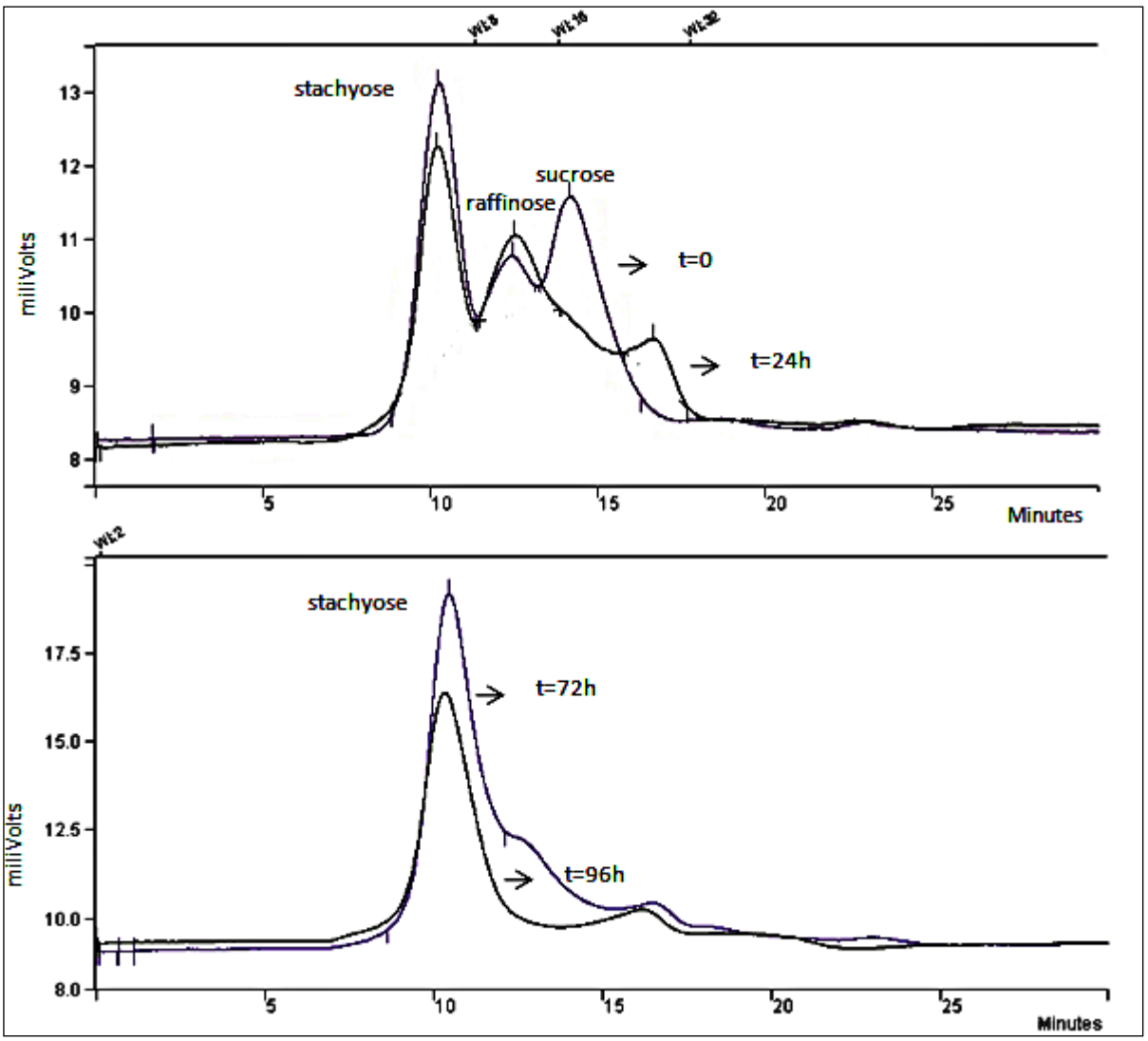

FIGURE 7 - UTILIZATION PROFILES OF STACHYOSE, RAFFINOSE, AND SUCROSE DURING *Bacillus atrophaeus* SPORULATION BY SOLID-STATE FERMENTATION ON SAND WITH SOY MOLASSES AS SUBSTRATE. FOR THE 0 h AND 24 h CHROMATOGRAMS, THE SAMPLES WERE DILUTED 1:2

SOURCE: The author (2012)

Sugar consumption after 72 h incubation may have been due the remaining vegetative cells that did not sporulate or from newly-germinated spores. The observed sporulation and initial total sugar profile were similar that obtained by Chen *et al.* (2010) for *B. subtilis* in submerged cultures after medium optimization. The

SSF sporulation advantages may be highlighted by observing that dry-fermented SSF should be directly diluted with the inert support to meet the dry-heat BI specification in regulatory standards (FDA, 2007; ISO- 11138-4, 2006; USP 31, 2008) (an initial microbial count of 10^6 CFU/unit), and that agar sporulation requires spore detachment, dilution, inoculation on suitable support, and drying steps (SELLA *et al*., 2012).

The kinetics of spore germination and cellular growth are shown in FIGURE 8. In all conditions, there was rapid cell growth from 0h –3 h after the start of incubation and then slower growth until 15 h. Most cell growth had occurred by 15 h, so this was designated as the maximum growth point. At 15 h of incubation, all samples in the commercial BI and 70% of samples in the developed soybean molasses-based BI had changed the color of the medium to yellow. After 21 h, all samples had changed the color of the medium from green to yellow, allowing visual confirmation of spore germination and growth. In the commercial BI, new spore formation was observed after 12 h incubation of heat-damaged spores and after 21 h incubation of unheated spores. In the optimized soybean molasses-based medium, spore formation was not observed.

The kinetics of total-sugar consumption showed different profiles between the two media but similar trends in the germination/growth of heated and unheated spores The consumption of the carbon source at 24 h was greater in the commercial medium (~ 90 g/L) than in the optimized medium (~ 20 g/L); this was expected due the differences in sugar composition between the two media. Germination kinetics demonstrated that the growth of the microorganisms in the soybean molasses-based BI was sufficient to meet the requirements for visual identification of a non-sterile BI (> 10^6 CFU/mL), as suggested by Gillis *et al.* (2010).

The differences observed between the newly-developed and commercial recovery media in the germination and growth patterns of the spores did not influence the performance of the medium. Nutrient limitation and high cell densities, both of which are sporulation inducers (WARRINER; WAITES, 1999), may explain the formation of new spores after 12 h – 21 h incubation in commercial media.

The three lots of newly-developed BI that were tested were produced with different batches of soybean molasses, and all three gave satisfactory results: viability = 100% growth, $D_{160°C}$ = 6.6 ± 0.1 min (commercial BI $D_{160°C}$ = 6.7 ± 0.2

min), N_0 = 3.3 ± 0.2 × 10^6 CFU/unit, 100% growth after 25 min exposure (survival time), and 0% growth after 60 min exposure (kill time) (FIGURE 9).

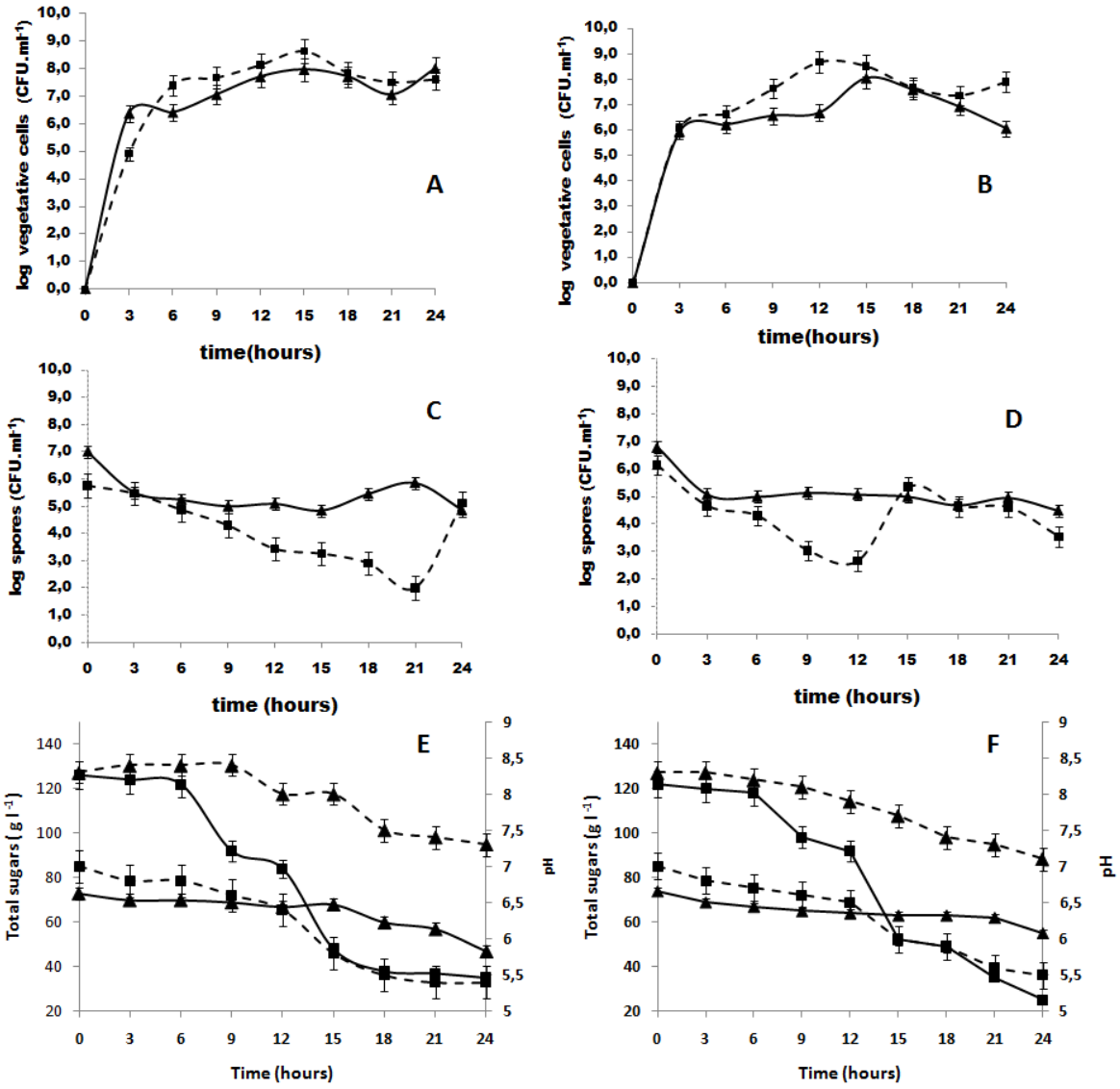

FIGURE 8 - GROWTH, TOTAL SUGAR CONSUMPTION, AND pH KINETICS OF *Bacillus atrophaeus* SPORES IN BIOINDICATOR RECOVERY MEDIUM BEFORE (A, C, and E) AND AFTER (B, D, and F) HEAT DAMAGE AT 160°C FOR 25 MIN. BACTERIA WERE ON OPTIMIZED SOYBEAN MOLASSES + TRYPTONE BI RECOVERY MEDIUM (▲) OR COMMERCIAL BI RECOVERY MEDIUM AS A REFERENCE (■). IN (E) AND (F), pH IS INDICATED BY DOTTED LINES

SOURCE: The author (2012)

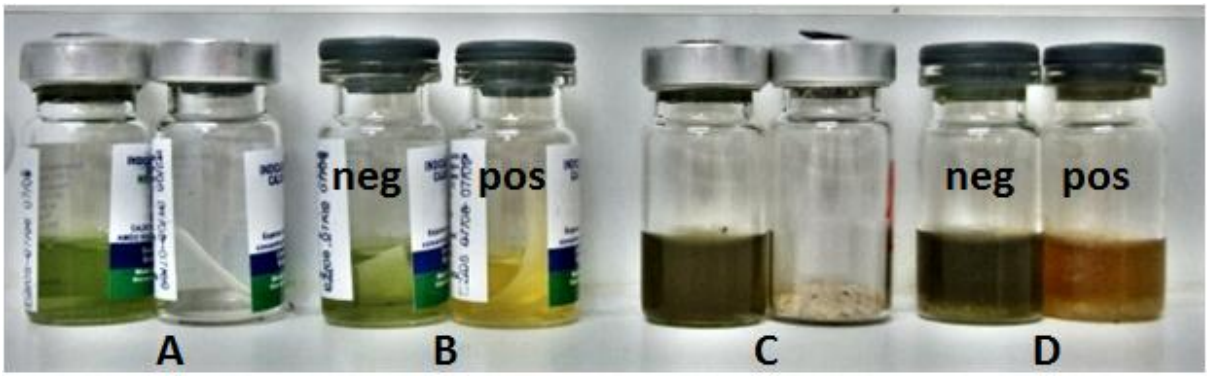

FIGURE 9 - COMMERCIAL *Bacillus atrophaeus* BIOLOGICAL INDICATOR SYSTEM (A AND B) AND SOYBEAN MOLASSES-BASED BIOLOGICAL INDICATOR SYSTEM (C AND D), BEFORE (A AND C) AND AFTER (B AND D) INOCULATION AND INCUBATION. POSITIVE RESULTS INDICATED THAT VIABLE SPORES REMAINED; THE MEDIUM CHANGED TO YELLOW AS THE ACIDIC METABOLIC PRODUCTS OF THE GROWING BACTERIA ACCUMULATED. IN NEGATIVE RESULTS, NO MICROBIAL GROWTH OCCURRED AND THE MEDIUM REMAINED GREEN AND CLEAR, INDICATING THAT THE SPORES WERE KILLED IN THE STERILIZATION PROCESS

SOURCE: The author (2012)

Calculated production costs (which included only the cost of the feedstock and quality control tests) were US$ 782/50,000 units (one batch) for BIS produced from optimized soybean molasses based-medium and US$ 1,134/50,000 units for BIS produced from commercial medium. Process economics was a minimum of 23.9% and the process cycle time was reduced from 29 days to 15 days (TABLE 8).

TABLE 8 - COMPARATIVE COSTS OF PRODUCTION TO OBTAIN 50,000 *B. atrophaeus* STERILIZATION BIOINDICATOR SYSTEM -BIS UNITS (CORRESPONDING TO ONE BATCH) BETWEEN THE DEVELOPED SOYBEAN-BASED BIS AND THE CONVENTIONAL BIS

Description	BI system		
	Soybean molasses-based	Conventional	Reduction (%)
Raw materials (US$)	520.2	684.0	23.9
Direct labor (hours)	58	106	45.3
Equipment (hours)	301	665	54.7
Quality control tests (US$)	261.5	450	41.9
Process cycle time (days)	15	29	48.3

4. CONCLUSIONS

The performance assays demonstrated that the newly-developed BI system could replace the commercial *B. atrophaeus* BI. The newly-developed BI could be widely used in developing countries. All data indicated that the process improved spore yield and heat resistance, and reduced production costs, suggesting that soybean molasses could be used for cost-effective sterilization BIS production. Further research is needed to reduce significantly the recovery medium raw material costs by studying new alternatives nitrogen sources. The inherent variability of agro-industrial by-products may mean that they cannot be reliably used as raw materials for health-care products. That is, the differences caused by weather, soil type, or processing can significantly affect the composition of the by-product. However, the results of this study suggested that using soybean molasses from different production lots did not affect the dry-heat resistance of the bacteria. The effects of variation among different lots of raw materials on the performance of the medium could be evaluated by determining the *D* values experimentally. If necessary, a CCD design could be used to determine the optimal concentrations of molasses and the nitrogen source. The use of agro-industry by-products helps prevent environmental contamination and allows the production of a cost-effective sterilization biological indicator with assured quality and performance.

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CHAPTER IV

RELATIONS BETWEEN PHENOTYPIC CHANGES OF SPORES AND BIOFILM PRODUCTION BY *Bacillus atrophaeus* ATCC 9372 GROWING IN SOLID-STATE FERMENTATION

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ABSTRACT

Bacillus spp. spores are usually obtained from strains cultivated in artificial media. However, in natural habitats, spores are predominantly formed from bacteria present in highly surface-associated communities of cells. Solid-state fermentation (SSF) is the culture method that best mimetizes the natural environment of many microorganisms that grow attached to the surface of solid particles. This study aims to confirm that sporulation through SSF of *Bacillus atrophaeus* occurs by biofilm formation and that this model of fermentation promotes important phenotypic changes in the spores. Sporulation on standard agar and by SSF with sand and sugarcane bagasse as support was followed by a comparative study of the formed spores. Growth characteristics, metabolic and enzymatic profiles confirmed that sporulation through SSF occurs by biofilm formation promoting important phenotypic changes. It was possible to demonstrate that spores coat had different structure and the presence of ridges only on SSF-spores' surface. The sporulation conditions did not affect the dry-heat spore resistance. The type of support evaluated also influenced in the phenotypic alterations; however, the used substrates did not cause interference. This work provides novel information about *B. atrophaeus* response when submitted to different sporulation conditions and proposes a new concept about bacterial biofilm formation by SSF.

Keywords: *Bacillus atrophaeus* sporulation; solid-state fermentation; biofilm; swarming motility; spores structure; bacterial adhesion

1. INTRODUCTION

Many bacterial species show elaborate and sophisticated tactics to survive

under the harshest conditions. Members of the *Bacillus* genus are able to form spores that are extremely resistant to a great variety of such stresses as heat and cold, UV radiation, and chemical assault (DRIKS, 2002). Because of these characteristics, *Bacillus atrophaeus* (formerly *Bacillus subtilis* var. *niger*) and related species have been established as industrial bacteria in the production of sterilization biological indicators and in studies of biodefense methods (FDA, 2007; FRITZE; PUKALL, 2001; GIBBONS *et al*., 2011; WEBER *et al*., 2003). Biological indicators (BIs) are routinely used by medical-device manufacturers and healthcare providers to monitor the efficacy of different sterilization processes – for example, dry heat, ethylene oxide, microwaves, gas-plasma; electro-thermal deactivation, peracetic acid (HALFMANN *et al*., 2007; ISO 11138-4, 2006; OLIVEIRA *et al*., 2010; ROTH *et al*., 2010; TURNBERG 1996). In most studies and in industrial BIs production, spores are obtained from laboratory standard strains cultivated in artificial media - fortified agar or liquid sporulation media (BUHR *et al*., 2008; ROSE *et al*., 2007). However, in natural habitats, spores are predominantly formed from bacteria present in highly surface-associated communities of cells in extracellular polymeric matrices called biofilms (DAVEY; O'TOOLE, 2000; HAMON; LAZAZZERA 2001). This ability to form multicellular complex structures on agar plates is often lost in laboratory strains as a result of planktonic growth in rich media for many generations (BRANDA *et al*., 2001; VEENING *et al*., 2006).

Solid-state fermentation (SSF) is the culture method that best mimetizes the natural environment of many microorganisms that grow attached to the surface of solid particles. SSF is a process culture that involves the growth of microorganisms (mainly fungi) on moist solid substrates in the absence of free-flowing water. SSF processes can use inert natural or artificial solid supports, such as sugarcane bagasse, perlite, amberlite, and polyurethane foam. The supports may act both as a base-attachment structure for the microorganism and as a nutrient source when it is metabolized by the cultivated microorganism (PANDEY, 1992). It is impregnated with a substrate (liquid medium which contains all the nutrients) according to their liquid retention capacity. The substrates are usually complex water-soluble materials from agriculture or by-products from food industry. The simulation of the natural environment with consequent better performance of cultivated microorganisms is one of the SSF advantages (HÖLKER *et al*., 2004; PÉREZ-GUERRA *et al*., 2003).

Sella *et al*. (2009, 2012) proposed the use of solid-state fermentation (SSF) with agro-industrial residues and sand as a cost-effective method for heat-resistant spore production of *B. atrophaeus*. They observed that different colony morphologies appeared when spores produced by SSF were germinated on tryptone soy agar (TSA).

It is well known that cultivation conditions may influence the gene expression of microorganisms (DE VRIES *et al*., 2005). Iwashita (2002) observed how different SSF conditions can lead to alteration of the expression of different genes, which can affect several phenotypes, such as growth, development, production, and other metabolic enzymes; however, few examples have been described.

The present study aimed to confirm that sporulation through SSF of *Bacillus atrophaeus* ATCC 9372 standard strain occurs by biofilm formation and that SSF promotes important phenotypic changes on germinated spores. There are several published protocols comparing sporulation based on industrialized or commercial sporulation media or its components (CAZEMIER *et al*., 2001; ROSE *et al*., 2007). However, there are no published reports about *B. atrophaeus* biofilm formation associated with phenotypic changes of spores produced by SSF.

2. MATERIAL AND METHODS

2.1 Bacterial strain

B. atrophaeus ATCC 9372, Bach-1403349, was obtained from the National Institute of Health Quality Control (INCQS/MS, Brazil).

2.2 Sporulation conditions

Spores were prepared from a suspension of vegetative cells grown for 18 h at 36°C in tryptone soy broth (TSB). For agar sporulation, the cultures were grown in Roux flasks containing 400.0 mL of sporulation medium and two substrates were utilized: (*a*) usual agar medium: 8.0 g/L yeast extract, 4.0 g/L nutrient broth, 0.05 g/L

$MnSO_4.4H_2O$, 0.05 g/L $CaCl_2.6H_2O$ and 30.0 g/L agar; (*b*) As control, to eliminate substrate influence in all studies, this sporulation agar medium was prepared using the same SSF substrate (20.0 g/L soybean molasses supplemented with 0.05 g/L $K_2HPO_4.H_2O$, 0.04 g/L $MnSO_4.4H_2O$, 0.04 g/L $CaCl_2.6H_2O$, and 0.05 g/L $MgSO_4.7H_2O$) added with 30.0 g/L agar. The media were autoclaved at 121°C for 15 min. Sporulation was carried out at 36°C for up to 7 days.

To produce spores by SSF, two supports type were utilized: sugarcane bagasse and sand. Sugarcane bagasse was supplied by Cocamar (Cianorte, Brazil). Sand was classified as medium size (0.4 mm – 2.0 mm). Both bagasse and sand were sieved to obtain a ~1.0-mm particle size (MESH 14-20). The sieved supports were washed once in tap water, twice in distilled water, and dried in trays for 24 h at 90°C in an air oven, before use. SSF was carried out in 250-mL Erlenmeyer flasks containing 78.0 mL of liquid substrate. The support, which was impregnated with substrate medium (20.0 g/L soybean molasses, supplemented with 0.05 g/L $K_2HPO_4.H_2O$, 0.04 g/L $MnSO_4.4H_2O$, 0.04 g/L $CaCl_2.6H_2O$ and 0.05 g/L $MgSO_4.7H_2O$), was weighed and added to the Erlenmeyer flask according to their liquid-retention capacity avoiding free-flowing water in the media: 12.0 g of sugarcane bagasse (water-holding capacity = 6.6 mL/g), and 300.0 g of sand (water-holding capacity = 0.3 mL/g). Due to the inert characteristics of the supports, we opted for keeping the substrate volume constant in order to provide the same nutritional status to the microorganism. Media were autoclaved at 121ºC for 15 min. The inoculum size was 4% (*v*/*v* substrate) corresponding to 10^7 CFU/g dry matter. SSF was carried out at 36ºC for 7 days.

Spores were harvested and suspended in cold and sterile 0.02 M calcium acetate solution adjusted to pH 9.7 with 1.4 g/L calcium hydroxide solution. The suspension was filtered through cotton and gauze tissue and centrifuged three times at 1,048 *g* for 20 min at 4°C. The spores' suspensions were subjected to a heat treatment (80°C, 10 min), which was lethal to vegetative cells but not to spores, and were stored at 4°C. Initial and final water activity (a_w) measurements were determined at 19.0 ± 2.0 ºC, using an Aqua Lab CX-2 water activity meter (Decagon Devices, USA). Viable spore counts were carried out using serial decimal dilutions in distilled sterile water, and 50 µL of each dilution was inoculated on a TSA plate surface in duplicate. The spore suspensions utilized for all assays were then

standardized in ~5.0 x 10^7 CFU/mL.

2.3 Growth in standing cultures

The experiment was carried out on 65-mm diameter x 15-mm deep with 10-mm grid Rodac plates (BD Diagnostics, Brazil) containing TSA agar. Each plate was toothpick-inoculated from an overnight colony and maintained at 36ºC during the growth study. The diameter of the colonies was measured with a pachymeter along two axes, and the mean values were recorded. For pellicle formation analysis, 100 µL of starting culture were added to 30.0 mL of TSB and 30.0 mL of E-medium in a glass tube and incubated without agitation at 36°C for 60 h. The E-medium contained 20.0 g/L L-glutamic acid, 12.0 g/L citric acid, 80.0 g/L glycerol, 70.0 g/L NH_4Cl, 0.5 g/L $MgSO_4.7H_2O$, 0.5 g/L K_2HPO_4, 0.2 g/L $CaCl_2.2H_2O$, 0.04 g/L, $FeCl_3.6H_2O$ and 0.15 g/L $MnSO_4.H_2O$. The pH was adjusted to 7.4 with 2M NaOH. A Nikon Coolpix L1 digital camera (Nikon, USA) was used to capture growth images. Cultures exhibiting distinct morphologies were repeatedly streaked to confirm stability of the phenotype.

2.4 Swarming motility assay

To investigate the swarming motility, 140-mm petri plates containing three different media were used: (*a*) LB (L-Broth or Luria Bertani) medium (1.0 g/L tryptone, 5.0 g/L yeast extract and 5.0 g/L NaCl); (*b*) minimum medium (MSgg) mod. (5.0 g/L glycerol, 5.0 g/L Na glutamate, 0.01 g/L NaH_2PO_4, 0.4 g/L KCl, 0.23 g/L $MgCl_2.4H_2O$, 0.1 g/L $CaCl_2.2H_2O$, 0.09 g/L $MnSO_4.H_2O$, 0.014 g/L $FeSO_4.7H_2O$, 0.003 g/L $ZnSO_4.7H_2O$) and (*c*) TSB medium (17.0 g/L tryptone, 3.0 g/L soytone, 5.0 g/L NaCl, 2.5 g/L dextrose, 2.5 g/L KH_2PO_4). To these media was added 7.0 g/L of bacto-agar. From each tested suspension, 10^6 spores were inoculated at a central location of the plate. Growth at various concentrations of agar in TSB (0.3% - 1.5%) was examined. After 24 h of incubation, plates were photographed against a black background. To quantify the swarming motility, the distance from the colony origin to

the swarm front was measured with a pachymeter along two axes, as a time function, and the mean values were recorded.

2.5 Microtiter plate biofilm assay

Biofilm formation was evaluated using the assay described by Merritt *et al*. (2011). The inoculum was obtained by growing *B. atrophaeus* spores (unattached to sand, sugarcane bagasse, or agar) in TSB medium and LB medium supplemented with 1.0 g/L glucose, 1.0 mM $MgSO_4$, 0.15 M ammonium sulfate and 34.0 mM citrate, pH 7.0, until the stationary phase (18 h). Then, the cell suspension was diluted (1:100) in the respective media. Samples of 100 µL were grown in sterile PVC 96-well microtiter plates (four wells). The negative control wells contained only the media. The plate was covered with a lid and incubated at 36ºC for 24–60 h. Every 24 h, the growth medium and non-adherent cells were removed by shaking the plate over a waste tray. Plates were then rinsed in tap water. Adherent cells were stained with a 1.0 g/L crystal violet solution (CV) for 10 min at room temperature. Excess CV was then removed, and the wells were rinsed in water. After drying, 200 µl of 33% (*v/v*) glacial acetic acid was added to the wells to dissolve the attached and colored cells. Biofilm formation was measured by determining the optical density of each well at 570 nm. Strains were classified into the four categories cited by Stepanovic *et al.* (2000): no biofilm producer, measured absorbance (A) = negative control absorbance (A_c); weak biofilm producer (+), $A > A_c$, A up to 2x A_c; moderate biofilm producer (++), $A > A_c$ from 2 x A_c up to 4 x A_c ; and strong biofilm producer (+++), A > Ac and $A > 4 \times A_c$.

2.6 Metabolic and enzymatic profile assay

The spore suspensions were subcultured once on tryptone soy agar- TSA agar plates for 24 h at 36°C before being tested using the VITEK 2 system (bioMérieux, France). A bacterial suspension was adjusted to a 0.5 McFarland standard in 2.5 mL of 0.45% sodium chloride solution with an ATB 1550

densitometer (bioMérieux, France). VITEK 2 uses fluorescence to monitor 41 metabolic reactions with the ID-GNB card and 43 metabolic reactions with the ID-GP card. The system automatically fills, seals, and transfers cards into an incubator. After 5–8 h of incubation at 35.5°C, the metabolic profile report was provided from the system's database. The analyses were done in triplicate aiming to reduce the test variation and reproducibility. Catalase activity was assayed by spotting drops of 3% hydrogen peroxide onto the isolated colonies' samples from the TSA plate. Bubble formation was considered catalase positive. Hemolytic activity was determined by culture on blood agar plates for 24–48 h at 36°C.

2.7 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Spore proteins were extracted from spores' suspensions. Vegetative cells proteins were extracted after spores' germination on TSB medium for 18h at 36ºC. The cells were washed four times (14,000 x *g* for 10 min) with purified water resulting in a 1.0-ml pellet. The pellet was resuspended with 1.0 mL extraction buffer solution, containing 0.25 M Tris-HCl pH 6.8, 100.0 g/L glycerol, 10 mM EDTA, 40.0 g/L SDS and 100.0 g/L mercaptoethanol, and boiled for 10 min. After centrifugation, the supernatant was filtered through an Amicon Ultra filter 10K device (Millipore, USA) at 14,000 *g* for 20 min. The protein extract was quantified using the Quant-iT Protein Assay (Qubit Fluorimeter, Invitrogen, USA), and aliquots from 23-µg proteins were applied directly to an SDS-polyacrylamide gel (15% and 20%). After the run, the gel was stained with silver staining. A Nikon Coolpix L1 digital camera was used to capture the gel images. The images were converted to negative form using Photoscape software (Mooii Tech Inc.).

2.8 Dry-heat resistance test

The dry-heat resistances of the spores were determined according ISO-11138-4 (2006) and expressed as *D* value. The *D* value is defined as the time taken—under specified conditions—to reduce the spore population by 90% or 1 log.

For this experiment, 300 strip vials of BIs were produced with each studied spore suspension. Sterile filter papers (48.0 g/m^2 strips, size 1.0 cm × 2.0 cm) were soaked in 0.02 M calcium acetate solution, pH 11, adjusted with 1.4 g/L calcium hydroxide solution, and kept at room temperature for about 18 h. The strips were spread on trays and dried at 45.0°C in a heated air oven. The trays were packed in sealed plastic papers bags, autoclaved at 121°C for 15 min, and dried at 45°C in a heated air oven for about 24 h. They were stored at room temperature. The spore suspensions were homogenized by vortex, and 10.0 µL of it was dispensed on each strip. The inoculated strips were dried at 45°C in a heated air oven for about 24 hours. Each strip was put into a 7.0-mL sterile glass vial, rubber-stoppered, and sealed with an aluminum seal. Prepared BIs were stored at 4°C. The recovery medium composition was 30.0 g/L TSB, 0.18 g/L $CaCl_2 \cdot 6H_2O$, 1.0 g/L soluble starch, and 0.02 g/L bromothymol blue.

The *D* value was assessed by fraction negative analysis—the limited Spearman–Kaber method (ISO- 11138-4, 2006). Dry-heat exposure conditions were 160°C at 25, 30, 35, 40, 45, and 50 min in a tabletop circulating air oven. The spores' germination and outgrowth were observed at 24 - 48 h' incubation by visual identification of a color change (green to yellow) and turbidity of the substrate media.

2.9 Scanning electron microscopy (SEM)

Scanning electron microscopy was has been widely used to study the spore's surface morphology not visible by transmission electron microscopy (CHADA *et al.,* 2003; MALKIN; PLOMP, 2011). The air-dried (65ºC, 24 h) fermented samples were fixed with 30.0 g/L glutaraldehyde in cacodilate buffer (0.1 M, pH 7.0) for a minimum of 24 h, rinsed in the same buffer, and dehydrated in an ethanol series (70, 90, and 100%) (Merck, Germany) and liquid CO_2, mounted in stubs and metalized with gold before being analyzed using the JEOL JSM-6360LV (JEOL USA Inc., USA). Morphology and spores' lengths and widths (µm) were measured, and the data presented are the average of over 100 spores.

+2.10 Transmission electron microscopy (TEM)

Transmission electron microscopy was used to examine the surface details and cross-sections of the spores. For visualization with the transmission electron microscope, the spore suspensions were centrifuged (14,000 *x g,* 10 min), and the pellets were fixed (25.0 g/L glutaraldehyde, 20.0 g/L formaldehyde in 0.1 M cacodylate buffer, pH 7.0), post fixed with osmium tetroxide (10.0 g/L in cacodylate buffer for 1 h), dehydrated in an ethanol series (30, 50, 70, 95, and 100%) (Merck, Germany) and propylene oxide, and embedded in PolyEmbed 812 resin (EMS, USA). After polymerization (60°C, 72 h), thin sections of 60–80 nm (Ultracut R, Leica Microsystems, Germany) were obtained and contrasted using 5% uranyl acetate and lead citrate (Reynolds). The sections were observed using the JEOL JEM-1200EXII (JEOL USA Inc, USA) transmission electron microscope.

2.11 General conditions

At least three different spore suspension batches from each culture media were evaluated. The assays were carried out in triplicate. Microbiological assays were conducted under aseptic conditions in a Good Manufacturing Practices (GMP) certified laboratory ISO 5 clean room. When spores only from SSF are described, this signifies that the results were identical within that using sugarcane bagasse and using sand as support. When spores only from agar are described, this signifies that the results were identical within that using soybean molasses or commercial substrates. For better understanding the methodology sequence is demonstrated in FIGURE 1.

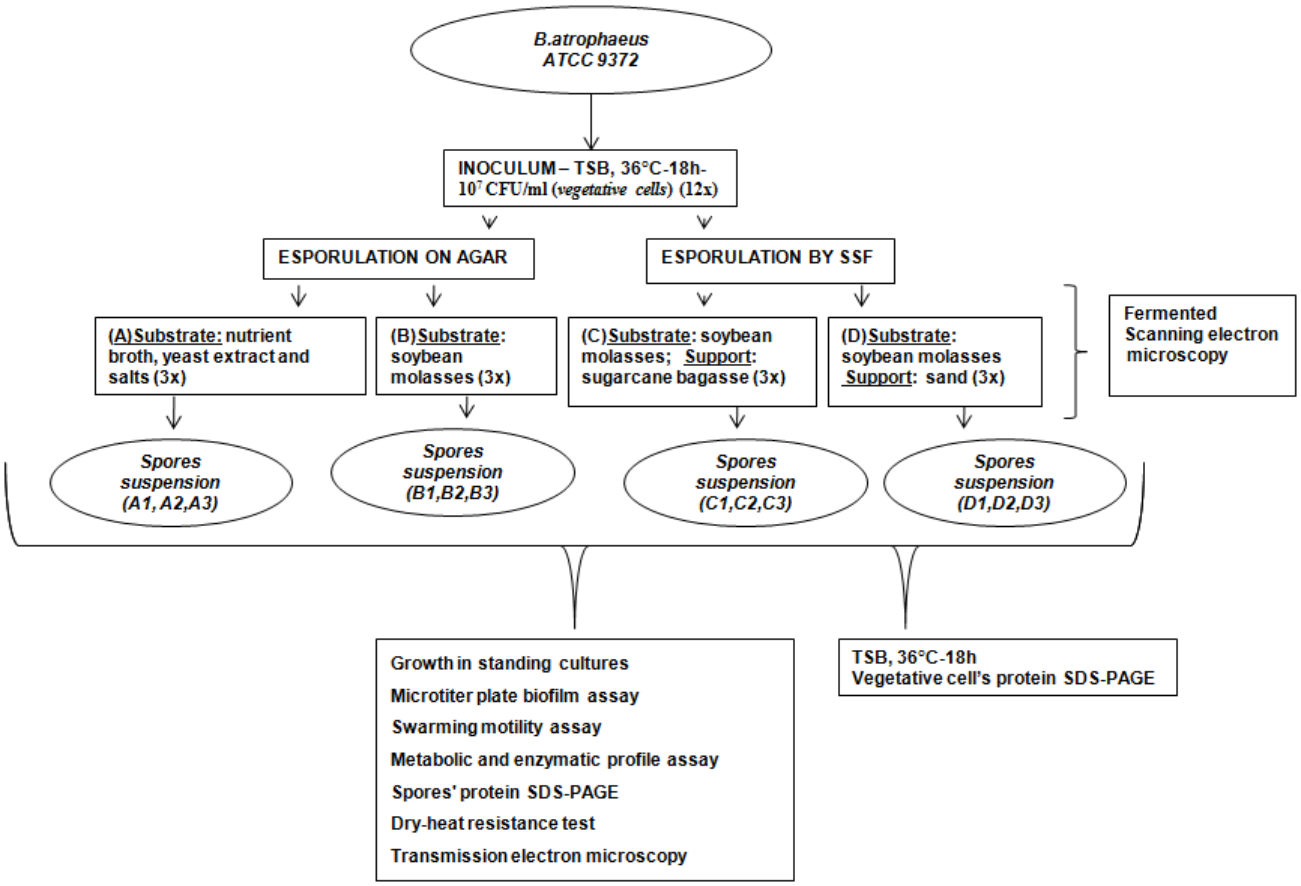

FIGURE 1 - FLOWCHART FOR METHODOLOGY SEQUENCE UTILIZED FOR THE STUDY OF PHENOTYPIC CHANGES IN *B. atrophaeus* IN RESPONSE TO SOLID-STATE FERMENTATION SPORULATION

SOURCE: The author (2011)

3. RESULTS

Solid-state fermentation was carried out with different supports to examine their effects on spore characteristics. The media water activity and moisture were measured before and after fermentation. The initial moisture content varied according to the support type and their water retention capacity: 85 ± 2% for sugarcane bagasse, and 20 ± 3% for sand. The initial a_w measured was 0.997 ± 0.003 for both supports and 0.998 ± 0.001 for sporulation agar. During the incubation periods, there were no observed a_w alterations in any media. However, the final moisture content showed a considerable decrease: 82 ± 2 % for sugarcane bagasse support medium and 6.0 ± 2% for sand support medium. The obtained spores yield

was 3.2 ± 1.8 x 10^9 CFU/g dry matter for bagasse as support and 2.3 ± 0.3x 10^9 CFU/g dry matter with sand; there was not a statistically significant difference between the means (*t*-test) and between the standard deviation (*F*-test) of the two samples at the 95.0% confidence level (*p*-value = 0.544 and *p*-value=0.096 respectively). Sporulation on agar showed the spores yield 1.1 ± 0.1 x 10^8 CFU/g was obtained with usual agar medium and 2.4 ± 2.0 x 10^8 CFU/g dry matter with soybean molasses as agar substrate, in this case was not a statistically significant difference between the means (*p*-value = 0.393), however there was difference between the standard deviations (*p*-value = 0.006), indicating that agar sporulation with commercial substrates showed less variation on spores' yield.

In the first test of the growth in standing cultures, which were carried out with the spores' growth in broth medium, only spores from SSF cultures revealed the formation of robust pellicles with intricate web-like structures. With Gram staining, it was observed that after 12–24 h of incubation, the cells at the air–medium interface began to sporulate and after 60 h more than 80% of cells had sporulate (FIGURE 2). No spores were detected in the liquid phase of either culture.

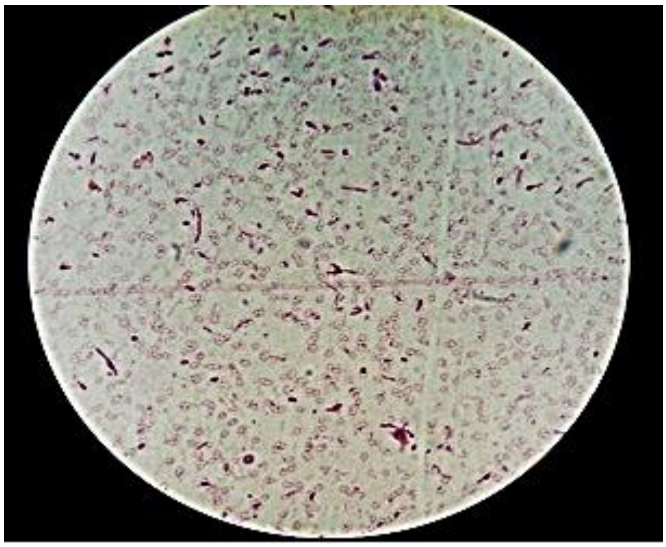

FIGURE 2 - GRAM STAINING OF *B. atrophaeus* GROWTH ON AIR-LIQUID INTERFACE OF E-MEDIUM BROTH ON STANDING CULTURE AFTER 60 HOURS INCUBATION TIME. MORE THAN 80% OF CELLS ARE SPORULATED (MAGNIFICATION: X 1,000)

SOURCE: The author (2010)

When spotted onto TSA plates, *B. atrophaeus* spore growth showed morphological features that were characteristic of those corresponding to pellicles in liquid medium. The agar colonies were smooth, small, and well defined, whereas the SSF colonies were mucoid, large, unlimited, and structurally complex (FIGURE 3, *lines 1 and 2*). Another observation was an increase in pellicle formation at the air/liquid interface when E-medium was utilized; this was more often seen in cultures from spores produced by SSF (FIGURE 3, *line 1-B*).

Colony radial growth rates were compared at 24–144 h' incubation time. The best colony radial growth rate was observed after 24 h' incubation (0.42 ± 0.2 mm/h) for SSF spores inoculation; this was far superior to the figure obtained for agar spores (0.17 ± 0.2 mm/h). FIGURE 4 shows the growth evolution represented by the colony diameter average up to 144 hours' incubation time.

Swarming motility was identified and characterized from spores produced by SSF in TSB and Luria-Bertani (LB) media plus 0.7% agar. Swarming was not found when grown was tested in minimal medium-MSgg (FIGURE 5). The response of the colony size to four different agar concentrations (0.3%, 0.7%, 1.2% and 1.5%) was examined over time, the mean values obtained from three different experiments are shown in FIGURE 3 (*line* 3), indicating that the agar concentration had a significant effect on the colony's radius (FIGURE 6).

Microtiter plate biofilm assay demonstrated reproducible adherence when using TSB medium after 24-h incubation. The reproducibility of this result decreased after 48-h and 60-h incubations, even when the medium was changed every 24 h after inoculation (data not shown). Growth in LB supplemented medium did not support reproducible adherence to the microtiter plate. A ring of stained cells attached to the wells was observed only in cultures obtained from SSF spores. Under the study conditions, *B. atrophaeus* spores obtained from SSF had great ability to form biofilms (++ up to +++), spores grown from agar did not show the same ability, as indicated above (FIGURE.3, *line 4*).

To evidence the effects of the sporulation culture method on the metabolic and enzymatic profiles, the strains were grown using separate carbon sources and enzyme substrates. The results of some metabolic reactions are summarized in TABLE 1, and they suggest some combined phenotypic alterations.

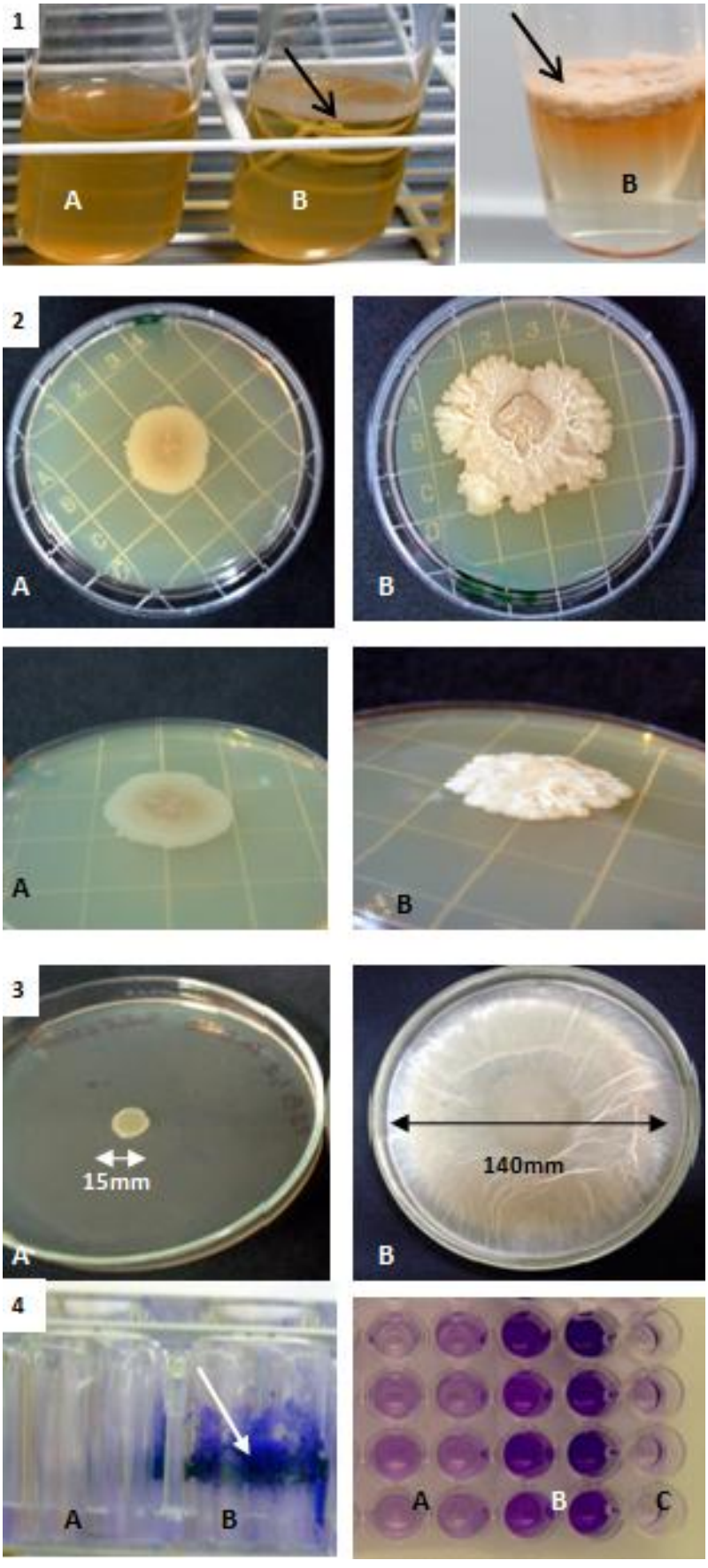

FIGURE 3 - *B.atrophaeus* PHENOTYPIC ALTERATIONS AND BIOFILM FORMATION.
GROWTH CHARACTERISTICS OF SPORES PRODUCED ON SPORULATION AGAR MEDIUM (**A**), AND SPORES PRODUCED BY SSF (**B**). *LINE 1*, THE *ARROWS* INDICATE THE PELLICLE AT THE AIR-LIQUID INTERFACE AFTER 60 h OF GROWTH IN STANDING LIQUID CULTURE -TSB (*LEFT*), AND E-MEDIUM (*RIGHT*); *LINE 2*, TSA (RODAC, 1.5% AGAR) GROWTH, SHOWING DIFFERENCES IN COLONY MORPHOLOGY OBSERVED AFTER 144 h ' INCUBATION; *LINE 3*, MOTILITY OBSERVED BY DEVELOPING COLONIES ON TSB PLUS 0.7% AGAR MEDIA, 48 h, 36ºC; *LINE 4*, BIOFILM ASSAY, THE *ARROW* SHOWS A RING OF CRYSTAL VIOLET STAINED, INDICATING *B. atrophaeus* CELLS ATTACHED TO THE MICROTITER PLATE WELLS (*LEFT*) AND READY FOR QUANTIFICATION AFTER 33% ACID ACETIC DILUTION (*RIGHT*); THE NEGATIVE CONTROL IS IDENTIFIED AS **C**

SOURCE: The author (2010)

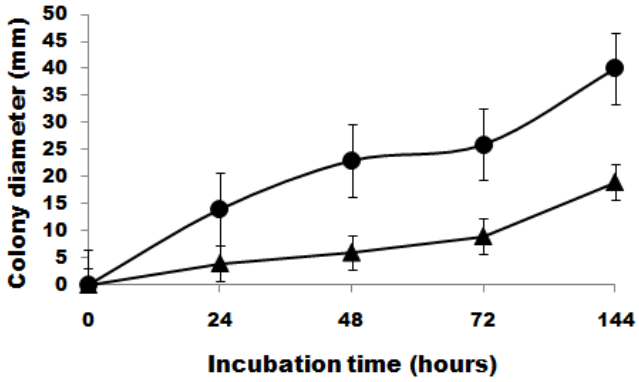

FIGURE 4 - COLONY RADIAL GROWTH RATE OF *B. atrophaeus* SPORES WHEN CULTIVATED IN TSA: (●) SPORES FROM SOLID-STATE FERMENTATION; (▲) SPORES FROM AGAR

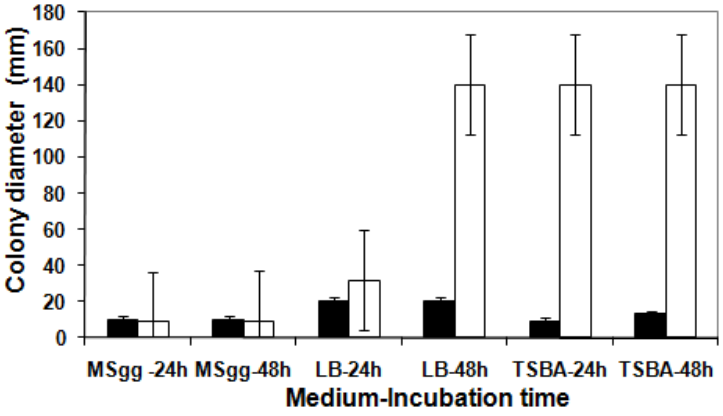

FIGURE 5 - COLONY DIAMETER OF *B. atrophaeus* SPORES CULTIVATED IN DIFFERENT AGAR MEDIA FORMULATIONS: (■) SPORES FROM SPORULATION AGAR; (□) SPORES FROM SSF

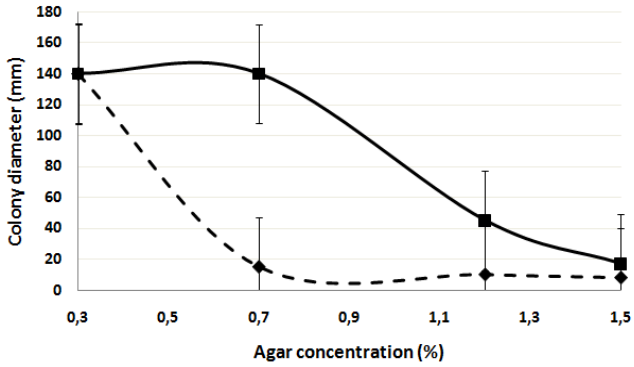

FIGURE 6 - THE EFFECT OF AGAR CONCENTRATION ON *B. atrophaeus* COLONY SIZE WHEN CULTIVATED IN TSB PLUS AGAR, 48 HOURS, 36ºC: (◆) SPORES FROM AGAR; (■) SPORES FROM SSF

FIG 4, 5 and 6, SOURCE: The author (2011)

TABLE 1 - METABOLIC AND ENZYMATIC PROFILES OF *Bacillus atrophaeus* CULTURE OBTAINED FROM SPORES CULTIVATED ON AGAR AND IN SSF WITH DIFFERENT SUPPORTS ANALYZED USING THE VITEK 2 SYSTEM (BIOMÉRIEUX)

ASSAY	*ABBREVIATION*	*Well concentration (mg)*	*AGAR Culture*	*Bagasse SSF Culture*	*Sand SSF Culture*
Amygdalin assimilation	AMY	0.192	+	±	+
Arginine dihydrolase	ADH1	0.111	+	±	±
Alpha-glucosidase	AGLU	0.036	+	±	±
Phosphatase	PHOS	0.050	-	±	-
Alpha-galactosidase	AGAL	0.036	-	±	±
Tyrosine arylamidase	TyrA	0.030	±	-	±
D- galactose	dGAL	0.300	-	±	±
D-Ribose	dRIB	0.,300	±	-	±
L- Lactate alkalinization	ILATk	0.150	±	-	-
Lactose	LAC	0.960	-	±	±
N-acetil-d-glucosamine	NAG	0.300	+	-	±
D-mannose	dMNE	0.300	+	±	±
Methyl-B-D-glucopyranoside	MBdG	0.300	+	±	+
Salicilin	SAL	0.300	+	±	+
D-trehalose	dTRE	0.300	+	-	±
Gamma-glutamyl -transferase	GGT	0.023	±	-	-
Beta-glucosidase	BGLU	0.040	+	±	+
Beta xylosidase	BXYL	0.030	-	±	-
Palatinose	PLE	0.300	±	±	+
L-Malato assimilation	IMLTa	0.040	-	±	-
Courmarate	CMT	0.130	+	±	±
Beta galactopyranosidase	BGAR	0.002	-	-	±
Cyclodextrin	CDEX	0.300	-	-	±
Catalase	-	-	+	+	+
Hemolysis	-	-	+	++	+

± = Only one of the triplicate sample showing different result.

SDS-PAGE analysis (FIGURE 7) showed no major differences in protein composition between vegetative cells from spores cultivated on agar and by SSF. Major differences were found when comparing spores proteins. Spores obtained from sugarcane bagasse in SSF showed fewer protein fractions, mainly at low molecular weights (<29 kDa).

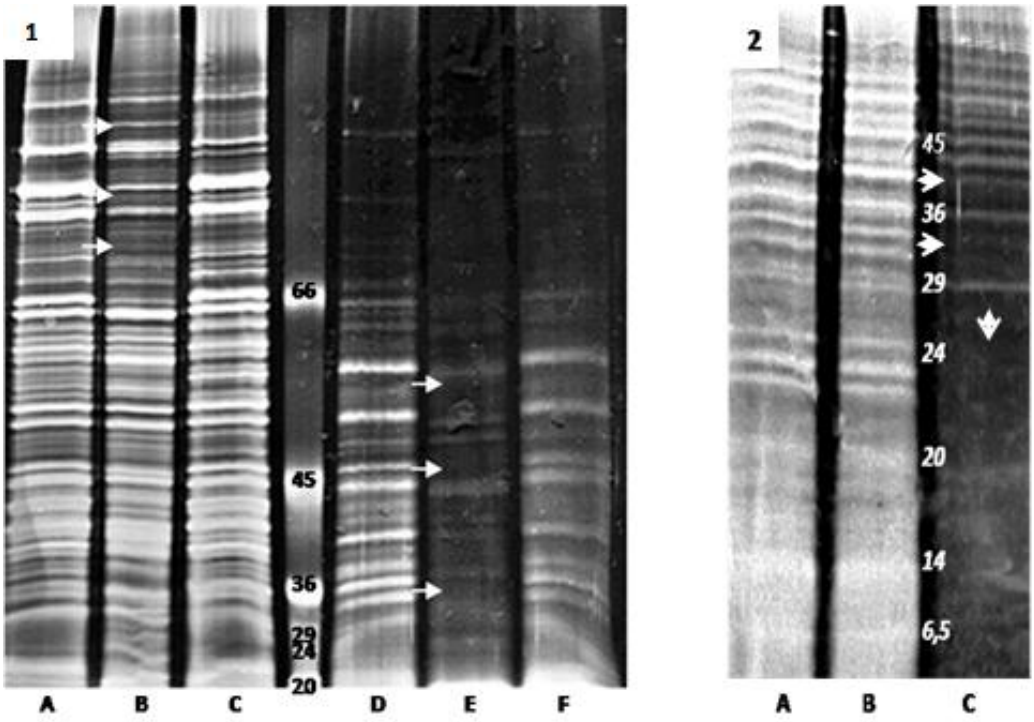

FIGURE 7 - SDS-PAGE ANALYSIS OF *B. atrophaeus* VEGETATIVE CELL WHOLE CELL PROTEINS AND SPORE CRUDE PROTEINS AFTER SPORULATION ON AGAR AND SSF. **GEL 1** (15% POLYACRYLAMIDE): *LANE* ***A***, VEGETATIVE CELLS OBTAINED FROM SPORES CULTIVATED ON AGAR; ***B***, VEGETATIVE CELLS OBTAINED FROM SPORES CULTIVATED ON SUGARCANE BAGASSE IN SSF; ***C***, VEGETATIVE CELLS OBTAINED FROM SPORES CULTIVATED ON SAND SSF; ***D***, SPORES CULTIVATED ON AGAR; ***E***, SPORES CULTIVATED ON SUGARCANE BAGASSE IN SSF; AND ***F***, SPORES CULTIVATED ON SAND SSF. **GEL 2** (20% POLYACRYLAMIDE) ***A***, SPORES CULTIVATED ON AGAR; ***B***, SPORES CULTIVATED ON SAND IN SSF; AND ***C***, SPORES PRODUCED ON SUGARCANE BAGASSE IN SSF. MOLECULAR MASSES ARE GIVEN IN KDa. THE *ARROWS* INDICATE THE MAJOR ALTERATIONS

SOURCE: The author (2011)

Scanning electron microscopy (SEM) was used to examine spore morphology and size. Measurements of 1.5 ± 0.1 µm for length and 0.8 ± 0.1 µm for width are observed for spores from agar sporulation, 1.5 ± 0.2 µm for length and 0.8 ± 0.1 µm for width and, 1.6 ± 0.2 µm for length and 0.8 ± 0.1 µm for width for spores from bagasse and sand SSF respectively. The statistical analysis demonstrated that there is only significant difference at the 95.0% confidence level among the means of the length for sand SSF spores (*p*-value = 0.000) and no significant difference among the means of the width (*p*-value = 0.399). Spores produced on agar were

uniformly smooth (FIGURE 8 A and B), whereas the development of a rough surface, with prominent ridges traversing longitudinally, was observed in spores produced by SSF (FIGURE 8 D and E, FIGURE 9).

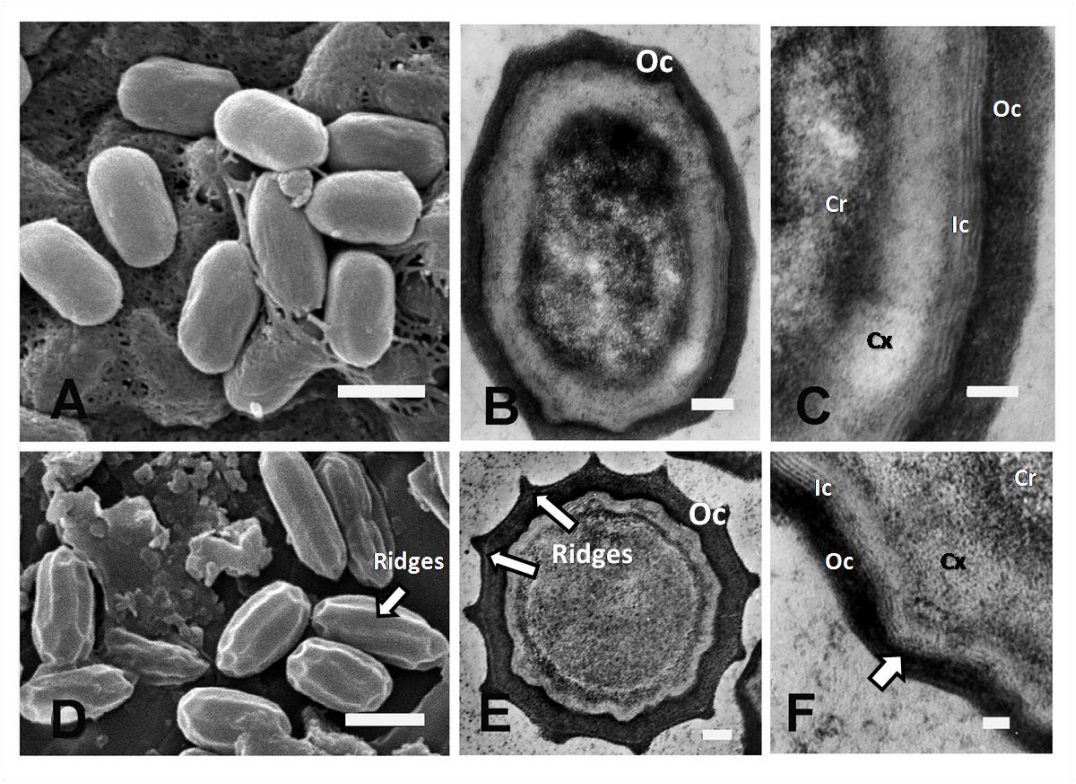

FIGURE 8 **-** ULTRA STRUCTURE OF *B. atrophaeus* SPORES.
SPORES PRODUCED ON AGAR MEDIUM. ***A***, ***B***, OBSERVE THE SMOOTH SURFACE OF THE SPORES' OUTER COAT (*OC*). THE SCALE BAR REPRESENTS 1 µm AND 100 nm. ***C,*** THE SPORES' OUTER COAT (*OC*), CORTEX (*CX*), AND CORE (*CR*). SCALE BAR 50 nm. THE SPORES WERE PRODUCED BY SSF. ***D, E,*** **O**BSERVES THE PROMINENT RIDGES TRAVERSING THE SPORES' SURFACE TRANSVERSALLY AND LONGITUDINALLY. THE SCALE BAR REPRESENTS 1 µm AND 50 nm. ***F,*** SPORE STRUCTURES WITH RIDGES REFLECTED IN THE ADJACENT LAYERS. INNER COAT (*IC*), CORTEX (*CX*). SCALE BAR 20 nm

SOURCE: The author (2011)

In thin section with transmission electron microscopy, the core was seen to be surrounded by a membrane and then a thick layer, called the cortex. The cortex is encased in a multilayered shell, called the coat. Folds in the outer coat were reflected slightly in the adjacent layers (inner coat and cortex), but not in the core (FIGURE 8 C and F).

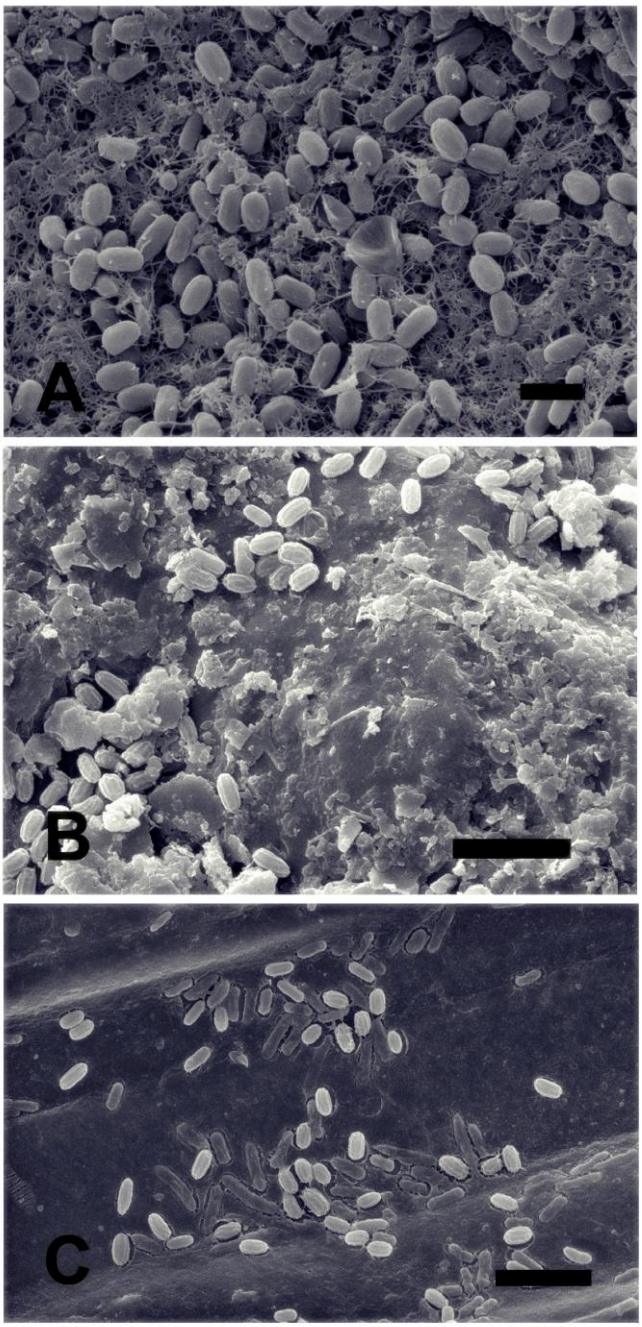

FIGURE 9 - SCANNING ELECTRON MICROSCOPY IMAGES OF *B. atrophaeus* SPORES': PRODUCED ON AGAR MEDIUM (***A***); PRODUCED ON SAND (***B***); AND, PRODUCED ON SUGARCANE BAGASSE (***C***). IN ***A*** WE OBSERVE THE SMOOTH SURFACE OF ALL THE SPORES, THE SCALE BAR REPRESENTS 2µm. IN ***B*** AND ***C*** WE OBSERVE SPORE SURFACE WITH RIDGES TRAVERSING LONGITUDINALLY. IT IS INTERESTING TO OBSERVE THE SUPPORTS DIFFERENT SURFACE CHARACTERISTICS, THE SCALE BAR REPRESENTS 5µm

SOURCE: The author (2011)

The sporulation conditions did not affect the dry-heat spore resistance: D_{160^oC} = 5.5 ± 0.3 min was obtained for BI produced from bagasse SSF spores; D_{160^oC} = 5.4 ± 0.3 min for BI from sand spores; D_{160^oC} = 5.2 ± 0.2 min for BI from usual agar medium spores and; D_{160^oC} = 5.3 ± 0.1 min from soybean agar medium spores

4. DISCUSSION

Water availability of a substrate is a critical factor on SSF process because its influence on growth and metabolism of microorganism (PANDEY, 1992). It was expected that the decrease in moisture content of the medium would lead to decrease spores' yield; however a_w remaining ≥ 0.994 during the sporulation process allowed accessible water for the SSF biomass development. The different characteristics and morphology of the support, which may favor or difficult the nutrients diffusion and the anchorage of the microorganism, didn't affects the spores biomass. Non-standardized and complex composition of soybean molasses may be the cause of the significant variation on spores' yield from agar plus soybean molasses.

Lemon *et al.* (2008) described some systems for biofilm formation. Three of them were used in the present study and confirmed the biofilm formation only by germinated spores from SSF: the formation of floating pellicles at the air-liquid interface of standing cultures, an easy study utilized for identification of biofilm producers (FRIEDMAN; KOLTER, 2004); colony growth on the surface of agar plates, to determine the presence of a macroscopically complex architecture, which is now widely recognized as a form of biofilm (BRANDA *et al.*, 2005); and the microtiter plate assay system, used to carry out high-throughput screening of microorganisms involved in biofilm formation (O'TOOLE; KOLTER, 1998). One of the best-characterized biofilms within the *Bacillus* genus is that from *B. subtilis*, the closest relative to *B. atrophaeus*; thus, we were able to use this as a reference comparison for our findings.

In standing liquid culture, *B. atrophaeus* spores originated from SSF showed the same behavior as that described by Branda *et al.* (2001) for wild strains of *B. subtilis*: a floating biofilm or pellicle on the surface of the medium; no sporulation in the liquid phase and dramatic differentiation with sporulation within the pellicle wrinkles after 5 days of incubation. These characteristics are also related to the formation of spores through special aerial structures called fruiting bodies. The wild *B. subtilis* strain forms colonies on agar plates that show a similar organization of cells and analogous architectural features to those observed in *B. atrophaeus* SSF spores grown on agar. Secondary colonies have very frequently been observed

beginning to grow within 24 h of the SSF spores' incubation on the surface of agar plates (personal observation), this growth being associated with sporulation at the fruiting body formation that could facilitate spore dispersal, as described by Veennig *et al.* (2006). Stanley and Lazazzera (2005) explained the observed increase in pellicle formation at the air/liquid interface when E-medium was utilized as being related to the fact that strains secreting plentiful amounts of *γ*-polyglutamic acid (PGA) tend to give rise to mucoid colonies, and they concluded that domestication may play a role in reducing or abolishing its occurrence.

The SSF germinated spores' results of the colony radial growth rates may indicate motility: motile strains can under ideal conditions growth toward a chemical signal, thereby increasing the colony size. Motility could promote biofilm formation by recruiting and improving bacterial access to surfaces and help it spread (HOURY *et al.,* 2010). Motility was confirmed by bacterial swarm assay. Plates fortified with 0.7% agar are called "swarm plates" since the reduced pore size precludes swimming through the agar, and extensive colonization of the plate can occur only by bacterial migration over the agar surface, what was observed also only in germined spores from SSF. At the lowest agar concentration (0.3%), *B*. *atrophaeus* cells presented a more sliding colony type irrespective of the spores' origin, as reported by Stecchini *et al.* (1998) for *B. cereus.* Swarming absence when all samples' growth was tested in minimal medium (MSgg) may demonstrate that culture medium utilized plays an important role in obtaining reliable and reproducible results. The use of complex media produced more consistent results by minimizing the impact of auxotrophy (PATRICK; KEARNS, 2009). All discussed results demonstrated that *B. atrophaeus* SSF spores exhibit swarming motility and do so in a robust manner; this behavior was not demonstrated in spores from sporulation agar (FIGURE10). These results confirm the finding of Patrick and Kearns (2009) that laboratory strains of *B. subtilis* do not swarm. Swarming growth after SSF-spore germination may be due to a response to environmental cues, surfactant production in response to cell population-density signals, and the formation of flagella in response to surface contact between the cells and the substratum (KEARNS; LOSICK, 2003), since SSF favors these conditions.

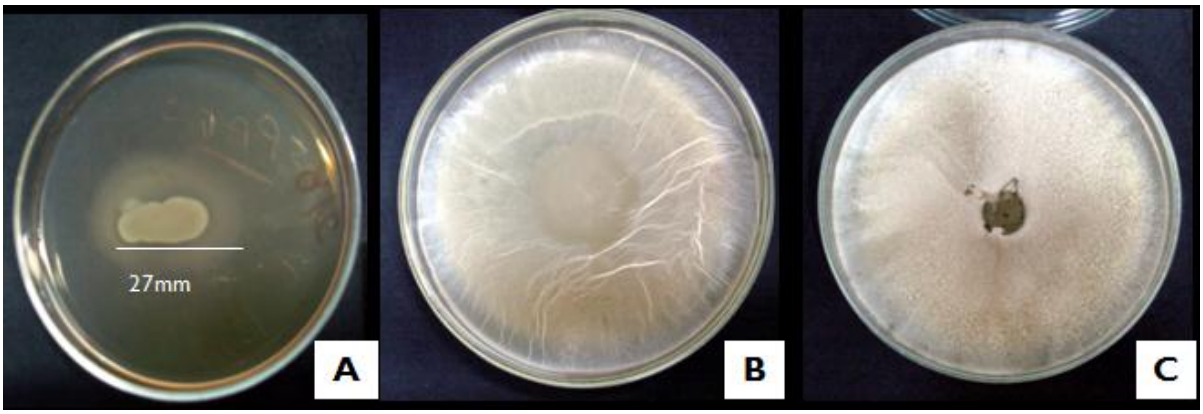

FIGURE 10 - *Bacillus atrophaeus* SWARMING MOTILITY IN 140mm PETRI PLATES CONTAINING TSB MEDIUM ADDED WITH 0.7% OF BACTO- AGAR, INOCULATED WITH (**A**) SPORES PRODUCED ON SPORULATION AGAR, (**B**) SPORES PRODUCED BY SSF WITH SUGARCANE BAGASSE AS SUPPORT AND (**C**) SPORES PRODUCED BY SSF WITH SAND AS SUPPORT

SOURCE: The author (2011)

Generally, the first step in biofilm formation is the adherence to a surface and the growth medium should be standardized according to the microorganism studied. *B. atrophaeus* growth in LB supplemented medium did not support reproducible adherence to the microtiter plates as described by Hamon and Lazazzera (2001) for *B. subtilis*. Adherence to a surface assay results confirmed that *B. atrophaeus* spores produced in SSF form a biofilm, as demonstrated by Sella *et al.* (2012). This suggests that SSF sporulation stimulates biofilm formation even after the spores have germinated. These results may be explained by the “memory” of the sporulation process under slow water loss during sporulation as a strategy to survive under environmental stress; however, it only can be comproved by the evaluation of the genes and mechanism of regulation of cell and spore structures. Veening *et al.* (2006), when studied *B. subtilis* sporulation, established that physiological state of the cell’s ancestor does affect the outcome of cellular differentiation and that the intergenerational ‘‘memory’’ may be important for the development of multicellular structures such as fruiting bodies and biofilms.

It is known that *Bacillus* genera are capable of assimilating numerous

carbohydrates as a source of carbon by secreting several enzymes to depolymerize complex nutrients and that the genes and operons that encode these enzymes are usually not expressed unless the respective carbon source is present in the medium (STULKE; HILLEN, 2000). Koburger *et al.* (2005) described that the *Bacillus* adaptation to glucose limitation (one inductor factor in the sporulation process) also involves changes in transcriptional activity. However, it is not completely clear how the sporulation conditions influence the metabolic profile of the resultant vegetative cells in the same germination and outgrowth conditions, as demonstrated by the metabolic and enzymatic profile assay results. Stulke and Hillen (2000) related that the synthesis of many extracellular degradative enzymes to the growth state of cultures requires distinct regulatory signals and that extensive research will be required to uncover all the factors involved in these regulatory pathways. Heterogeneity in spore behavior due to conditions during spore formation and maturation and its influence on bacterial spore outgrowth were described by Hornstra *et al.* (2009) and may be considered in the metabolic and enzymatic profile responses.

The catalase and hemolysis results in the present study differ from those found by Gibbons *et al.* (2011) for the same ATCC 9372 and its variant (the dominant morphotype). However, it is known that catalase presence is an identifying feature of *B. atrophaeus* and hemolytic activity is associated with lipopeptide production. These features were retained after sporulation with both sporulation methods used in the present study.

The surface of *B. atrophaeus* spores is composed of a multilayered protein shell called coat, which represents 50 to 80% of the total protein in mature spores (DRIKS, 1999). The differences found in SDS-PAGE with the spores' coat proteins need to be elucidated using specific protein coat-defective strains. The low band intensity of SSF protein spores, which was more pronounced with spores cultivated on sugarcane bagasse, demonstrates that extraction was easier than with spores prepared on agar. This finding may suggest that spores produced on agar have more soluble proteins. Complementary biochemical and molecular methods should be used for better characterization of the expression and structure of proteins of spore-coat of *B. atrophaeus* in these different culture conditions.

There are two major explanations for the presence of accentuated folds or

ridges extended along the long axis of the spore surface revealed by electron microscopy: nutrient limitation during spore formation and dehydration of the core in the dormancy state due to coat flexibility (CHADA *et al*., 2003; MALKIN; PLOMP 2011). Both factors are present in SSF. The major characteristic of SSF is a medium with low water content, which suggests that the ridge formation is a mechanical alteration resulting from environmental humidity. Dricks (2003) proposed that the coat and the cortex are sufficiently flexible to accommodate some volume changes without impairing the spore's integrity and that the gradient of cross-linking in the cortex somehow allows it to swell in conditions of high humidity without compromising the dryness of the core. Since *B. atrophaeus* spores are described with a length of 1.2-2.1 *μ*m and a diameter of 0.6-0.8 *μ*m, varying according the sporulation medium, the hydration state and the measurement technical (BUHR *et al.*, 2008; YANG *et al*., 2010; MALKIN; PLOMP, 2011), all spores sizes observed are within these values independent from the media source. The spores originated from sand SSF presented ridges on their surface, indicating a state of dehydration, and also had a width slightly larger, suggesting that in this case the hydration status did not affect the spore size.

It well knows that spore heat resistance is due to a number of different factors, including the dehydration of the spore core and the spore coat structure (DRIKS, 1999). It was not observed in this study. Maybe these alterations were too slight to be detected in the dry heat resistance test or the other factors that are not affected significantly by sporulation parameters and the composition of the medium, like the spore coat mineralization, the levels of dipicolinic acid and small acid soluble proteins (MELLY *et al.*, 2002; ROSE *et al.,* 2007), had carried greater influence than the first ones.

In conclusion, all the assays reported here demonstrated substantial heterogeneity among spores formed through SSF cultivation using different supports and agar sporulation, and no alterations were observed between usual agar sporulation medium and agar with soybean as substrate. These finds may be partially explained by Eijlander *et al.* (2011) when describes that environmental conditions influence gene transcription in bacterial populations and it can result in differences in gene expression levels in forespores of the same, clonal population of cells during the sporulation process. Differences in spore properties, such as give

rise to a heterogeneous spore population that may respond differently to germination triggers, or nutrients, which leads to heterogeneous germination and/or outgrowth behavior.

Although some papers have presented phenotypic alterations after culture in different media, no previous studies have compared spores of a standard strain using different sporulation methodologies and germination characteristics on the same substrates. The present study attempted an overview of *B. atrophaeus* SSF sporulation and its influence on germinative cells' phenotypes, and it proposes a new concept about this methodology involving bacterial adhesion and biofilm formation associated to phenotypic alterations. SSF support physical characteristics, water content, high-yield cell concentration, quorum sensing, microorganism starvation and multicellularity are some of the factors that may influence all the described alterations. Elucidation of the mechanisms remains to be carried out. It would be very interesting to determine these mechanisms owing to their potential application for industrial production improvement or new-products development by exploring new metabolic profiles from bacterial growing in solid state fermentation associated to biofilm production.

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CHAPTER V

GLYCEROL-BASED STERILIZATION BIOINDICATOR SYSTEM FROM *Bacillus atrophaeus*: DEVELOPMENT, PERFORMANCE EVALUATION, AND COST ANALYSIS

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ABSTRACT

The development of new value-added applications for glycerol is of worldwide interest because of the environmental and economic problems that may be caused by an excess of glycerol generated from biodiesel production. A novel use of glycerol as a major substrate for production of a low-cost sterilization biological indicators system (BIS; spores on a carrier plus a recovery medium) was investigated. A sequential experimental design strategy was applied for product development and optimization. The proposed recovery medium enables germination and outgrowth of heat-damaged spores, promoting a D_{160^oC} value of 6.6 ± 0.1 min. *Bacillus atrophaeus* spores production by solid-state fermentation reached a 2.3 ± 1.2 x 10^8 CFU/g dry matter. Sporulation kinetics results allowed this process to be restricted in 48 h. Germination kinetics demonstrated the visual identification of nonsterile BIS within 24 h. Performance evaluation of the proposed BIS against dry-heat and ethylene oxide sterilization showed compliance with the regulatory requirements. Cost breakdowns were from 41.8 (quality control) up to 72.8% (feedstock). This is the first report on sterilization BIS production that uses glycerol as a sole carbon source, with significant cost reduction and the profitable use of a biodiesel byproduct.

Keywords Glycerol; *Bacillus atrophaeus*; sterilization bioindicator; spores; recovery medium; solid-state fermentation

1. INTRODUCTION

Biological indicators systems (BIS) are used globally to monitor the efficacy of sterilization processes. BIS is a microbiological test system providing a defined resistance to a specified sterilization process. The system consists of a known

number of microorganisms, with a determinate resistance to the mode of sterilization, in or on a carrier and enclosed in a protective package plus a recovery medium that promotes the growth of any surviving test organism cells (FDA, 2007). BIS allows the qualitative evaluation, by visual identification, of a color change or turbidity of the substrate media caused by the growth of the inactivated bacteria. They are used primarily in the health care industry, but also in many other commercial and industrial applications. It is often necessary to monitor the effectiveness of the processes used to sterilize equipment, such as medical and non-medical devices, instruments, foods, medical or other microbial contaminated wastes, and other articles and materials (GILLIS *et al.,* 2010). The periodic utilization of bioindicators to monitor the sterilization processes used in the medical and industrial sectors has been a major requirement of regulatory agencies.

Conventional BIS has used dormant spores of some gram-positive bacteria such as *Bacillus* sp. (ISO 11138-4, 2006). Spores of *Bacillus atrophaeus* are used as a biological indicator (BI) to assess antiseptic and sterilizing products and procedures, such as low-temperature steam, dry-heat, plasma, UV radiation, electrothermal deactivation and microwaves (HALFMANN *et al.*, 2007; OLIVEIRA *et al.,* 2010), and in the development of dry heat sterilization cycles for spacecraft (KEMPF *et al.,* 2008). These spores also have biotechnological applications in water and wastewater treatments and systems (SZABO *et al.*, 2007), as a surrogate for *Bacillus anthracis* in biodefense (WEBER *et al.,* 2003) and as a potential adjuvant for veterinary rabies vaccine (OLIVEIRA-NASCIMENTO *et al.,* 2012).

It is known that all of the BI system components, such as spore characteristics, the carrier material on which the spores are deposited and the primary packaging and recovery media, have an impact on the BI performance (GILLIS *et al.,* 2010). The sporulation conditions significantly affect the spore properties, including germination capacity and resistance to heat treatment (NGUYEN THI MINH *et al.,* 2008) and different composition of the recovery medium may not have the same degree of ability to promote the growth of injured spores, both factors interfering with the efficacy of the BI system.

Glycerol or 1,2,3-propanetriol, a clear, odorless, viscous liquid, is a by-product from the biodiesel industry. Biodiesel, a renewable fuel, is produced from vegetable oils or animal fats through transesterification, generating about 10% *(w/w)*

glycerol. World biodiesel production is increasing exponentially and was at 19 billion liters in 2010, a 12% increase from 2009 (SHRANK; FARAHMAND, 2011). The excess of glycerol generated has a consequence in that its price is low and the substance may become an environmental problem since it cannot be disposed of in the environment (DA SILVA *et al.* 2009). It is a growing global concern to reduce the future environmental problems caused by the accumulation of glycerol and to make biodiesel production more profitable. So the development of new value-added applications for glycerol is of worldwide interest. Glycerol is a good source of carbon and energy for the growth of several microorganisms, and it may be suitable for the biotechnological production of bioindicators.

Solid-state fermentation (SSF) using sand as support was first described by Sella *et al.* (2012) as a process that provided a high-yield of *B. atrophaeus* spores. Sand was considered an inert support that enables the direct use of fermented material in BI, making the final product less expensive.

The current work proposes the development of a new and economical bioprocess for the production of a *B. atrophaeus* sterilization BI system (spores on a carrier plus a recovery medium) by using a glycerol as the sole carbon source.

2. MATERIALS AND METHODS

This study was executed in three steps: (a) development and optimization of the recovery medium; (b) development and optimization of spore production by SSF, and (c) performance evaluation of the developed BIS by comparing sporulation and germination kinetics as well as resistance characteristics and process costs, with a commercial BI (FIGURE 1).

2.1 Bacterial strain, spores suspension, and glycerol source

B. atrophaeus ATCC 9372 (batch: 1403349) was obtained from standard strains supplied by Instituto Nacional de Controle de Qualidade em Saúde (INCQS/MS, Brazil). Spores suspensions were utilized as seed and prepared with a

standard agar sporulation method. The inoculum was prepared from a suspension of vegetative cells grown for 18 h at 36°C in tryptic soy broth (TSB) and the cultures were grown in Roux flasks containing 400.0 mL of sporulation medium - 8.0 g/L yeast extract, 4.0 g/L nutrient broth, 0.05 g/L $MnSO_4.4H_2O$, 0.05 g/L $CaCl_2.6H_2O$ and 30.0 g/L agar. Sporulation was carried out at 36ºC for 7 days. Spores were harvested and suspended in cold and sterile 0.02 M calcium acetate solution adjusted to pH 9.7 with 1.4 g/L calcium hydroxide solution. The suspension was filtered through cotton and gauze tissue and centrifuged three times at 1,048×*g* for 20 min at 4°C. The spores' suspensions were subjected to a heat treatment (80°C, 10 min), which was lethal to vegetative cells but not to spores, and were stored at 4°C.

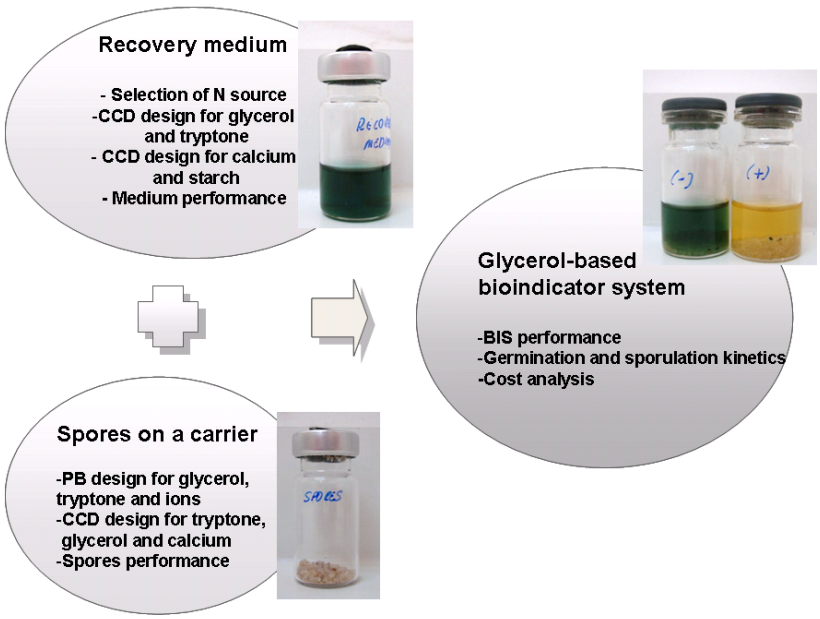

FIGURE 1 - ILLUSTRATIVE CHART FOR THE DESIGN AND PERFORMANCE EVALUATION OF GLYCEROL-BASED BIOINDICATOR (CCD= CENTRAL COMPOSITE DESIGN, PB= PLACKETT-BURMAN DESIGN, BIS=BIOINDICATOR SYSTEM)

SOURCE: The author (2012)

Three different brands of glycerol were used: (a) glycerol P.A. (Nuclear, Diadema, Brazil), ≥ 99,5% purity (b) glycerol for electrophoresis (Sigma-Aldrich, St. Louis, MO ,USA), ≥ 99,0% purity; and (c) glycerol commercial grade (Rioquimica,

São José do Rio Preto, Brazil), ≥ 95% purity.

2.2 Development and optimization of the BI recovery medium

2.2.1 Selection of the nitrogen source

Five different media with 10.0 g/ L glycerol, 0.02 g/L bromothymol blue, and different organic and inorganic nitrogen sources were formulated: (1) 5.6 g/L tryptone, (2) 4.8 g/L casein peptone, (3) 4.0 g/L ammonium sulfate, (4) 2.0 g /L ammonium sulfate, and 2.0 g /L ammonium nitrate, (5) 4.0 g/ L ammonium nitrate. The pH was adjusted with NaOH 0.1N to 7.0 and the media were autoclaved at 121°C for 15 min. These media were loaded in 3.0 mL- aliquot into borosilicate glass vials, stoppered with silicone rubber, and sealed with an aluminum seal. These studied recovery media were submitted to a viability assay and dry-heat resistance tests with commercial BI spore on strips (CPPI, Piraquara, Brazil).

2.2.2 Experimental designs for optimization

a) A 2^2 full-factorial central composite design (CCD) with four axial points (α=1.682) and two replications at the center points was employed to search for the optimal nitrogen source and glycerol concentrations. The amounts of the main components were as follows: 5.0 - 15.0 g/L glycerol and 2.0 - 10.0 g/L tryptone. The values of bromothymol blue (0.002g %) was fixed, and the pH was adjusted before sterilization to 7.0 (design and results are shown in TABLE1

b) A second 2^2 full-factorial central composite design (CCD) was employed to evaluate the soluble starch and $CaCl_2$ influence and their optimum concentrations. The amounts of the evaluated components were as follows: 0.5 - 2.0 g/L soluble starch and 0.1 - 0.3 g/L $CaCl_2$. The values of glycerol, tryptone, and bromothymol blue were fixed at the optimum, and the pH was adjusted before sterilization to 7.0.

TABLE 1 - 2^2 FULL-FACTORIAL CCD WITH UNCODED VALUES FOR GLYCEROL AND TRYPTONE, AND EXPERIMENTAL RESULTS FOR THE FIRST OPTIMIZATION OF *B. atrophaeus* BIOLOGICAL INDICATOR RECOVERY MEDIUM

	Variables		Results	
Assays	Glycerol *(g/L)*	Tryptone *(g/L)*	Viability *(%)*	$D_{160ºC}$ *(min)*
1	5.0	2.0	40	0
2	5.0	10.0	100	5.6 ± 0.1
3	15.0	2.0	70	0
4	15.0	10.0	100	5.6 ± 0.2
5	3.0	6.0	70	0
6	17.0	6.0	100	5.5 ± 0.1
7	10.0	0.3	20	0
8	10.0	12.0	100	6.9 ± 0.1
9(CP)	10.0	6.0	100	6.6 ± 0.2
10(CP)	10.0	6.0	100	6.6 ± 0.2
11(CP)	10.0	6.0	100	6.6 ± 0.1
CM	-	-	100	6.6 ± 0.2
F-ratio	4.73	34.31		
p-value	0.0818	0.0021		
R^2=91,99%		CP=Central point	CM=Commercial medium	

2.3 Development and optimization of spore production

2.3.1 Fermentation process

SSF was carried out in 250-mL Erlenmeyer flasks containing 50.0 g of support each. Washed commercial grade sand, washed once in tap water, twice in distilled water, dried in trays for 24 h at 90°C in an air oven and sieved to obtain a ~1.0-mm particle size (mesh, 14-20) was used as inert support. The support was impregnated with 13.0 mL of the tested medium, according to the sand's water-holding capacity. The pH was adjusted after sterilization with 0.1 M sterile NaOH to 7.0. Flasks were autoclaved at 121ºC for 15min. The inoculum size was 4% (*v/v* substrate). The inoculum was produced in 30 mL of TSB medium, inoculated with 100 µL of 10^9 colony-forming units per gram (CFU/g) spores suspension and incubated for 18 h at 36°C. Sporulation was carried out at 36ºC for 7 days. The

fermented material was dried in trays for 24 h at 65°C in an air oven. Spore detachment for counting was performed as previously described (SELLA *et al.,* 2012). SSF yield was available as CFU/g dry matter and spores resistance was determined by the $D_{160°C}$ value (decimal reduction time).

2.3.2 Experimental designs for optimization

a) A Plackett-Burman design (PLACKETT; BURMAN, 1946) was used to evaluate the effects of medium components on the spores yield and heat resistance. The variable factors- glycerol, tryptone, KH_2PO_4, $MnSO_4$, $CaCl_2$ and $MgSO_4$- were examined at two coefficient levels, low (-) and high (+), using a set of orthogonal contrasts. Central points were investigated in triplicate to identify any experimental anomalies (design and results are demonstrated in TABLE 2).

b) A 2^3 full-factorial CCD was used to optimize the three main components of the sporulation media: glycerol, tryptone, and calcium chloride. Independent factors were studied at five different levels with two replications at the center points. The amounts of the main components were as follows: 10.0 - 70.0 g/ L glycerol, 5.0 - 35.0 g/L tryptone, and 0.01 - 0.20 g/L $CaCl_2$.

2.4 BIS performance determination

The effectiveness of the developed formulation was checked by evaluating three different BIS batches using different brands of the raw material.

(a) Recovery medium

Performance was determined by spores growth viability and dry-heat resistance ($D_{160°C}$ value) after inoculation with *B. atrophaeus* commercial spore strips (CPPI, Piraquara, Brazil).

TABLE 2 - PLACKETT–BURMAN DESIGN MATRIX WITH UNCODED VALUES AND EXPERIMENTAL RESULTS TO INVESTIGATE THE EFFECTS OF THE SPORULATION MEDIUM COMPOSITION ON *Bacillus atrophaeus* SPORE YIELD AND HEAT RESISTANCE

	Variables						Results	
Assays.	Glycerol *(g/L)*	Tryptone *(g/L)*	KH_2PO_4 *(g/L)*	MnSO 4 *(g/L)*	$CaCl_2$ *(g/L)*	$MgSO_4$ *(g/L)*	Spores *(CFU/g dry matter)*	D_{160^oC} *(min)*
1	40.0	10.0	0.1	0	0	0	$2.1 \pm 0.2 \times 10^5$	1.5 ± 0.2
2	40.0	40.0	0	0.1	0	0	$1.6 \pm 0.4 \times 10^6$	4.2 ± 0.1
3	10.0	40.0	0.1	0	0.1	0.1	$9.1 \pm 0.6 \times 10^5$	3.5 ± 0.2
4	40.0	10.0	0.1	0.1	0	0.1	$5.5 \pm 0.8 \times 10^2$	1.5 ± 0.2
5	40.0	40.0	0	0.1	0.1	0	$1.5 \pm 0.1 \times 10^6$	4.5 ± 0.3
6	40.0	40.0	0.1	0	0.1	0.1	$3.1 \pm 0.3 \times 10^5$	3.3 ± 0.2
7	10.0	40.0	0.1	0.1	0	0.1	$1.6 \pm 0.4 \times 10^2$	3.5 ± 0.1
8	10.0	10.0	0.1	0.1	0.1	0	$9.4 \pm 0.3 \times 10^5$	3.8 ± 0.2
9	10.0	10.0	0	0.1	0.1	0.1	$1.5 \pm 0.2 \times 10^6$	4.4 ± 0.3
10	40.0	10.0	0	0	0.1	0.1	$2.1 \pm 0.4 \times 10^6$	4.0 ± 0.1
11	10.0	40.0	0	0	0	0.1	$1.3 \pm 0.2 \times 10^6$	4.5 ± 0.1
12	10.0	10.0	0	0	0	0	$1.9 \pm 0.1 \times 10^6$	4.0 ± 0.3
13(CP)	25.0	25.0	0.05	0.05	0.05	0.05	$9.0 \pm 0.6 \times 10^5$	3.4 ± 0.2
14(CP)	25.0	25.0	0.05	0.05	0.05	0.05	$8.6 \pm 0.4 \times 10^5$	3.5 ± 0.1
15(CP)	25.0	25.0	0.05	0.05	0.05	0.05	$9.4 \pm 0.3 \times 10^5$	3.5 ± 0.2
Effect (*D*)	-1.1630	-0.8128	-7.5293	-1.4954	2.6393	-1.5598		
F-ratio	1.3526	0.6606	56.6899	56.6900	6.9659	2.4330		
p-value	0.2829	0.4431	0.0001	0.1785	0.0335	0.1628		

R^2=*94.1* CP=Center Point.

(b) Fermented medium with spores

Biological indicators were prepared by diluting the dried fermented product with sterilized dry sand to obtain ~10^6 CFU spores/g and filling 7.0 mL borosilicate glass vials with 1.0 g of material. The loaded vials were closed with silicone rubber stoppers and sealed with aluminum seals. After the dry-heat resistance test, the vials

were cultured with 3.0 mL of commercial recovery medium (CPPI, Piraquara, Brazil). Germination and growth of the spores were observed over 24 up to 48 h incubation time.

(c)Biological indicator system

Dry-heat and ethylene oxide resistance was tested for developed BIS, and a commercial BI system (CPPI, Piraquara, Brazil) was used as a control.

2.4.1 Viability

For the viability assay, germination and growth, the recovery medium was inoculated with spores (10 units) that was incubated at 36°C and observed over 24 to 48 h incubation time. Positive results were indicated by changes in the color (green to yellow) and turbidity of the recovery medium.

2.4.2 Dry-heat resistance test

The *D*-value is defined as the time taken, in a specified set of conditions, for the spore population to decrease by 90% or 1 log (FDA, 2007). The *D*-value was determined by fraction-negative analysis, using the Limited Spearman–Kaber Method (LSKM) (USP 31, 2008). Dry heat conditions were as follows: 160°C for 8, 12, 16, 20, 24, 28, 32, 36, and 40 min in a tabletop air oven. Temperature was monitored using a K-type thermocouple 1.5 x 400mm coupled to a digital thermometer SALCASTERM mod1200-2 (SALCAS, São Paulo, Brazil). A survivor curve was constructed for BIS resistance determination based on the spores' direct enumeration. The survival/kill times were determined by the following formulae: Survival Time (log of population –2) x *D*-value, and Kill Time = (log of certified population +4) x *D*-value.

2.4.3 Ethylene oxide resistance test

SSF glycerol-based dry fermented were diluted with sterile inert silica sand-quartz to achieve 10^6 CFU/g and 1.0 g was conditioned into a gas-permeable package and sealed. Ethylene oxide (EtO) sterilization was performed using 650 mg/L at 55°C, 55% humidity, with a partial cycle time of 15 min. D_{EtO} values (decimal reduction time) were calculated based on logarithmic curve inactivation, after determining the number of surviving spores. A commercial EtO BIS (BROWNE, Leicester, UK) was used as control and had the following labeled characteristics: D_{EtO}= 3.4 min and initial number of spores N_0 = 1.8 x 10^6 CFU/unit.

2.4.4 Sporulation kinetics

SSF was carried out under the optimum conditions. Standard agar sporulation was done as previously described in “Bacterial strain, spores suspension and glycerol source”. Growth was monitored by total cell and spore counts (total cells – spores = vegetative cells). Total cells and 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°C. For spore counts the sample was submitted to heat treatment (80 - 85°C, 10 min) to eliminate vegetative cells. Time course results of spores formation was compared with sporulation on agar. Glycerol consumption profiles on SSF were analyzed by high performance liquid chromatography in a Varian HPLC using a Shodex KS 801 column, with refraction index detector, mobile phase purified water, 0.5 mL/min, 70 °C. The samples were centrifuged at 10,000 × *g* and filtered with 0.22 µm PVDF membranes, diameter 13 mm (Millipore, Billerica, MA). Glycerol was identified by comparing retention times with a standard. For pH determination, 1.0 g sample was diluted with 9.0 mL distilled water, blended for 2 min, and then centrifuged. The supernatant liquid was used for the assay.

2.4.5 BI recovery kinetics

Unheated BI (spores in vials), and those that were subjected to sublethal heating (160°C for 15 min),were inoculated into developed recovery medium and cultured for 24 h at 36°C. Measurements of colony forming units were used to monitor the time course of growth in recovery medium. The 160°C dry-heat exposure time of 15 min was calculated based on the optimized medium *D*-value obtained for unheated spores as the time to kill ~50% of the initial population (10^6 spores). Glycerol consumption profiles were observed by high performance liquid chromatography as described.

2.5 General conditions and statistical analysis

The assays were carried out in triplicate. Microbiological assays were conducted under aseptic conditions in a Good Manufacturing Practices (GMP) certified laboratory ISO 5 clean room. Experimental designs and analyses were carried out using Statistic 8.0 software (Stat Soft, Tulsa, OK) and the SGWIN program (Stat Graphic Plus for Windows version 5.0, Statistical Graphics, Cheshire, CT). Analysis of variance was used to evaluate the model at 95.0% of significance level. The quality of the polynomial model equation was judged statistically using the coefficient of determination (R^2), and its statistical significance was determined by an *F* test. The validation of the optimization of medium composition was carried out by the analysis of three tests using the optimized conditions and the results analyzed statistically. Commercial *B. atrophaeus* Biological Indicators (IB Calor Seco, CPPI, Piraquara, Brazil) were used as controls in all assays and for comparative results. The commercial BI samples had the following labeled characteristics: $D_{160°C}$ = 6.5 ± 0.2 min and initial number of spores $N_0 = 3.6 \pm 0.8\ 10^6$ CFU/unit.

3. RESULTS

3.1 Development and optimization of the BIS recovery medium

For glycerol-based medium development the results of nitrogen sources selection demonstrated that only organic sources have a positive effect over heat injured spores recovery after 48 h incubation time. Besides, all media had promoted the germination and outgrow of unheated spores. Glycerol media Glycerol media with ammonium sulfate and ammonium nitrate promoted later heat damaged spores growth (only after 7 days of incubation time). The spores D_{160^oC} values observed with the addition of organic nitrogen sources were 5.8 ± 0.1 min for glycerol plus tryptone medium, 5.4 ± 0.2 min for glycerol plus casein peptone medium and 6.5 ± 0.1 min for the control medium (TABLE 3).

TABLE 3 - *B. atrophaeus* SPORES' VIABILITY AND DRY-HEAT RESISTANCE IN GLYCEROL BASED-MEDIA WITH DIFFERENT NITROGEN SOURCES

Glycerol (10 g/L) Based Media with addition of:	pH after terilization	Heat-damaged spores viability 48h (%)	Viability 48h *(%)*	D_{160^oC} *(min)*
Tryptone (5.6 g/L)	7.1	100	100	5.8 ± 0.1
Casein peptone (4.8 g/L)	6.9	100	100	5.4 ± 0.2
Ammonium sulphate (4.0 g/L)	6.8	0	100	0
Ammonium sulphate (2.0 g/L) and ammonium nitrate(2.0 g/L)	6.9	0	100	4.5 ± 0.2
Ammonium nitrate (4.0 g/L)	6.9	0	90	0
Commercial medium	7.0	100	100	6.5 ± 0.1

The optimum glycerol concentration in the recovery medium (the main carbon source) and nitrogen source concentration (tryptone) were determined. The results are shown in TABLE 1. The value of R^2 was 91.99% suggesting the strong significance of the model. The best result obtained was D_{160^oC} = 6.9 ± 0.1min for the concentrations of 12.0 g/L of glycerol and 10.0 g/L of tryptone. The relationship between the experimental variables and the heat resistance of spores was plotted on a three-dimensional surface graph (FIGURE 2a) and fitted to a second-order polynomial equation describing the surface:

$$D_{160^\circ C} \text{ value} = -8.63 + 1.35.\text{glycerol} + 1.50.\text{ tryptone} - 0.06.\text{glycerol}^2 + 0.001\text{glycerol}.\text{tryptone} - 0.07.\text{tryptone}^2 \quad (1)$$

Where the optimum calculated $D_{160^\circ C}$-value was 6.9 min.

The starch and $CaCl_2$ optimization results showed a significant effect of starch (*p*-value=0.0001) and no significant effects of $CaCl_2$ presence in the developed medium (*p*-value =0.9678) (TABLE 4).

TABLE 4 - A 2^2 FULL-FACTORIAL CCD WITH UNCODED VALUES FOR SOLUBLE STARCH AND $CaCl_2$, AND EXPERIMENTAL RESULTS FOR THE SECOND OPTIMIZATION OF *B. atrophaeus* BIOLOGICAL INDICATOR RECOVERY MEDIUM

Assays	Variables		Results	
	Soluble Starch *(g/L)*	$CaCl_2$ *(g/L)*	Viability *(%)*	$D_{160°C}$ *(min)*
1	5.0	3.0	100	5.9 ± 0.1
2	5.0	1.0	100	5.8 ± 0.2
3	20.0	1.0	100	6.7 ± 0.1
4	20.0	3.0	100	5.9 ± 0.1
5	12.5	3.4	100	5.9 ± 0.2
6	23.1	2.0	100	6.6 ± 0.2
7	12.5	0.6	100	6.6 ± 0.1
8	1.8	2.0	100	5.9 ± 0.1
9 (CP)	12.5	2.0	100	5.9 ± 0.2
10(CP)	12.5	2.0	100	5.8 ± 0.2
11(CP)	12.5	2.0	100	5.8 ± 0.1
CM	-	-	100	6.6 ± 0.2
Effect	0.4724	-0.4284		
F-ratio	141.77	0.0018		
p-value	0.0001	0.9679		

R^2= 97,06%
CP=Central point CM=Control medium

The contour response surface plot illustrate the behavior of the $D_{160°C}$ value (FIGURE 2b). Application of the response surface methodology-RSM gave the following regression equation, which is an empirical relationship between the $D_{160°C}$-value and the tested variables:

$$D_{160°C} = 5.83 + 1.02\ starch - 0.29\ starch^2 \qquad (2)$$

Where the optimum calculated $D_{160°C}$-value was 6.7 min for 2.0 g/L soluble starch without $CaCl_2$ addition.

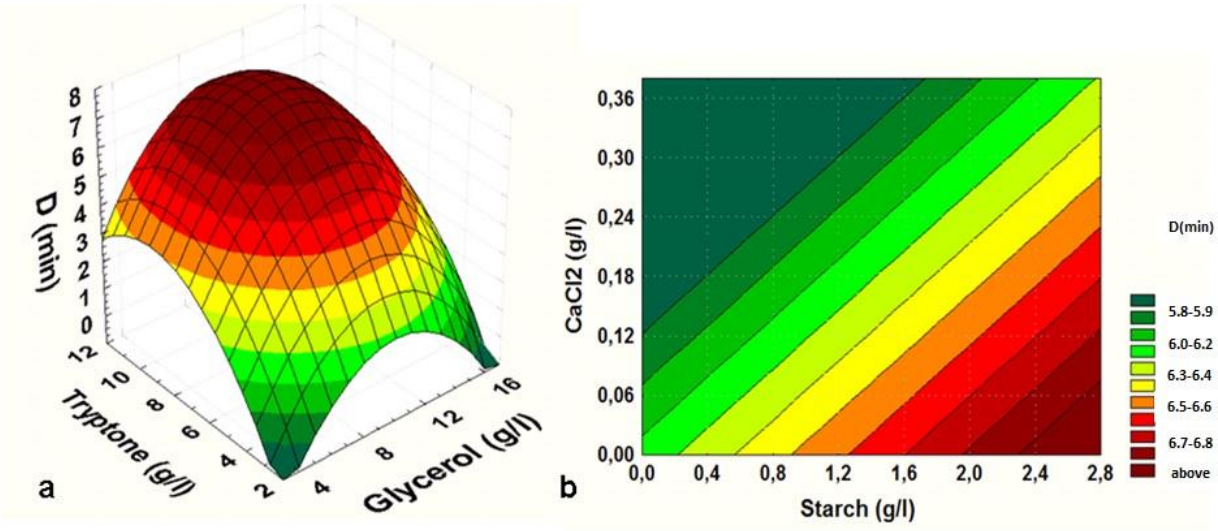

FIGURE 2- ESTIMATED RESPONSE SURFACE PLOTS SHOWING THE EFFECTS OF TRYPTONE AND GLYCEROL (A) AND $CaCl_2$ AND SOLUBLE STARCH (B) ON THE RECOVERY OF *Bacillus atrophaeus* SPORES, AS DETERMINED BY $D_{160°C}$
SOURCE: The author (2012)

In order to check the model adequacy, three experiments were performed with the optimized medium: 12.0 g/L glycerol, 10 g/L tryptone, 2.0 g/L soluble starch, and 0.025 g/L bromothymol blue, and their results were compared with those of the reference medium. The optimized medium attained a $D_{160°C}$ value of 6.6 $\pm$ 0.1 min, which is relatively close to the theoretically predicted value and the industrialized control recovery medium (6.7 $\pm$ 0.1 min).

3.2 Development and optimization of spores' production

The Plackett-Burman assays were conducted to study influence of the composition of the sporulation medium. The results demonstrated spore yield variation from 10^2 up to 10^6 CFU/g and the dry-heat resistance ($D_{160°C}$ value) variation within 3.0 min (TABLE 2). The ANOVA analysis demonstrated the statistical significance of the components of the sporulation medium on spore yield and dry-heat resistance by observing the mean square and estimating the experimental error. For spores yield, only potassium and calcium showed significant influence,

negative (*p*-value = 0.0001 and effect = -7.53) and positive (*p*-value = 0.0335 and effect = 2.64), respectively. In the case of heat resistance, four factors presented *p*-values < 0.05 (potassium, calcium, glycerol, and tryptone) indicating that they produce significant effects on the spores dry-heat resistance (TABLE 5). Potassium and glycerol demonstrated a negative influence (FIGURE 3; FIGURE 4). In this way, the first one was removed from the medium and the glycerol concentration was reduced in the next experiments.

TABLE 5 - ANALYSIS OF VARIANCE FOR $D_{160°C}$ DETERMINED FROM THE PLACKETT-BURMAN DESIGN RESULTS FOR MINERAL COMPOSITION OF THE SPORULATION MEDIUM

Source	Sum of Squares	Df	Mean Square	*F*-ratio	*p*-value
Glycerol	2.00	1	2.00	19.89	0.0029
Tryptone	1.55	1	1.55	15.39	0.0057
KH_2PO_4	5.72	1	5.72	56.80	0.0001
$MnSO_4$	0.08	1	0.08	0.83	0.3924
$CaCl_2$	1.69	1	1.69	16.83	0.0046
$MgSO_4$	0.08	1	0.08	0.83	0.3935
Total error	0.70	7	0.10		
Total (Corr.)	12.07	13			

R-squared = 94.17%

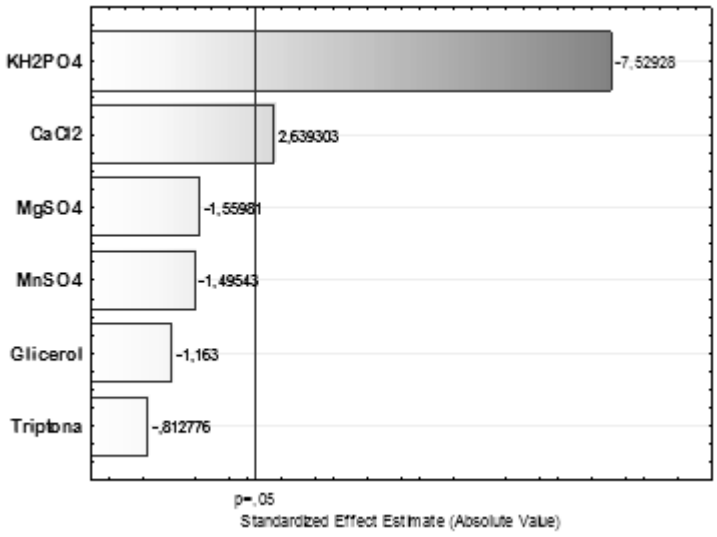

FIGURE 3 - PARETO CHART OF THE EFFECTS OF SOLID-STATE FERMENTATION VARIABLES ON *Bacillus atrophaeus* SPORE PRODUCTION (R-SQUARED = 91.44%)
SOURCE: The author (2012)

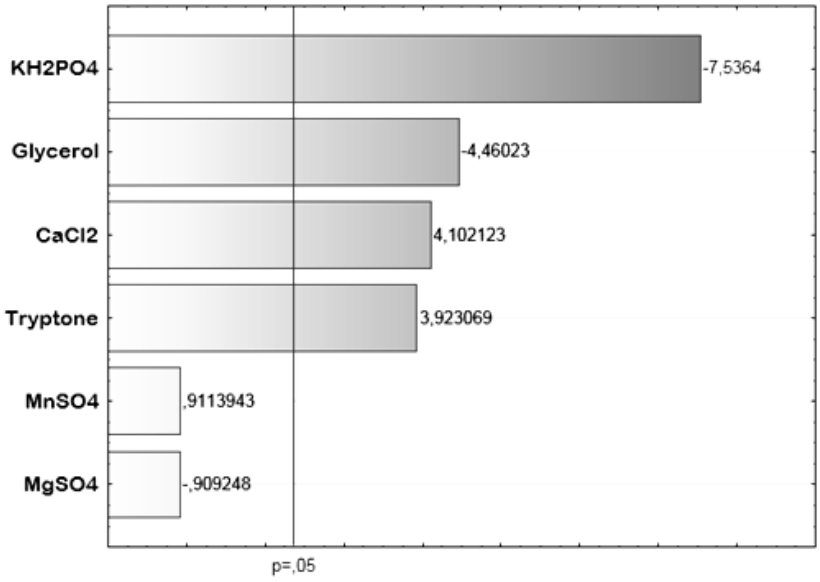

FIGURE 4 - PARETO CHART OF THE EFFECTS OF SOLID-STATE FERMENTATION VARIABLES ON *Bacillus atrophaeus* ON SPORES' DRY-HEAT RESISTANCE

SOURCE: The author (2012)

For sporulation medium evaluation, it is important to consider quantitative aspects such as spores yield and qualitative aspects such as heat-resistance. A central composite design was employed to determine the optimal levels of glycerol, tryptone, and $CaCl_2$ and the effect of their interactions (TABLE 6). The ANOVA analysis of results indicated that among the independent variables, $CaCl_2$ had no significant effects (*p*-value = 0.0714 for spore yields and 0.7937 for heat-resistance) and negative coefficients, suggesting that its absence increased spore production and resistance. In this model, R^2 was 91.83% and 95.79% for spores yield and heat-resistance, respectively. The results were plotted (FIGURE 5) and fitted with the following second-order polynomial:

Spores (CFU/g dry matter) = $3.3 \times 10^7 + 6.7 \times 10^5$glycerol $- 1.6 \times 10^4$tryptone $- 1.9 \times 10^4$ glycerol^2 $+ 5.3 \times 10^3$ glycerol.tryptone $- 7.7 \times 10^3$ tryptone^2 *(3)*

and,

$D_{160°C}$ = 4.7 + 0.03 glycerol – 0.017 tryptone – 0.00087 glycerol^2 + 0.00014 glycerol.tryptone + 0.00012 tryptone^2 *(4)*

The models predicted that the optimal values for the test factors of variables were glycerol = 25.0 g/L and tryptone = 12.0 g/L. The maximum predicted value of spore yield 3.8 x 10^7 CFU/dry matter and for heat-resistance $D_{160°C}$ = 4.9 min.

TABLE 6 - A 2^3 FULL-FACTORIAL CCD WITH UNCODED VALUES FOR GLYCEROL, TRYPTONE AND CALCIUM, AND EXPERIMENTAL RESULTS FOR THE OPTIMIZATION OF *B. atrophaeus* SPORULATION MEDIUM

	Variables			Results	
Assay	Glycerol *(g/L)*	Tryptone *(g/L)*	Calcium chloride *(g/L)*	Spores *(CFU/g dry matter)*	$D_{160°C}$ *(min)*
1	5.0	10.0	0.01	4.0 x 10^7	4.6 ± 0.1
2	5.0	10.0	0.20	2.1 x 10^7	4.6 ± 0.2
3	5.0	70.0	0.01	3.4 x 10^6	4.3 ± 0.1
4	5.0	70.0	0.20	8.0 x 10^6	3.6 ± 0.3
5	35.0	10.0	0.01	3.1 x 10^7	4.6 ± 0.2
6	35.0	10.0	0.20	1.5 x 10^7	4.8 ± 0.1
7	35.0	70.0	0.01	6.7 x 10^6	4.2 ± 0.3
8	35.0	70.0	0.20	8.9 x 10^6	4.4 ± 0.1
9	0.00	40.0	0.11	1.3 x 10^7	3.5 ± 0.2
10	45.2	40.0	0.11	3.5 x 10^7	4.0 ± 0.3
11	20.0	0.0	0.11	3.3 x 10^7	4.9 ± 0.1
12	20.0	90.5	0.11	3.5 x 10^5	4.3 ± 0.2
13	20.0	40.0	0.00	2.3 x 10^7	4.9 ± 0.1
14	20.0	40.0	0.27	1.3 x 10^7	5.0 ± 0.1
15(CP)	20.0	40.0	0.10	3.5 x 10^7	4.3 ± 0.2
16(CP)	20.0	40.0	0.10	3.7 x 10^7	4.2 ± 0.3
17(CP)	20.0	40.0	0.10	3.3 x 10^7	4.3 ± 0.1

CP=Center point

Finally, three tests were carried out under the optimized conditions to validate the model. The experimentally derived $D_{160°C}$ value was 4.8 ± 0.2 min and N_0 = 2.3 ± 1.2 x 10^8 CFU/g dry matter. The results of the validation of the regression equations obtained with the response surface methodology-RSM demonstrated a not statistically significant variation among the three batches produced with different brands of glycerol (*p*-value = 0.7075).

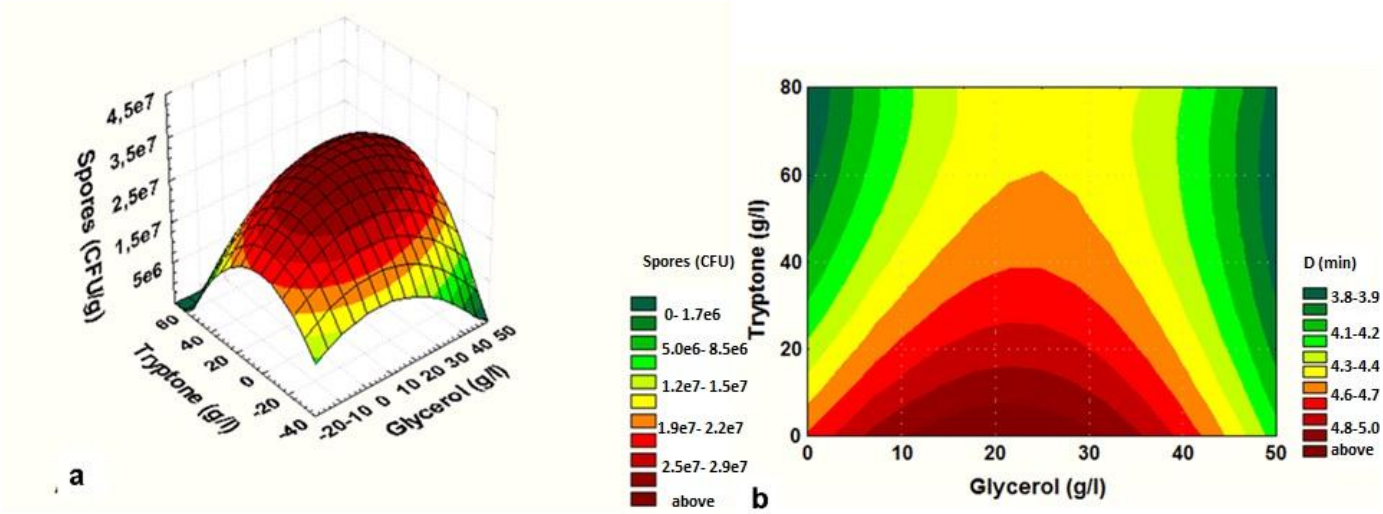

FIGURE 5 - ESTIMATED RESPONSE SURFACE PLOTS SHOWING THE EFFECTS OF TRYPTONE AND GLYCEROL ON SPORES YIELD (A) AND DRY-HEAT RESISTANCE (B) OF *Bacillus atrophaeus* SPORES, AS DETERMINED BY CFU/g DRY MATTER - AND $D_{160°C}$ RESPECTIVELY

SOURCE: The author (2012)

3.3 Performance evaluation of the developed BI system'

There are international references regulations recommending BI requirements (ISO 11138, 2006; FDA, 2007; USP 31, 2008). Besides the recommended parameters (viable spore population assay ~ 10^6CFU/unit and resistance characteristics: $D_{160°C}$ value ≥ 3.0 min and survival time ≥12.0 min, and D_{EtO} value ≥ 3.0 min and survival time ≥15.0 min), the sporulation and germination kinetics were determined. The survivor curve was also plotted to elucidate the process characteristics and to evaluate the effect of the sterilization process on the developed BIS.

3.3.1 Sporulation kinetics

A time course of spore production using the optimized medium composition was performed and compared with the sporulation on standard agar medium (FIGURE 6).

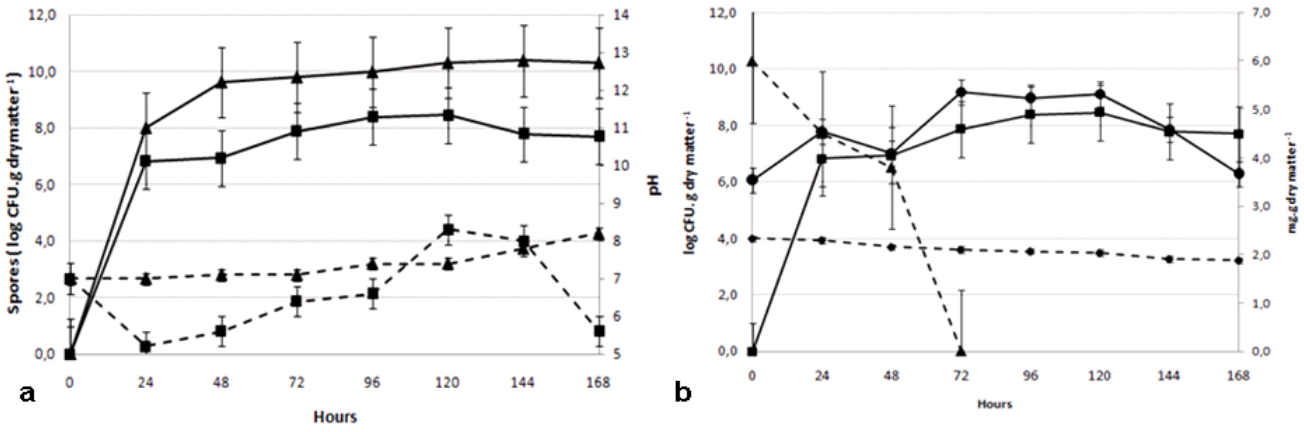

FIGURE 6 - SPORULATION KINETICS OF *B. atrophaeus:* (a) SOLID-STATE FERMENTATION ON OPTIMIZED GLYCEROL BASED SPORULATION MEDIUM (■) AND ON STANDARD AGAR MEDIUM (▲), AND pH MEASUREMENT (--), (b) OPTIMIZED SOLID-STATE FERMENTATION PROCESS SHOWED SPORES (■) AND GERMINATIVE CELLS (●), AND GLYCEROL (--▲--) AND PROTEINS (--◆--) CONCENTRATION

SOURCE: The author (2012)

It was observed that maximal spore yield 2.9 x 10^8 CFU/g dry matter was achieved after 120 h of SSF; 2.5 x 10^{10} CFU/g dry matter of spores were reached at 144 h for standard agar medium. A pH increase during all incubation time was observed only for agar sporulation. The HPLC analysis indicated that the strain is able to consume it completely in the first 72 h (FIGURE 6 b).

3.3.2 Germination kinetics

The kinetics of spore germination and cellular growth is presented in

FIGURE 7. In all conditions, there was a rapid cell growth from 0 to 3 h after the start of incubation and then a slower growth until 15-18 h. Visual detection of color change and turbidity caused by microbial growth and/or cumulative metabolic activity in the glycerol-based recovery medium was observed after 18 h for no damaged spores (4.1 x 10^7 CFU/mL) and 24 h for injured spores inoculum (3.7 x 10^7 CFU/mL). For the control medium, the corresponding times were 12 h and 15 h, respectively.

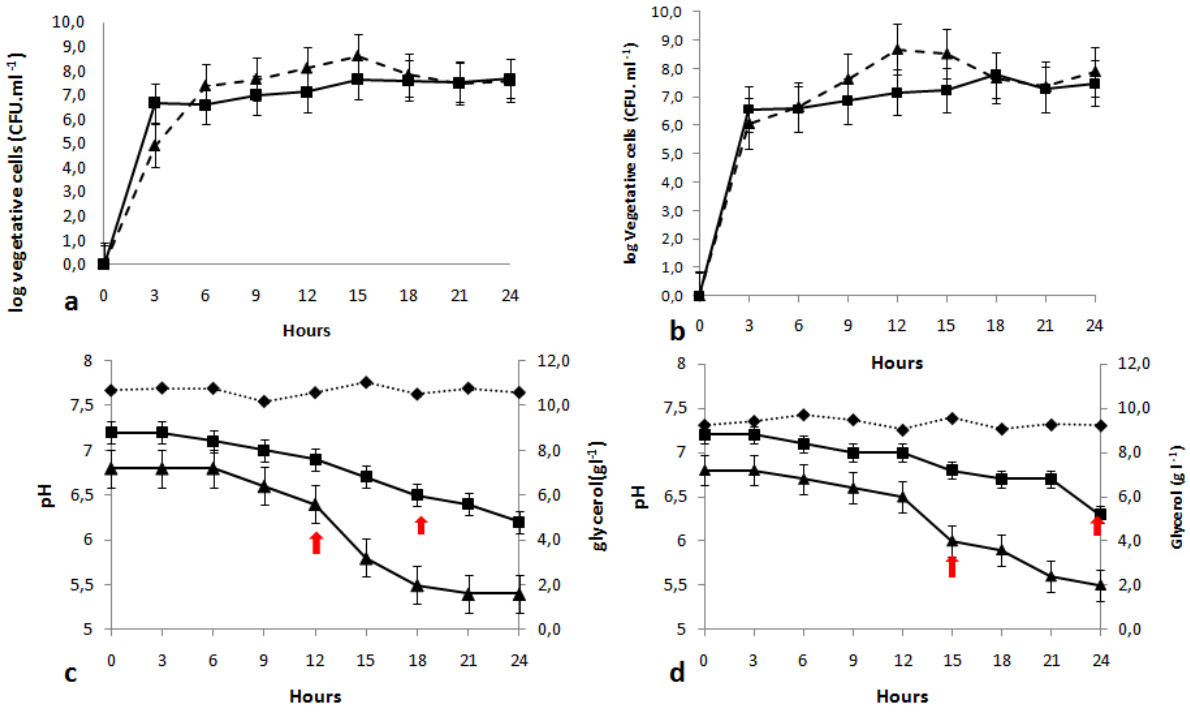

FIGURE 7 - GROWTH, GLYCEROL CONSUMPTION, AND pH KINETICS OF *Bacillus atrophaeus* SPORES IN THE BIOLOGICAL INDICATOR RECOVERY MEDIUM BEFORE (**a** AND **c**) AND AFTER (**b** AND **d**) HEAT DAMAGE AT 160°C FOR 15 MIN. BACTERIA WERE ON OPTIMIZED GLYCEROL + TRYPTONE BI RECOVERY MEDIUM (■) OR COMMERCIAL IB RECOVERY MEDIUM AS A REFERENCE (▲). IN (C) AND (D), GLYCEROL IS INDICATED BY DOTTED LINES. THE ARROWS INDICATE VISUAL DETECTION OF COLOR CHANGE AND TURBIDITY DUE TO MICROBIAL GROWTH

SOURCE: The author (2012)

3.3.3 BIS sterilization resistance

Three lots of newly developed IB were produced and tested, and all three gave satisfactory results: N_0 = 1.2 ± 0.6 × 10^6 CFU/unit and viability = 100% growth

in a 24 h incubation time. The determination of the $D_{160°C}$ -value was performed by the Limited Spearman-Kaber (LSK) and Survival Curve Methods (FIGURE 8) and the obtained results were $D_{160°C}$ = 3.1 ± 0.1 min, survival time = 12.2 min and kill time = 31.3 min, and $D_{160°C}$ = 4.6 ± 0.1 min , survival time = 18.8 min and kill time = 46.4 min, respectively. Both results are in accord with the recommended parameters ($D_{160°C}$ value ≥ 3.0 min). Control commercial BIS results were $D_{160°C}$ = 6.5 ± 0.2 min, survival time = 29.9 min and kill time = 68.9 min for LSK method and $D_{160°C}$ = 6.1 ± 0.1 min, survival time = 26.2 min and kill time = 62.8 min for survival curve method.

The oxide ethylene resistance was demonstrated by D_{EtO}= 5.6 ± 0.6 min, survival time = 21.2 min and kill time = 64.5 min results. Control commercial BIS results were D_{EtO}= 3.1 ± 0.1 min, survival time = 12.8 min and kill time = 32.8 min.

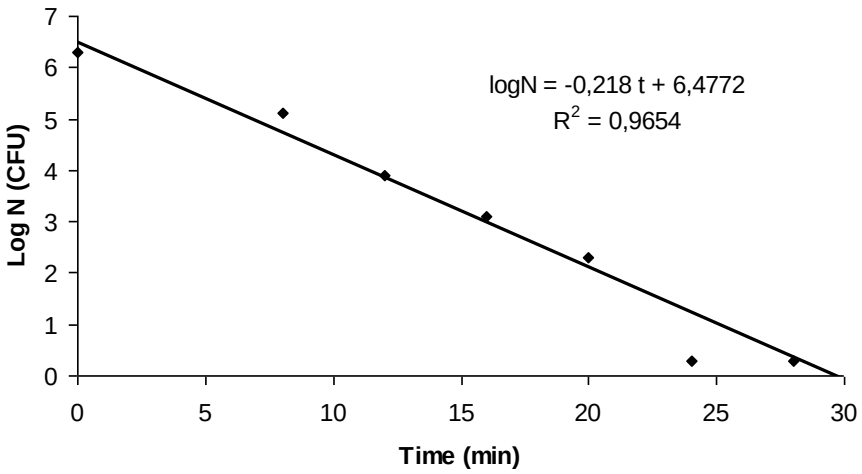

FIGURE 8 - DRY-HEAT LOG LINEAR SURVIVAL CURVE OF *Bacillus atrophaeus* BIOINDICATOR SYSTEM PRODUCED WITH GLYCEROL AS ONLY CARBON SOURCE WHEN SUBMITTED TO 160°C

SOURCE: The author (2012)

3.4 Cost analysis

Preliminary comparative production costs between conventional BIS and the developed glycerol BIS are demonstrated in TABLE 7. The minimum costs reduction

was 41.8% for the quality control tests and the maximum was for the feedstock (72.8%). The process cycle time was reduced by 44.8% with corresponding reduction in direct labor and use of equipment.

TABLE 7 - COMPARATIVE PRELIMINARY COST OF PRODUCTION TO OBTAIN 50.000 *B. atrophaeus* STERILIZATION BIOLOGICAL INDICATOR SYSTEM -BIS UNITS (CORRESPONDING TO ONE BATCH) BETWEEN THE DEVELOPED GLYCEROL-BASED BIS AND THE CONVENTIONAL BIS

Description	Sporulation Process			Recovery medium			BI system		
	SSF/ Glycerol based medium	Agar/ Comm. medium	Cost Reduction (%)	Glycerol based medium	Comm. medium	Cost Reduction (%)	Developed	Conv.	Cost Reduction (%)
Raw materials (US$)	10.8	137.0	92.2	175.0	547.0	68.0	185.8	684,0	72.8
Direct labor (hours)	50	98	48.9	8	8	0	58	106	45.3
Equipment (hours)	273	613	55.5	52	52	0	325	665	51.1
Quality control (US$)	217	405.5	46.5	44.5	44.5	0	261.5	450	41.8
Process cycle time (days)	16	29	44.8	-	-	-	16	29	44.8

Comm. =Commercial Conv. =Conventional

4.0 DISCUSSION

Recovery conditions have been shown to influence the heat resistance of *Bacillus* spores. It has been recognized that the recovery of damaged bacterial spores depends on the medium in which the spores are cultured (LÒPEZ *et al.,* 1997). Commercial recovery growth media are typically composed of water, protein extracts or digests, carbohydrates, minerals, and a pH indicator to create optimal conditions for injured spores' outgrowth, so the alternative glycerol-based recovery medium composition should provide the same conditions, as demonstrated by organic nitrogen source addition to the medium. Since the killing of *Bacillus* spores

by dry heat is accompanied by the accumulation of DNA damage (SETLOW, 2007), a mild thermal treatment may affect some enzymes synthesis. Elmerich and Albert (1971) reported that *Bacillus*, which lacks glutamate dehydrogenase, glutamate synthase, and either glutamine synthetase, were unable to consume ammonium as nitrogen source. DNA repair systems involved in the repair of dry-heat damage to spore's DNA may explain the later assimilation of the inorganic nitrogen sources. Calcium, because of its capacity to induce acid-soluble spore proteins formation, helps in regenerating the DNA of damaged cells (SETLOW, 2007). Starch, as an absorbent of free acids and some inhibitors, is reported as a favorable component supply for a recovery medium (PENNA *et al.*, 2000). The improvement of the germination and outgrow capacity of the glycerol-based recovery medium by the soluble starch addition is in agreement with Brown and Gaze (1988), Lopez *et al.* (1997) and Penna *et al.* (2000). The nonsignificant effects of calcium that were observed in the present study, contradicting Sasaki *et al.* (2000), may have been caused by the additional calcium, which is present in the tested spore strips. Dry-heat resistance results obtained from the developed BI recovery medium are in agreement with the official standards for dry-heat sterilization BI, which recommend a D_{160^oC} value ≥ 3.0 min (FDA, 2007; ISO-11138, 2006; USP 31, 2008). This fact demonstrates the glycerol-based recovery medium could replace the commercial one.

An optimum sporulation medium has to provide the maximization of spores yield associated with thermal resistance improvement. Under nutrient sources limitation and high cells density *B. atrophaeus* can sporulate by forming a highly resistant endospore, which is later released by lyses of the mother cell (PIGGOT; LOSICK, 2002). The sporulation conditions such as heat resistance and germination capacity determine the characteristics of the formed spores (ROSE *et al.*, 2007). In addition, it was cited that divalent ions induce sporulation and increase the thermal resistance: calcium as key element for the development of resistance by inducing specific genes involved in the synthesis of the spore coat and potassium, and manganese by stimulating the uptake of potassium (CAZEMIER *et al.,* 2001; HORNSTRA *et al.,* 2009). The negative effect of potassium, manganese, and magnesium on spores yield and resistance are in disaccord with Shi and Zhu (2006), which produced *B. subtilis* in a distillery effluent and Cazemier *et al.* (2001) for

spores' resistance, which were produced in nutrient agar supplemented with ions. However, the observed calcium influence is in agreement with Hornstra *et al.* (2009), who produced spores of *B. subtilis* 168 cells in minimal defined medium with an increasing amount of Ca^{2+}. It was demonstrated that ion traces, which are present in tryptone are enough to provide *B. atrophaeus* heat resistant spores' formation. The results from the obtained model for spores' production are in agreement with the predicted value, since a regulatory standard (USP 31, 2008) allows a 50% up to 300% variation on spore count, and it influences the *D* value calculation. No similar studies were published with which the obtained results could be compared. The optimized process of spore production resulted in high heat-resistant spores' level that can be used for *B. atrophaeus* spores production for other applications, e.g., water and wastewater treatments.

Sporulation kinetics' demonstrated that SSF promoted a drastic pH decrease in the first 24 h corresponding to the exponential growth period, due to organic acids released during initial glycerol metabolization (similar to the carbohydrate metabolization pathway), followed by an increase until 120 h, corresponding to the maximum spore yield. This increase in pH is justified by the reassimilation of the acid products and increase in protein turnover (caused by the extensive degradation and resynthesis of the proteins from the mother cell) (DE VRIES *et al.*, 2005; MANDELSTAM; WAITES, 1968). A similar sporulation profile was described by Monteiro *et al.* (2005) for *B. subtilis* cultivated in a commercial sporulation medium. The glycerol consumption kinetics confirmed the *B.atrophaeus* suitability to employ the glycerol as the sole carbon source. The high number of vegetative cells after 72 h up to120 h incubation time demonstrates that not all cells produced spores; but there is the possibility of spore germination. According to Veening *et al.* (2006), *Bacillus* sp. uses a bet-hedging strategy, whereby in the same culture only some cells enter into the sporulation process, whereas others use alternative metabolites to continue growth, and it leads to two distinguishable cell types: sporulating and nonsporulating cells. The number of vegetative cells declined after 144 h, but this was not accompanied by an increment in the number of spores, indicating cell death, which may explain the decrease in pH during the same period. Sporulating cells are able to kill the cells that have not yet initiated this process, and this phenomenon is usually responsible for the reduction in cell density of a sporulating culture

(GONZALEZ-PASTOR *et al.,* 2003). The continuous decrease of protein concentration may be caused by its consumption, although it was not possible to differentiate the presence of proteins in the medium derived from produced enzymes and/or cell lyses. Sporulation kinetics results also allowed the sporulation process to be restricted in 48 h.

When evaluating a BI system for detection of its efficacy it is critical to understand that stressed microorganisms (post sublethal sterilization exposition) do not have the same responses to growth as the nonheat-damaged microorganisms. It is not unusual for heat-damaged microorganisms to exhibit an inhibition of growth or a retarded growth (WESCEIE *et al.*, 2009). The spores' germination kinetics demonstrated that recovery medium allowed the conditions for injured spores germination and outgrowth. The visual color change detects acid metabolites produced during growth of the *B. atrophaeus*. This growth produces a pH change in the medium, which causes a change of the color of the medium from green to yellow. It requires an average of cell density greater than 10^6 cells/mL (GILLIS *et al.*, 2010). The results indicated that the medium had heat-damaged spores' recovery capacity within a 24 h incubation time.

The nonglycerol consummation in germination and outgrowth was not expected. According to Burk and Tempest (1990) the glycerol kinase activity may be decreased when the extracellular glycerol concentration was close to a cell-saturating level, suggesting that glycerol kinase synthesis might be repressed by the excess of glycerol from the recovery medium plus the glycerol from fermentation. However, the germination might occur in response to environmental signals, including amino acids and cell wall peptidoglycan muropeptides (degradation products of the peptidoglycan cell wall) as described by Shah *et al.* (2008). The germination of a single spore might lead to the germination of most of the population (SETLOW, 2008). In this case, the starch present in the recovery medium may have been used as the initial carbon source since *B. atrophaeus* is an amylase producer. The microorganism multiplication might be supported by the spore-derived proteins released during the early germination, when 20% of the spore proteins are degraded, providing the source of amino acids for the biosynthesis of new proteins and nucleotides (SETLOW; PRIMUS, 1975). Since no glycerol was consumed by *B. atrophaeus* after spore germination, glycerol might have another particular

mechanism to improve the dry-heat resistance, and more work should be performed in order to further elucidate the positive variation in *D*- value with the presence of glycerol.

The newly developed bioindicator system performance results meets international standards and regulations. The difference observed in results of $D_{160°C}$ –value (1.5min) was not an artifact of the method as suggested by the USP (2008). It is clear that the LSK method evaluated the IB system (spores plus recovery medium), while the survival curve method only evaluated the spores performance that were similar with that obtained after sporulation optimization, which was tested with a commercial recovery medium. This indicates that the recovery medium could be optimized for a specific use (with spores in the dried glycerol-based fermented). The obtained ethylene oxide resistance results are also in accord with the recommended parameters. The developed BIS showed a greater resistance to ethylene oxide than commercial BI; it may be reported as a positive factor to improve EtO process monitoring. Since the regular cycle time is 180 min, a BI with a kill time of approximately 60 min better demonstrates the sterilization security. Experimental evidence and scientific modeling may allow the modification of EtO sterilization process time or parameters. In this case, the developed BI, suitability for use in this specific sterilization process, should be established through new studies.

The preliminary cost analysis showed the process efficacy. The use of highly available raw material associated to the efficient use of energy and water and no emission of wastes, through solid-state fermentation utilization reduces cost and makes the process environmentally friendly.

The results of this study show that glycerol could be used to produce a high-quality, low-cost bioindicator system that meets international standards and regulations. The glycerol-based newly developed bioindicator system (FIGURE 9) could replace the commercial one. The use of glycerol as a carbon source for sporulation and as a recovery medium presents new opportunities for the development of a BIS product. The advantages of such applications include the profitable use of a biodiesel byproduct, and a decrease in the production cost of BI systems. Studies of the direct use of crude glycerol remains to be carried out and its purification cost must be considered.

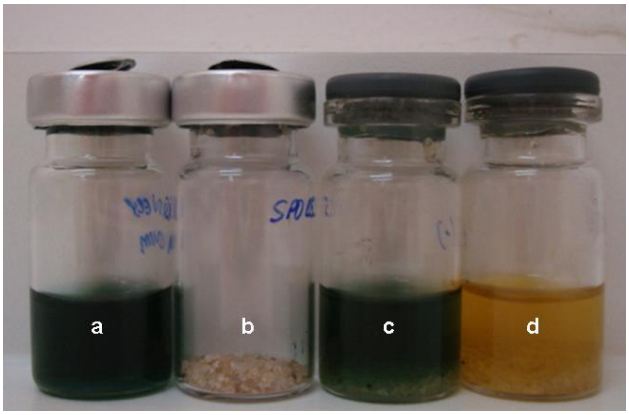

FIGURE 9 - GLYCEROL-BASED BIOLOGICAL INDICATOR SYSTEM (BIS): (***a***) RECOVERY MEDIUM, (**b**) SPORES ON A CARRIER, (***c*** AND ***d***) BIS AFTER INOCULATION AND INCUBATION. POSITIVE RESULTS (***d***) INDICATED THAT VIABLE SPORES REMAINED; THE MEDIUM CHANGED TO YELLOW AS THE ACIDIC METABOLIC PRODUCTS OF THE GROWING BACTERIA ACCUMULATED. IN NEGATIVE RESULTS (***c*)**, NO MICROBIAL GROWTH OCCURRED AND THE MEDIUM REMAINED GREEN AND CLEAR, INDICATING THAT THE SPORES WERE KILLED IN THE STERILIZATION PROCESS

SOURCE: The author (2012)

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CHAPTER VI

TESTING THE PERFORMANCE OF COMMERCIAL, SOYBEAN MOLASSES-BASED AND GLYCEROL-BASED BIOLOGICAL INDICATORS FOR STERILIZATION DURING DRY-HEAT, ETHYLENE OXIDE AND MICROWAVE TREATMENTS

ABSTRACT

Biological indicator systems (BISs) are designed to provide a challenge to the disinfection or sterilization process that exceeds the process resistance of the natural bioburden in or on the product. To present a true challenge, these BISs must meet specific standard requirements as set out by international standards or regulatory agencies. The performances of two *B. atrophaeus* BISs formulated from inexpensive raw-material (soybean molasses and glycerol) were assessed by determining its resistance characteristics to dry-heat (160°C) and ethylene oxide (EtO) sterilization, along with microwave disinfection. Performance evaluation of the BIS against dry-heat and ethylene oxide sterilization showed compliance with regulatory requirements. Dry heat resistance was observed in the following decreasing order: commercial BIS, soybean molasses-based BIS, glycerol-based BIS for the Survival Curve Method and soybean molasses-based BIS, commercial-BIS and glycerol-based-BIS for the Limited Spearman–Kaber Method, which demonstrates the influence of the recovery medium during *D*-value determination. For EtO, the greatest resistance was observed for soybean molasses-based BIS followed by glycerol-based BIS, which was increased by 2.5 minutes compared to the control commercial BIS. Microwave disinfection tests demonstrated that all of the tested BISs were more resistant than the control. Additional studies are necessary to determine if this is a positive factor for microwave medical waste treatment monitoring or if it may cause a false process failure indication.

Keywords: *Bacillus atrophaeus,* spores, biological indicators, dry-heat sterilization, ethylene oxide sterilization, microwave disinfection, medical waste

1. INTRODUCTION

A biological indicator (BI) is a test system containing viable microorganisms that provide a defined resistance to a specified sterilization process (ISO 11139, 2006d). BIs contain known numbers of bacterial spores (10^3 to 10^9 colony-forming units - CFU) that are highly resistant to the sterilization process for which they are

designed. The spores are placed on or in a carrier material such as paper, glass or liquid solution and packaged in order to protect the BI. After being subjected to sterilization, the indicator is cultivated to determine the effectiveness of the evaluated process. Biological indicator systems allow a qualitative evaluation by visual identification of a color change or turbidity of the substrate media containing a pH indicator. The visual color identification method detects acid metabolites produced after germination and growth of the spores. The acid metabolites are the result of a series of enzyme-catalyzed reactions that occur during growth, producing a pH change in the medium. This pH change causes the medium to visually change color. The BI must be considered as a system, with the spore characteristics, spore carrier, packaging, and recovery medium all contributing to its effectiveness (GILLIS *et al.*, 2010).

"*The use of Biological Indicators provides evidence of efficacy by challenging the sterilizer with a large number of highly resistant bacterial spores. Biological monitoring provides the only direct measure of the lethality of a sterilization cycle* (AAMI, 2008)". Due to their high resistance to certain sterilization agents, the most recognized application of *B. atrophaeus* spores is as a biological indicator for sterilization to monitor ethylene oxide (EtO), dry heat and microwave disinfection or sterilization (FDA, 2007; ISO 11138-4, 2006c; RUTALA; WEBER, 2008)

Dry-heat sterilization has proven effective in both medical and nonmedical applications, including medical prosthesis, implants, dry chemicals, oils, glycerin and even spacecrafts. The process involves exposing the product to hot air circulated in a closed chamber (batch sterilizers) or in tunnels (continuous sterilizers). The effectiveness of the process is based on both the temperature and exposure time, but uniform airflow distribution and load configuration also have an influence. The method has been applied to heat stable products that are sensitive to moisture, resistant to penetration by steam heat or prone to radiation damage (NIGHSWONGER, 2002). The most common time-temperature relationships for sterilization with hot air sterilizers are 170°C or 180°C for 60 minutes, 160°C for 120 minutes, and 150°C for 150 minutes (RUTALA; WEBER, 2008). The primary lethal process is the oxidation of cell constituents. The transfer of heat energy to objects upon contact results in dehydration and protein coagulation, which prevents the cell from reproducing, either by direct effects on the genetic system or disrupting the

metabolic systems that provide the required stimulation and nutrient environment for reproduction (RUTALA; WEBER, 2008)

Ethylene oxide (EtO) sterilization is a gaseous chemical process that is commonly utilized to sterilize medical and pharmaceutical products that cannot support the conventional high temperatures of dry-heat and steam sterilization, as well as radiation or peroxide. This includes disposable healthcare products, devices that incorporate electronic components, plastic packaging and plastic containers. EtO is a molecule in which two carbon and four hydrogen atoms are joined to one oxygen atom in a highly strained ring. Because of its very low boiling point (10.4ºC), it vaporizes and permeates rapidly through packaging, dissolving in substances such as plastic and rubber. It is an alkylating agent which adds alkyl groups to proteins, enzymes, DNA, and RNA in microorganisms and binds to sulfhydryl, hydroxyl, amino, and carboxyl groups, preventing normal cellular metabolism and reproduction and rendering affected microbes nonviable (MENDES *et al.,* 2007). The essential parameters for EtO sterilization are gas concentration (450 to 1200 mg/L), temperature (37^{o}C to 63^{o}C), relative humidity (40% to 80%) to allow the water molecules to carry EtO to reactive sites, and exposure time (1 h to 6 h) (JOSLYN, 2001). The EtO sterilization cycle takes approximately 2.5 h excluding aeration time and consists of five steps: a) preconditioning and humidification, b) gas introduction, c) exposure, d) evacuation, and e) air washes. Mechanical aeration is necessary to allow desorption of the toxic EtO residues contained in exposed absorbent materials (AAMI, 1999).

Microwaves, or radio-frequency waves, have been used for microbial destruction in the food industry with great potential in the pasteurization of foods (ROSENBERG; BOGL, 1987), the treatment of ballast water (BOLDOR *et al.,* 2008) and the disinfection of soft contact lenses, dental instruments, dentures, milk, and urinary catheters (RUTALA; WEBER, 2008). However, microwave treatment has mainly been used for terminal disinfection of medical waste. An automatic microwave waste treatment system consists of the following: (1) an all-weather steel enclosure, (2) an automatic mechanical lift-and load system, and (3) a shredding system where waste is ground into tiny particles. The grinding process reduces the waste volume and makes the waste unrecognizable and more homogeneous. Additionally, the system includes (4) a series of air filters, including the High Efficiency Particulate Air

(HEPA) filter, to avoid environmental contamination; (5) a microwave disinfection system composed by a stainless steel screw conveyor, where the waste is moistened with steam to intensify the heating process and passed by a series of microwave generation units; (6) a waste compactor or dumpster; and (7) an onboard microprocessor (FIGURE 1). Using this treatment, the volume of the medical waste product can be reduced by as much as 80% (EDLICH *et al.,* 2006).

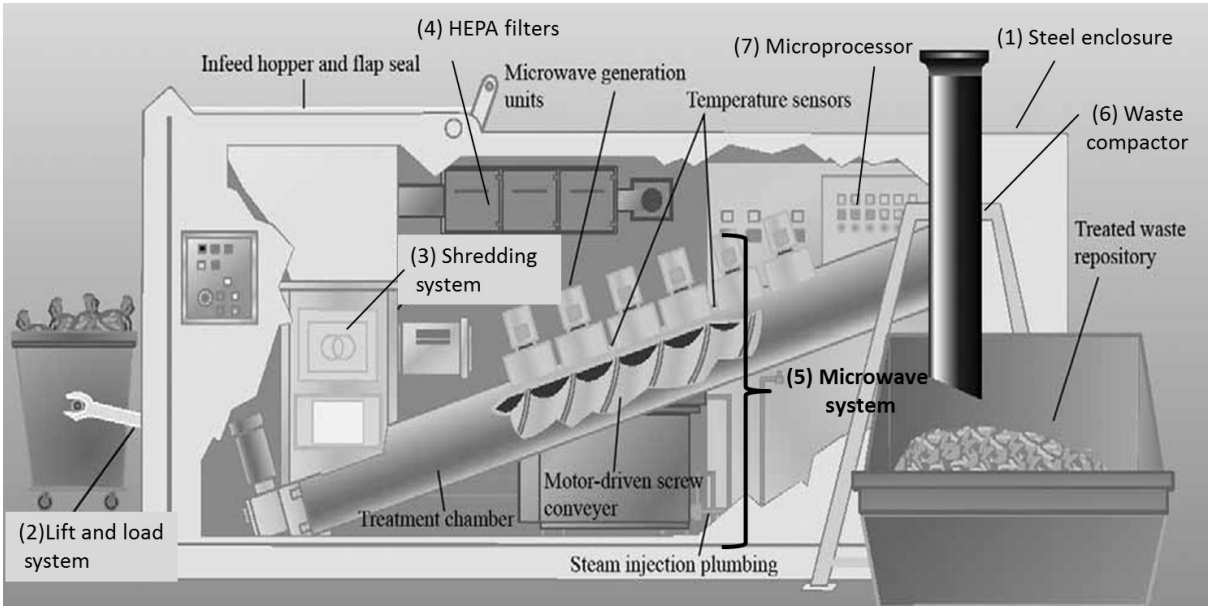

FIGURE 1 - SCHEMATIC DRAWING OF A MICROWAVE TECHNOLOGY UNIT
SOURCE: Adapted from Devine *et al.* (2007)

A microwave disinfection system usually consists of a series of six microwave generators (magnetrons), each with a 2450 MHz frequency and 1,400 W power. Therefore, the total power of the equipment is 8,400 W. The microwave generators input energy to maintain uniform heating of the waste at a minimum temperature of 95°C. The waste is transported along the screw so that the final exposure time is a minimum of 30 minutes. The units are designed to treat 250 kg/hour up to 460 kg/hour of waste at 62 W/kg up to 93 W/kg potency/mass ratio. Steam in the range of 150°C to 172°C is injected at four locations to maintain uniform heating and humidity (50%-56%) of the waste at a minimum temperature of 95°C (DEVINE *et al.,* 2007; TONUCI *et al.,* 2008; SANITEC, 2012). Important parameters in this

processes are frequency and wavelength of radiation, exposure time, composition, moisture content, the mass and calorific capacity of the treated substrate, and temperature. The microbial inactivation standard for medical waste is Level III (a 6 log reduction or greater for bacteria, fungi, viruses, parasites, and mycobacteria and a 4 log reduction or greater for *Bacillus* or *Geobacillus* spores) (STAATT, 1994).

There has been controversy over the mechanism of microwave sterilization and its thermal and non-thermal effects. Celandroni *et al.* (2004) demonstrated that microwave fields induce changes in the structural and/or molecular components of spores that differ from those attributable only to heat. Kim *et al*. (2009) described that 2.0 kW microwave irradiation ruptures the spore coat and inner membrane and is significantly different from boiling. However, Jeng *et al.* (1987), Welt *et al.* (1994) and Kang *et al*. (2011) found that the effect of microwave energy on the viability of spores was indistinguishable from the effect of conventional heating. Known concentrations of a biological indicator, *B. atrophaeus* spore strips (MESON *et al.,*1993) or spore suspensions in a vial (CETESB, 2007), were added to test waste loads under routine operating conditions to evaluate the efficacy of the microwave treatment on medical waste. Samples were processed and then returned to an independent microbiology laboratory where they were cultured and monitored for population reduction of the BIS (CETESB, 2011).

Commercially available biological indicators systems (BISs) are designed to provide a challenge to the disinfection or sterilization process that exceeds the process resistance of the natural bioburden in or on the product. These BIs must meet specific standards as set out by international standards or regulatory agencies to present a true challenge.

Commercial BISs are produced in industrialized or chemically defined media (SCHAEFFER *et al.* 1965; CAZEMIER *et al.* 2001). However, Sella *et al.* (2012a, 2012b) proposed the use of alternative raw materials, such as soybean molasses and glycerol, for the cost-effective production by solid-state fermentation (SSF) of *a* biological indicator system *for B. atrophaeus* sterilization. SSF process can be defined as microbial growth on solid particles without the presence of free water (PANDEY *et al.,* 2000). SSF makes the BI production less expensive than others process by allowing the direct use of the dry fermented as product, reducing the production steps.

The present study aimed to evaluate the performance of glycerol-based, soybean-based and commercial *B. atrophaeus* biological indicators for dry-heat, EtO sterilization and microwave waste treatment.

2. MATERIAL AND METHODS

The analyses were carried out based on the United States Pharmacopeia (USP 29, 2006), Food and Drug Administration (FDA, 2007), and International Standard ISO 11138-2 and ISO 11138-4 (2006 b,c) standards, which guide BI production.

2.1 Biological Indicators

Soybean-molasses based and glycerol-based BISs were prepared from *B. atrophaeus* ATCC 9372 standard strains supplied by the Instituto Nacional de Controle de Qualidade em Saúde (INCQS/MS, Brazil).

2.1.1 Glycerol-based biological indicator system

Glycerol-based spores were prepared by solid-state fermentation using sand as a support and 25.0 g/L glycerol plus 12.0 g/L tryptone as substrates. A dry fermentation containing ~10^6 CFU spores/g was conditioned in 7.0 mL borosilicate glass vials with 1.0 g of material for dry-heat assays and in a sealed gas-permeable package (medical grade paper + polyethylene/polypropylene film) for EtO assays. For microwave tests, ~10^4 CFU spores were conditioned into a gas-permeable package. Glycerol-based recovery medium was composed of 12.0 g/L glycerol, 10.0 g/L tryptone, 2.0 g/L soluble starch and 0.025 g/L bromothymol blue, pH 7.0. This medium was loaded into borosilicate glass vials in 3.0 mL aliquots, stoppered with a silicone rubber, sealed with an aluminum seal and autoclaved at 121°C for 15 min (SELLA *et al.,* 2012b) (See CHAPTER V).

2.1.2 Soybean molasses-based biological indicator system

Soybean molasses-based spores were prepared by solid-state fermentation using sand as a support and 0.08 g/L $MnSO_4$, with 24.0 g/L soybean molasses as the substrate. Soybean molasses-based recovery medium was composed by 30.0 g/L soybean molasses (80° Brix), 40.0 g/L tryptone, 0.02 g/L bromothymol blue, and 1.0 g/L soluble starch, pH 8.5 (SELLA *et al.,* 2012a). The number of spores/units and the packaging were as described in 2.1.1 (See CHAPTER III).

2.1.3 Commercial BIS

For dry-heat sterilization, a commercial *B. atrophaeus* Biological Indicator (IB Calor Seco, CPPI, Piraquara, Brazil) was used, which was composed of strips conditioned in glass vials and recovery medium, and had the following labeled characteristics: $D_{160°C}$ = 6.5 min, initial number of spores N_0 = 3.6 x 10^6 CFU/unit. For EtO sterilization, the following were used: (a) the BI as described above, with the strips conditioned in a gas-permeable package, and (b) a self-contained commercial EtO BIS (BROWNE, Leicester, UK) with the following labeled characteristics: D_{EtO}= 3.4 min, initial number of spores N_0 = 1.8 x 10^6 CFU/unit. For microwave treatment, (a) the BI was as described above, with the strips conditioned in a gas-permeable package (medical grade paper + polyethylene/polypropylene film), with (b) 1.0 mL of a spore suspension conditioned into a 2.0 mL polypropylene locked vial with an initial number of spores N_0 = 3.6 x 10^6 CFU/unit (CPPI, Piraquara, Brazil).

2.2 General BIS performance evaluation

The BIS performance evaluation was carried out as described below.

2.2.1 Sterility

Ten biological indicator units of each evaluated BI were incubated at 36°C for

48 h to 7 days and examined visually (for change in the color indicator or turbidity) and microscopically (for the absence of microbial growth).

2.2.2 Purity

Appropriate morphological, cultural and biochemical characteristics of the *B. atrophaeus* strain were tested according to the description in USP 22 (1990).

2.2.3 Spore viability

Ten BI units were cultivated at 36°C to check the viability of the spores. Spore germination and outgrowth were observed after 24 h and 48 h incubation times by visual identification of a color change (green to yellow) and turbidity of the substrate media.

2.2.4 Growth promotion

Ten units of each evaluated BI were inoculated with 100 CFU of spores, and the other ten were inoculated with 1,000 CFU of spores to test the growth promoting ability of the recovery medium. The samples were incubated at 36°C. Spore germination and growth were observed daily for up to 7 days.

2.2.5 Total viable spore count

Three BIs were randomly selected (inoculated paper on carriers and dry-fermented) from each lot to be assayed and placed into sterile tubes with glass beads and 10.0 mL of sterile, purified water with tween 80, 0.01%. The tubes were vortexed for 5 minutes. Serial decimal dilutions with sterile distilled water were prepared, and 50.0 µL of each dilution was inoculated in duplicate on a tryptone soy agar plate surface. The plates were incubated overnight at 36°C. Colony-forming units (CFU) per unit were calculated by colony counts.

2.2.6 Incubation time

A partial dry-heat sterilization cycle in which 30-80% of the BIs survive was identified, and 100 BIs from each of the 3 different lots were exposed to 3 partial sterilization cycles. These BIs were placed in recovery media after removal from the sterilization oven and then incubated for 7 days. The growth was monitored daily. Using the number of BIs that test positive on day 7 as the base of 100% grow out, it was determined that more than 97% of BIs tested positive in each partial cycle. The greatest number of days of incubation time to obtain more than 97% positive BIs (based on the 7 day incubation time) in any one of the partial cycles was considered the minimum incubation time for the BIS (FDA, 2007).

2.3 Dry-heat resistance

The *D*-value is defined as the time taken, in a specified set of conditions, for the spore population to decrease by 90% or 1 log of its initial value (FDA, 2007). The *D*-value was determined by the following: (*a*) fraction negative analysis, using the Limited Spearman–Kaber Method (LSKM), according equations (1) and (2) (USP 29, 2006). Dry heat conditions were as follows: 160°C for 4, 8, 12, 16, 20, 24, 28, 32, 36, 40 and 48 min in a tabletop air oven; and (*b*) a survivor curve based on direct enumeration of the spores using the Stumbo-Murphy Cochran formula (equation 3).

For D value determination by the Limited Spearman–Kaber Method:

$$D_{160^{o}C} = \frac{U_{sk}}{\log N_o + 0.2507} \qquad (1)$$

where:

N_o = initial number of organisms on BI

U_{sk} = Spearman–Kaber heating time/dosage estimate

$$U_{sk} = U_k - \frac{d}{2} - \left(\frac{d}{n} . \sum_{i=1}^{k-1} \frac{r_i}{n} \right) \quad (2)$$

where:

U_k = first heating time with all units negative

d = time interval (minutes)

n = BIS per exposure time

r_i = number of units negative

For D value determination, the Stumbo-Murphy Cochran formula was used:

$$D = \frac{t}{\log No - log N} \quad (3)$$

where:

N_o = initial number of organisms on BI

N = final number of organisms on BI

t = exposure time

The survival/kill times were determined by the following formula (equations 4 and 5):

Survival Time = (log of population – 2) x *D*-value *(4)*

Kill Time = (log of population + 4) x *D*-value *(5)*

2.4. Ethylene oxide resistance

Ethylene oxide (EtO) sterilization was performed using 650 mg/L at 55°C and 55% humidity, with (*a*) a partial cycle time of 15 min, (*b*) a half-cycle time of 90 min, and (*c*) a total cycle time of 180 min. The cycles were performed in triplicate, and at least ten units of each evaluated BI were exposed. The D_{EtO} values (decimal reduction time) were calculated based on the Stumbo-Murphy Cochran formula.

2.5 Microwave treatment resistance

The BIS microwave treatment resistance was determinate as described below.

2.5.1 Waste preparation

The simulated medical waste was prepared according to described by Oliveira *et al.* (2010) (TABLE 1; FIGURE 2). A substitute medical waste can be used for microwave treatment evaluation if it has the same characteristics of gravimetric composition (%), moisture and bulk density (kg/m^3) (OLIVEIRA *al.,* 2010). The waste was prepared with particles, 90% of which ranged from 3.0 mm to 38.0 mm.

TABLE 1 - COMPOSITION AND BULK DENSITY OF SIMULATED MEDICAL WASTE

Material	Mass (g)	% in mass (dry basis)
Paper/fabric	785.2	39.3
Hard plastic	493.9	24.7
Plastic	263.8	13.2
Latex/Rubber	210.6	10.5
Metals	88.1	4.4
Grass	42.0	2.1
Bone	31.2	1.6
Wood	21.3	1.1
Polystyrene foam	12.7	0.6
Others: soil, fibers, dust...	51.3	2.6
Total	2000,0	100
Bulk density for 56% moisture content		128 kg/m^3 (wet basis)

FIGURE 2 - SIMULATED MEDICAL WASTE

SOURCE: The author (2012)

The moisture content of the simulated medical waste was determined gravimetrically by drying a sample in an oven at 105°C for approximately 24 h and then it was adjusted to 56% with purified water. Three units of each tested BI was mixed with the simulate waste before its humidification so that samples remain homogeneous into the waste. The humid simulated waste was spread on a 30.0 cm diameter rotating glass plate. The waste height was ~ 3.0 cm.

2.5.2 Calibration of the microwave oven

The experiments were conducted in an Electrolux (Manaus, AM, Brazil) domestic microwave oven with a frequency of 2450 MHz, capable of up to 1000 W of microwave output power and 45 L of capacity. The microwave oven was set to operate at 10%, 30%, 50% and 70% of its nominal power, according to the manufacturer's instructions. Initially, 1600 g of purified water was placed in 26.4 x 5.8 cm (diameter x height) glass plate to obtain ~3.0 cm of water column height. The initial and final temperature of the water was measured using a K-type 1.5 x 400 mm thermocouple coupled to a SALCASTERM mod1200-2 digital thermometer (SALCAS, São Paulo, Brazil). The thermometer was placed at a central position in the water, and the oven was set to operate for 120 s at the desired power (FIGURE

3- LEFT).

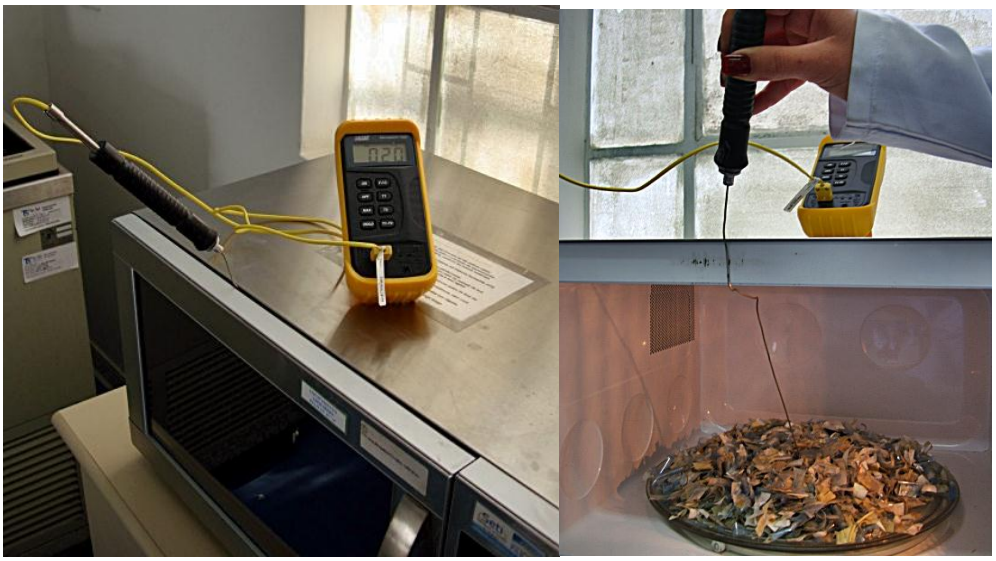

FIGURE 3 - MICROWAVE OVEN WITH A K-TYPE THERMOCOUPLE COUPLED TO A DIGITAL THERMOMETER: INSIDE (RIGHT) AND OUTSIDE (LEFT) OF THE OVEN

SOURCE: The author (2012)

The experiment was carried out in triplicate. The actual dissipated power was calculated by the principle of the conservation of energy, according to the following equation (6):

$$P = \frac{m_a.C_{pa}.\Delta T}{t} + \frac{m_v.C_{pv}.\Delta T}{t} \qquad (6)$$

where:

P = real power absorbed by the sample (W);

m_a = mass of the water (kg);

m_v = mass of beaker (kg);

C_{pa} = calorific capacity or the specific heat of the water (J/kg.ºC), assumed 4,181 J/kg.ºC;

C_{pv} = calorific capacity or the specific heat of the glass (J/kg.ºC), assumed 840 J/kg.ºC;

t = exposure time (s), 120 s;

ΔT = temperature difference (ºC).

The temperature of the glass was hypothetically assumed to be the same as that of the water in each calibration test.

The power/mass ratio for each assay was calculated based on the actual power dissipated between 10% and 40% of the nominal power according to the mass of waste used for each assay (320g up to 360g), as shown in equation (7):

$$P.m^{-1} = \frac{P_{real}}{m} \qquad (7)$$

where:

m = mass of the waste (kg);

P_{real} = real power absorbed by the sample (W);

$P.m^{-1}$ = power by mass (W/kg).

2.5.3 Inactivation conditions

The first inactivation experiments were performed with potency/mass ratios in the range of 7 W/kg up to 790 W/kg for 40 minutes as the total exposure time. In these conditions, the thermometer was placed at a central position in the waste, and the waste temperature profile was determined (FIGURE 3- RIGHT). The inactivation fraction (X) was calculated from equation (8).

$$X\ (\%) = \frac{No-N}{No}\ .100 \qquad (8)$$

where:

X = inactivation fraction (%);

No = number of viable microorganisms before treatment;

N = number of viable microorganisms after treatment.

The microbial inactivation efficacy was also equated to log kill, which is defined as the difference between the decimal logarithms of the number of viable microorganisms (spores) before (No) and after treatment (N):

Log kill= Log No – Log N *(9)*

The influence of inactivation time (10, 20, 30 and 40 minutes) on the reduction of the BI population was evaluated at the minimum P/m ratio that was able to reduce the spore suspension population by 4 logs (level III of disinfection).

2.6 General conditions and statistical analysis

The assays were carried out in triplicate. Three different lots of each tested BIS were utilized. Microbiological assays were conducted under aseptic conditions in a Good Manufacturing Practices (GMP) certified laboratory ISO 5 clean room. Statistical analyses were carried out using Statistic 8.0 software (Stat Soft, USA) and the SGWIN program (Stat Graphic Plus for Windows version 5.0, Statistical Graphics, USA). Analysis of variance was used to evaluate the model to obtain a *D*-value at a 95% significance level.

3. RESULTS AND DISCUSSION

The initial performance results indicated that all of the tested BI met the legal and basic regulatory requirements as demonstrated in TABLE 2. The viable spore population count of the commercial BIS showed that the procedure of recovery of spores from the carrier and the culture methodology was valid due to obtaining 77.7% - 87.5% recovery. Results are in accordance with as USP 24 (2000a) that has as acceptance criteria for spores count not less than 50% or more than 300% of the labeled certified population for the spore strips. According to ISO 11138-2 (2006b), replicate determinations of the viable spore count shall be either within ± 50% of the nominal population.

The growth promotion and incubation time assays proved the effectiveness of the recovery medium to promote the germination and outgrowth of non-damaged and heat-damaged spores. If heat-damaged viable spores' growth not occurs, the effects of the sterilization process should be overestimated, as surviving

microorganisms should be unable to grow after exposure to the process.

TABLE 2 – RESULTS OF THE PERFORMANCE DETERMINATION OF THE *B. atrophaeus* BIOLOGICAL INDICATOR SYSTEM

Assays	Glycerol-based BIS	Soybean molasses-based BIS	Spores on strips	Spores suspension	Commercial dry-heat BIS (CPPI)	Self-contained commercial EtO BIS (BROWNE)	Regulatory recommendations
Population (a) for dry-heat and EtO	$2.5 \pm 0.2 \times 10^6$	$1.4 \pm 0.1 \times 10^6$	-	-	$3.6 \pm 0.8 \times 10^6$	$1.5 \pm 0.1 \times 10^6$	$10^5 – 10^9$ CFU/unit
(b) for microwave (CFU/unit)	$5.1 \pm 0.5 \times 10^4$	$3.4 \pm 0.2 \times 10^4$	$4.0 \pm 0.2 \times 10^4$	$3.6 \pm 0.2 \times 10^6$	-	-	$10^4 - 10^6$ CFU/unit
Viability	100% in 24 h	100% in 24 h	100% in 24 h	100% in 24 h	100% in 24 h	100% in 24 h	100% after 7 days of incubation time
Purity	100%	100%	100%	100%	100%	100%	100%
Recovery medium sterility	Absence of growth	Absence of growth	-	-	Absence of growth	-	Absence of growth
Growth promotion	positive	positive	-	-	-	-	positive
Incubation time	48 hours	48 hours	-	-	48 hours (labeled)	48 hours (labeled)	up to 7 days

3.1 Dry-heat resistance

Three lots of each newly developed BIS were tested for dry-heat resistance and the results were compared with commercial BIS. ISO 11138-1:1994(2006a) allows the use of both a Survivor Curve Method and Fraction Negative Method to determine a *D*-value of a BIS. Microbial inactivation by dry-heat sterilization is considered to follow first-order kinetics, and according to the first-order kinetics approach, the plot of the logarithm of the number of surviving microorganisms versus time is a straight line at isothermal conditions (FIGURE 4). The graph of the Survival Curve Method indicates a superior dry-heat resistance for the commercial BIS (spore strip),

followed by soybean molasses-based BIS and glycerol-based BIS, respectively. However, all obtained $D_{160°C}$ values (6.1 ± 0.1 min, 4.6 ± 0.1 min and 5.7± 0.5 min) are in accordance with the recommended parameters ($D_{160°C}$ value ≥ 3.0 min).

The determination of the $D_{160°C}$-value was also performed by the Limited Spearman-Kaber (LSK) method and the obtained results (TABLE 3) demonstrated that soybean molasses-based BIS had similar resistance to commercial BIS: $D_{160°C}$ value was within 20% of the commercial BIS *D* value (6.7 ± 0.2 min and 6.5 ± 0.2 min, respectively). The glycerol-based BI had the lowest resistance, 3.1 ± 0.1 min, but was also in accordance with regulatory recommendations.

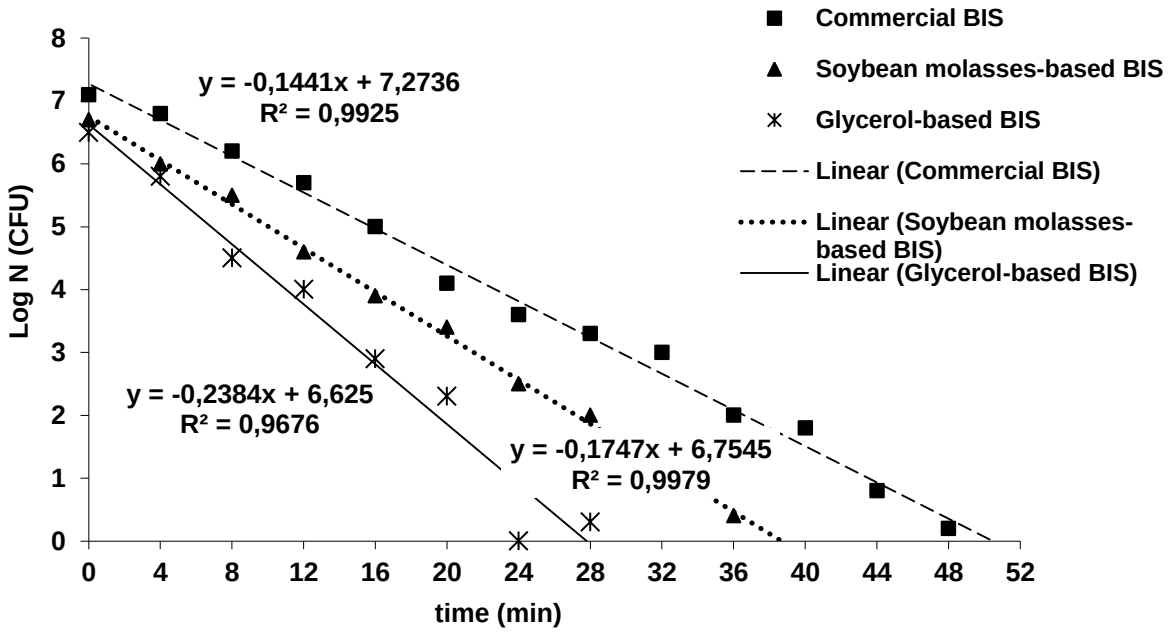

FIGURE 4 - DRY-HEAT (160°C) SURVIVAL CURVE OF THE *B. atrophaeus* SOYBEAN MOLASSES-BASED BIOLOGICAL INDICATOR SYSTEM (BIS) AND GLYCEROL-BASED BIS, COMPARED TO COMMERCIAL BIS

SOURCE: The author (2012)

TABLE 3 – RESULTS OF THE DRY-HEAT RESISTANCE DETERMINATION OF THE *B. atrophaeus* BIOLOGICAL INDICATOR SYSTEM BY THE SURVIVAL CURVE AND SPEARMAN-KABER (LSK) METHODS

Dry-heat Resistance (min)	Commercial BIS		Glycerol-based BIS		Soybean molasses-based BIS		Regulatory Recomendations
	LSK Method	Survival Curve Method	LSK Method	Survival Curve Method	LSK Method	Survival Curve Method	
$D_{160°C}$	6.5 ± 0.2	6.1 ± 0.1	3.1 ± 0.1	4.6 ± 0,1	6.7 ± 0.2	5.7± 0.5	≥ 3.0 min
$Usk_{160°C}$	44.6 ± 1.3	40.0 ± 0.7	20.6 ± 0.7	30.6 ± 0.7	45.4 ± 1.3	36.5 ± 3.2	≥ 12.0 min
Survival time	29.9 ± 1.4	26.2 ± 0.4	13.6 ± 0.5	20.2 ± 0.4	30.3 ± 0.9	23.7 ± 2.1	≥ 4.0 min
Kill time	68.9 ± 3.2	62.8 ± 1.0	32.2 ± 1.1	47.8 ± 1.0	70.5 ± 2.0	57.9± 5.1	≥ 10.0 min

The Analysis of Variance – ANOVA testing of LSK and SCM methods (TABLE 4) resulted in $F<F_c$ and p-value>0.05, indicating that there was not a statistically significant difference between the averages of the tested methods. However, a significant difference was demonstrated among the types of BISs. A novel ANOVA analysis between the commercial BIS and the soybean molasses-based BIS (TABLE 5) confirmed that there was not a statistically significant difference between the averages of these tested BISs. It was possible to conclude that glycerol-based BIS results are significantly different.

TABLE 4 – ANOVA FOR DIFFERENT TYPES OF BIS RESISTANCE IN *B. atrophaeus* DETERMINED BY $D_{160°C}$ VALUES OBTAINED FROM LIMITED SPEARMAN-KABER (LSK) AND SURVIVAL CURVE (SCM) METHODS

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
LSK x SCM methods	0.7076	17	0.0416	0.0788	1.0000	1.9332
BIS type	69.8726	2	34.9363	66.1596	1.9016E-12	3.2759
Error	17.9541	34	0.5281			
Total	88.5343	53				

TABLE 5 – ANOVA for COMMERCIAL AND SOYBEAN MOLASSES-BASED RESISTANCE IN *B. atrophaeus* DETERMINED BY $D_{160°C}$ VALUE

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.1111	1	0.1111	0.4333	0.5148	4.1300
Within Groups	8.7189	34	0.2564			
Total	8.83	35				

Only standardized BIS can be used to determine the capacity of sterilization or disinfection treatments. Appropriate BIS must have a stable thermal resistance to the sterilization process greater than the bioburden of the product or device to be sterilized (PENNA *et al.* 2002). Shintani (2011) described that the most modern way to demonstrate sterility is by using a BIS as a way to ensure an appropriate "sterility assurance level" (SAL). Typically, a SAL of 10^{-6} is recommended for medical products intended to contact compromised tissue (i.e., tissue that has lost the integrity of the natural body barriers) (RUTALA; WEBER, 2008). A SAL of 10^{-6} indicates a one in one million probability of a single viable microorganism remaining on the product after sterilization. Knowing the thermal resistance of each specific BIS, characterized by the *D*-value and its spore population number, it was possible to calculate the sterilization time to achieve the desired SAL. The minimum required $D_{160°C}$ value to meet regulations is 3 min for a 10^6 CFU BIS; in this case, to achieve a SAL of 10^{-6}, 12 (6+6) log reductions in the cycle time are required, which should take at least 36 minutes. For the recommended dry-heat sterilization cycle (120 minutes at 160°C), a BIS with a higher $D_{160°C}$ value, such as commercial spore strips or soybean molasses-based BI, may be reported as a positive factor to improve process monitoring as the overkill cycle time could be enlarged.

The difference in the observed $D_{160°C}$–value determined by the different methods may be an artifact of the technique as suggested by the USP 32 (2008) or may be due to the characteristics of the methods. Considering that the LSK method evaluated the BI system (spores plus recovery medium) while the Survival Curve Method only evaluated spore performance, the results suggest that glycerol recovery medium could be optimized for improving heat-damaged spore germination and growth, while heat-damaged spores from soybean molasses-based BI are better

recovered in broth (recovery medium) than in TSA medium. This is consistent with the results of Smith *et al.* (1982), who found different *D*-values for spores recovered in broth medium (TSB) and agar plates (TSA). However, the *D*-values obtained by the survival curve method were higher than that obtained by the fraction negative method. Pflug *et al.* (1981) and Leguérinel *et al.* (2006) reported that heated spores have an increased sensibility to a nutritional environment, so a $D_{160°C}$ determination should be carried out whenever the recovery conditions are changed.

3.2 Ethylene oxide resistance

The use of BIS in the EtO sterilization process is an important part of sterility assurance compliance. The BIS is used for EtO validation cycles as a microbial challenge, as a tool for its comparative resistance with the bioburden resistance of the device and for routine cycle monitoring. None of the tested BISs showed any growth when exposed to EtO sterilization for a half-cycle time of 90 min and a total cycle time of 180 min, either in recovery medium or on TSA agar plates. The ethylene oxide resistance calculated after a 15 minute exposition time is demonstrated in TABLE 6.

TABLE 6 – DETERMINATION OF THE RESISTANCE OF THE *B. atrophaeus* BIOLOGICAL INDICATOR SYSTEM TO ETHYLENE OXIDE

Ethylene oxide Resistance (min)	Commercial BIS	Soybean molasses-based BIS	Glycerol-based BIS	Regulatory Recommendations
D_{EtO} (min)	3.1 ± 0.1	6.1 ± 0.1	5.6 ± 0.5	≥ 2.5 min
Usk_{EtO} (min)	20.3 ± 0.7	43.6 ± 1.3	35.6 ± 3.8	≥ 15.0 min
Survival time (min)	12.9 ± 0.9	29.9 ± 0.5	22.9 ± 2.5	≥ 10.0 min
Kill time(min)	32.8 ± 1.0	66.5 ± 1.1	56.5 ± 9.2	≥ 25.0 min

The ANOVA results are presented in TABLE 7. The analysis showed $F>F_c$ and p-value<0.05, indicating that there was a statistically significant difference between the resistances of the tested BISs. A novel ANOVA analysis between the glycerol-

based and soybean molasses-based BIS (TABLE 8) confirmed that there was no statistically significant difference between the averages of these tested BISs, indicating that only the results of the commercial BIS are significantly different.

TABLE 7 - ANOVA for *B. atrophaeus* RESISTANCE TO COMMERCIAL, GLYCEROL-BASED AND SOYBEAN MOLASSES-BASED BIS AS DETERMINED BY D_{EtO} VALUE

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	47.2363	2	23.6182	647.401	1.32E-21	3.4028
Within Groups	0.8756	24	0.0365			
Total	48.1119	26				

TABLE 8 - ANOVA for *B. atrophaeus* RESISTANCE TO GLYCEROL-BASED AND SOYBEAN MOLASSES-BASED BIS AS DETERMINED BY D_{EtO} VALUE

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1.125	1	1.125	21.9512	0.0003	4.4940
Within Groups	0.82	16	0.0513			
Total	1.945	17				

The D_{EtO} value of the commercial paper strip BIS was determined according to the manufacturer's label (3.4 min), as ISO 11138-2 (2006b) allows an accuracy of ±0.5 min. The observed difference between the D_{EtO} values of the commercial BIS and the other BISs may be due to the carrier type. Pinto *et al.* (1994) also observed a significant difference in spore resistance between paper and other carriers and questioned the use of paper as a universal carrier for BIs to monitor EtO sterilization. As discussed for dry-heat sterilization, the greater resistance of the BIS produced in sand allows a better EtO sterilization cycle assurance due to a kill time of 56-66 min during the monitoring of a 180 minute cycle time. In contrast, the kill time of the commercial BIS is approximately 32 min. Because of operational difficulties, the high costs of the EtO cycles and the comparative objective of this study, the D_{EtO}-value was calculated from the partial cycle method. However, the BIS manufacturer should utilize the limited Spearman-Karber procedure according to ISO recommendations.

3.3 Microwave resistance

Actual microwave output should be determined in inactivation studies to yield reproducible results, as domestic microwave ovens are not intended for use in sterilization. The calibration data are demonstrated in TABLE 9.

TABLE 9 – RESULTS OF MICROWAVE OVEN CALIBRATION

Nominal Potency	Real Potency (W)
10% (100W)	2.1
20% (200W)	94.8
30% (300W)	187.5
40% (400W)	280.2
50% (500W)	463.5
60% (600W)	465.6
70%(700W)	558.3

The determination of the temperature as a function of time for powers of 7 W/kg, 270 W/kg, 530 W/kg and 790 W/kg is demonstrated in FIGURE 5. Tonuci *et al.* (2008) obtained higher temperatures at 100 W/Kg potency/mass ratios in similar experiment compared to the higher potency/mass ratio temperatures of this study; this may be due to the different ways that the waste was placed inside the microwave oven. Product shape, volume and surface area, as well as dimension and other electromagnetic characteristics of the oven, are critical factors in microwave heating (FDA, 2000).

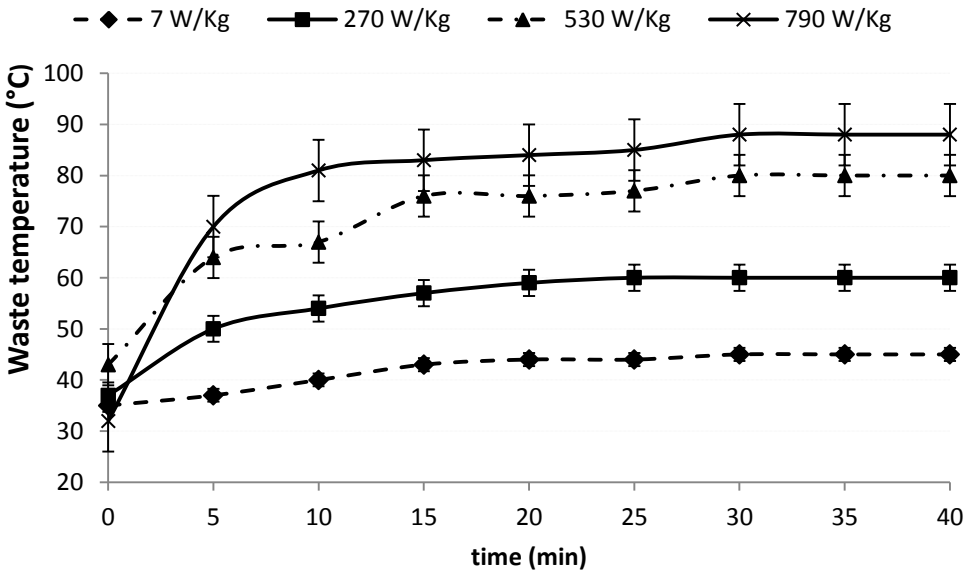

FIGURE 5 – TEMPERATURE AT THE CENTER OF THE WASTE AS A FUNCTION OF EXPOSURE TIME AND POTENCY/MASS RATIO

Losses in the waste moisture content with increases in microwave power density were demonstrated. The moisture losses measured after a 40 min microwave exposure at different power densities are shown in TABLE 10. These results are in accordance with those observed by Abdul *et al.* (2011), who described the increase of the microwave power density results in greater moisture loss in sawdust blocks.

TABLE 10 – WATER LOSS OF 56% MOISTURE CONTENT IN WASTE AFTER EXPOSURE TO DIFFERENT POTENCY/MASS RATIOS

Microwave Potency/Mass (W/kg)	Water loss (%)	Waste final Moisture (%)
790	34.9	36.4
530	28.5	40.0
270	11.4	49.6
7	1.9	54.3

Log reductions after 40 minutes of exposure time for each studied BIS when submitted to different potency/mass ratios are shown in TABLE 11 and FIGURE 6.

TABLE 11 - EXPERIMENTAL RESULTS BY BI TYPE (IN DRY STATE AND 56% HUMIDITY) FOR THE NUMBER OF MICROORGANISMS *(N*) AND PERCENTAGE OF INACTIVATION *(X*) AS A POWER FUNCTION (W/kg) AFTER A 40 MINUTE OF MICROWAVE EXPOSURE TIME

BIS	Power (W/kg)	*N* (CFU)	Log *N*	*Log Kill*	*X* (%) *calc*
Soybean molasses- based in dry state				-	
	0	$3.4 \pm 1.8 \times 10^4$	4.5		0
	7	$3.2 \pm 1.6 \times 10^4$	4.5	0	18.6 *
	270	$2.6 \pm 1.3 \times 10^4$	4.4	0.1	28.4 *
	530	$1.5 \pm 0.7 \times 10^3$	3.2	1.3	95.6 ± 2.1
	790	$7.0 \pm 3.8 \times 10^2$	2.8	1.7	97.9 ± 1.2
Soybean molasses- based humid				-	
	0	$3.4 \pm 1.8 \times 10^4$	4.5		0
	7	$2.6 \pm 0.8 \times 10^4$	4.4	0	23.5 *
	270	$7.7 \pm 3.2 \times 10^3$	3.9	0.6	74.9 ± 6.4
	530	$6.7 \pm 3.1 \times 10^3$	3.8	0.7	80.3 ± 9.1
	790	$1.1 \pm 0.6 \times 10^3$	3.0	1.5	96.8 ± 1.8
Glycerol- based in dry state					
	0	$5.1 \pm 1.9 \times 10^4$	4.7	0	0
	7	$4.6 \pm 1.4 \times 10^4$	4.7	0	15.7 *
	270	$2.3 \pm 0.7 \times 10^4$	4.4	0.3	54.9 ± 13.7
	530	$8.0 \pm 3.0 \times 10^3$	3.9	0.8	84.3 ± 5.9
	790	$5.8 \pm 1.8 \times 10^3$	3.8	0.9	88.6 ± 3.6
Glycerol- based humid					
	0	$5.1 \pm 1.9 \times 10^4$	4.7	0	0
	7	$3.8 \pm 1.4 \times 10^4$	4.6	0.1	17.0 ± 12.0
	270	$2.3 \pm 0.8 \times 10^4$	4.4	0.3	54.9 ± 12.8
	530	$8.0 \pm 3.2 \times 10^3$	3.9	0.8	84.3 ± 5.1
	790	$4.2 \pm 1.8 \times 10^3$	3.6	1.1	91.8 ± 2.9
Strip in dry state				-	
	0	$4.0 \pm 1.2 \times 10^4$	4.6		0
	7	$3.1 \pm 0.8 \times 10^4$	4.5	0.1	22.5 ± 16.3
	270	$2.9 \pm 0.6 \times 10^4$	4.5	0.1	27.5 ± 12.3
	530	$2.6 \pm 0.8 \times 10^4$	4.4	0.2	35.0 ± 16.3
	790	$5.5 \pm 2.0 \times 10^3$	3.7	0.9	86.3 ± 4,2
Strip humid				-	
	0	$4.0 \pm 1.2 \times 10^4$	4.6		0
	7	$3.1 \pm 0.8 \times 10^4$	4.5	0.1	22.5 *
	270	$2.7 \pm 0.9 \times 10^4$	4.4	0.2	32.5 ± 18.4
	530	$2.4 \pm 0.6 \times 10^4$	4.4	0.2	40.0 ± 12.3
	790	$6.4 \pm 1.9 \times 10^3$	3.8	0.8	84.0 ± 3.9
Spores suspension (control)				-	
	0	$3.6 \pm 2.3 \times 10^6$	6.6		0
	7	$3.3 \pm 3.0 \times 10^6$	6.5	0.1	21.3 *
	270	$3.2 \pm 0.6 \times 10^6$	6.5	0.1	13.0 ± 11.4
	530	$2.1 \pm 1.1 \times 10^6$	6.3	0.3	41.7 ± 24.9
	790	$3.5 \pm 0.1 \times 10^2$	2.5	4.1	99.9 ± 0.09

(*) The uncertainty observed in these standard deviation calculations may be generated by the accuracy of plate count method associated to a low spores ‘inactivation.

The level of BIS microbial destruction was susceptible to microwave power density. A 4 log reduction in spore suspension (utilized as the BI control) was observed only at 790 W/kg. For all treatments, differences ≥1 log reduction was not observed between the humidified and dry BI. The ANOVA results from the analysis of the inactivation percentage of the dry and wet state of the BIS confirmed that there was no significant difference between the means (p=0.9854) at 790 W/Kg. However, at 530 W/kg and 270 W/kg, humid soybean-molasses BISs were less resistant than the dry ones. Vela and Wu (1979) reported that microorganisms, including spores, were inactivated only in the presence of water and that dry or lyophilized organisms were less affected even by extended exposures. Heating by microwave energy occurs by both dielectric and ionic mechanisms, dielectric heating occurs by the oscillation of water molecules, and ionic heating is caused by the migration of ions that transfer energy to water molecules by acceleration and collision (FDA, 2000). The sample hydration may be lost during the heating process due to the permeable characteristics of the BIS pack in not allowing the evaluation of the hydration effect and justifying the lack of difference found in the highest potency/mass ratio studied. It was observed that the spores inactivation variation decrease with potency/mass ratio increment. At the lower studied potency/mass ratio (7 W/kg) there was no significant inactivation for the most of BIs samples due the low percentage of inactivation associated to the colony count method accuracy.

Figure 7 shows the studied BIS inactivation at 790 W/kg. The inactivation, as expected, increases as a function of exposure time. All BISs studied showed resistance greater than the spore suspension (control). In increasing order, the resistance of each BIS type is as follows: spores on strips, glycerol-based BI, soybean molasses-based BI and spore suspension. The high resistance of the BIS may cause false process failure demonstration. The results indicated that the control BIS resistance may be not high enough to efficiently monitor the process or that the process conditions are inadequate to achieve 4 log spores reduction.

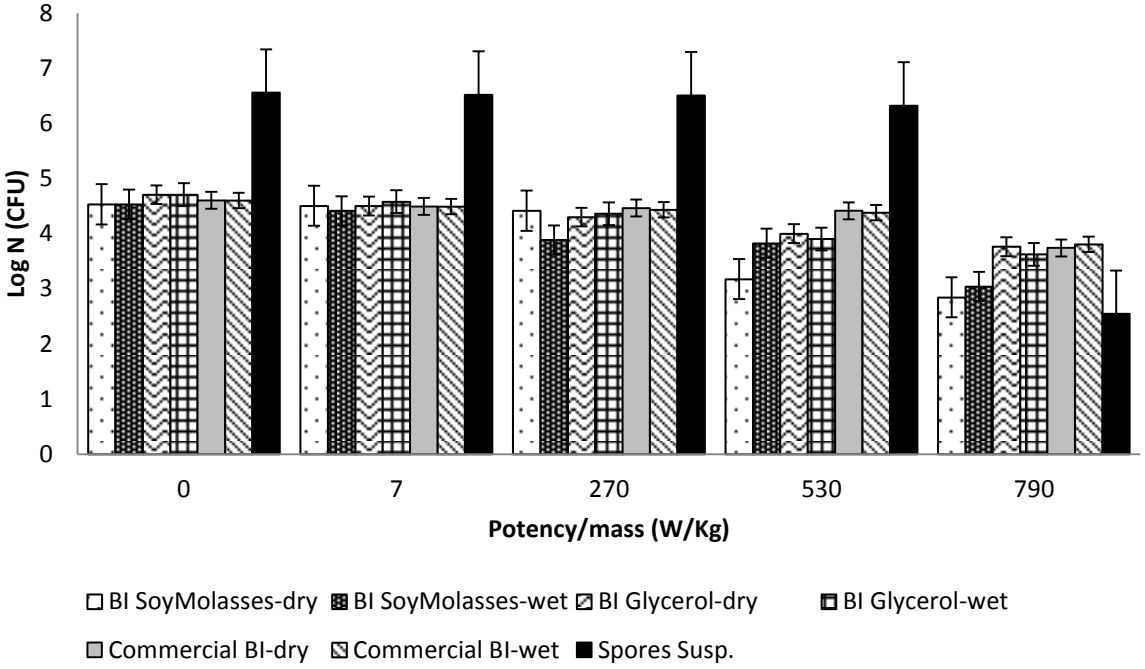

FIGURE 6 – *Bacillus atrophaeus* BIOLOGICAL INDICATOR SYSTEM (BIS) LOG REDUCTION AFTER 40 MINUTES MICROWAVE EXPOSURE TIME AT DIFFERENT POTENCY/MASS RATIOS

SOURCE: The author (2012)

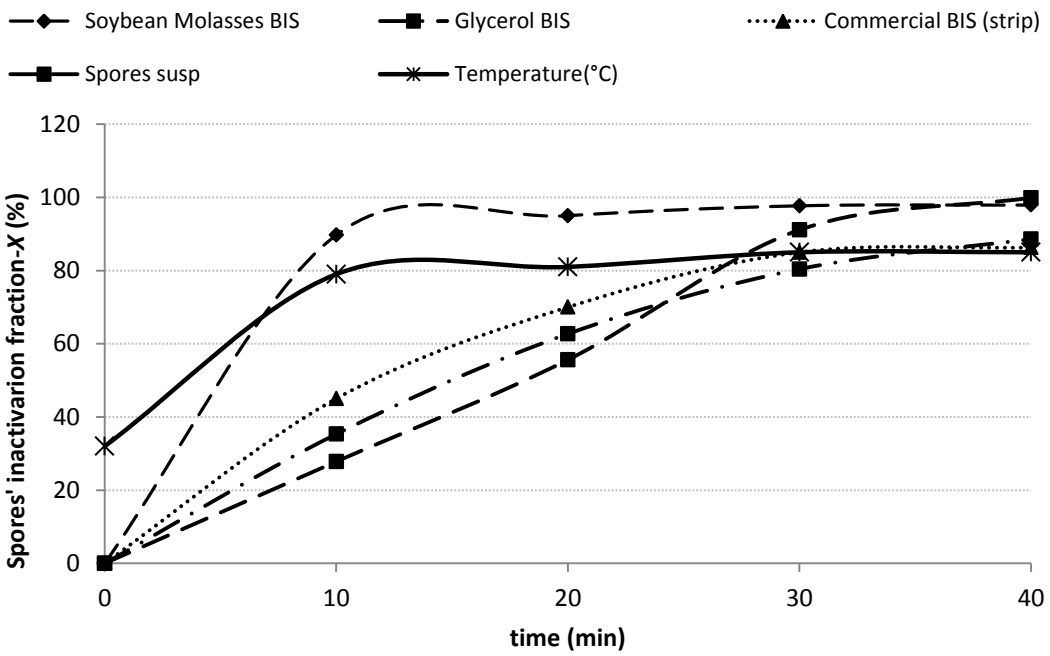

FIGURE 7 – FRACTION OF *B. atrophaeus* SPORES INACTIVACTION FRACTION AS A FUNCTION OF EXPOSURE TIME WHEN SUBMITTED TO 790 W/kg MICROWAVE RADIATION
SOURCE: The author (2012)

These results are in accordance with Jeng *et al.* (1987), who demonstrated that microwave heating at temperatures below 117°C was not effective for the sterilization of dry medical devices because of the low lethality rate and because a minimum 45 min exposure time was required to inactivate 10^5 dry spores at 137°C.

The use of spore suspensions in sealed ampoules or flasks as a medical waste sterilization BIS is controversial. The Brazilian Company of Environmental Technology and Sanitization (CETESB, 2007) recommends its use; however, it is not accepted by the U.S. Environmental Agency-EPA (MESON *et. al* 1993), as this treatment may create artificial conditions of temperature and pressure that do not accurately reflect actual waste conditions. Oliveira et al. (2010b), when comparing the microwave inactivation of spore suspensions in sealed ampoules with spores inoculated in healthcare waste, concluded that the exposure time necessary to kill 4 log of *B. atrophaeus* spores in sealed ampoules did not result in the same inactivation of inoculated spores. They reported that the real process exposure time (30 minutes) did not sufficiently promote a high level disinfection for healthcare waste According to Kim *et al. (*2009), microwave can be used to kill microbial pathogens but not for sterilization of spore-forming microorganisms within a short exposure time.

4. CONCLUSIONS

The results of this study showed that soybean-based and glycerol-based BIS met international standards and regulations and could replace the commercial BIS for dry-heat and ethylene oxide sterilization. These BISs were more resistant than the control (spores in an ampoule) when submitted to microwave sterilization. Additional studies should be performed to elucidate whether the proposed control is suitable. An inactivation curve of *B. atrophaeus* spores should be performed in microwave medical waste treatment equipment to evaluate the actual process capacity to inactivate spores at different exposure times on strips, sand and spores inoculated in the waste.

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CONCLUDING REMARKS

The production of *Bacillus atrophaeus* spores by solid-state fermentation (SSF) using sand as a low-cost support is viable and was successful. This approach was successful because of the spores' ability to attach to sand and because of bacterial adhesion and surface colonization through biofilm formation prior to sporulation. These properties allowed for the reduction of downstream processing steps, which was achieved by the direct use of the fermented material, thus reducing cost and process cycle time.

The observed SSF sporulation influence on the phenotypes of germinating cells suggests a new methodology involving bacterial adhesion and biofilm formation associated with phenotypic alterations. The mechanistic basis remains to be elucidated. It would be interesting to determine whether these mechanisms can be applied to industrial production improvement or new product development by exploring new metabolic profiles from bacterial growth during solid state fermentation associated with biofilm production.

Soybean molasses may be used for the production of cost-effective biological indicator systems (BISs) for sterilization, although further research on alternative nitrogen sources is needed to significantly reduce the raw material costs of the recovery medium. The results of this study also showed that glycerol could be used to produce a high-quality, low-cost bioindicator system. However, studies of the direct use of crude glycerol remain to be carried out, and its purification cost must be considered.

The use of agro-industry and biodiesel production byproducts helps prevent environmental contamination and allows the production of a cost-effective biological indicator for sterilization with assured quality and performance. The inherent variability of agro-industrial and biodiesel production byproducts may mean that they cannot be reliably used as raw materials for healthcare products. The effects of variation among different lots of raw materials on the performance of the medium could be evaluated by determining the experimental *D*-values. If necessary, a central composite design could be used to determine the optimal concentrations of these materials and the sources of nitrogen.

The performance results of the two newly developed bioindicator systems meet international standards and regulations and could replace the commercial *B. atrophaeus* BI for dry-heat and ethylene oxide sterilization. Microwave disinfection tests demonstrated that the developed BISs were more resistant than the control BIS. The high resistance of BIS may cause false process failure indications, indicating that the resistance of the BIS control is not strong enough to efficiently monitor the process or that the process exposure time and conditions are inadequate. Additional studies are necessary to determine if it is a positive factor for microwave medical waste treatment monitoring or if it may cause false process failure indications.

The developed process may be utilized for *B. atrophaeus* spore production, other applications and the cost-effective production of spores from other *Bacillus* species.