In Silico analysis of Bacillus Stearothermophilus as a Bioindicator for Sterilization

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Abstract:

To kill every kind of viable bacterial cells, sterilization in food and pharmaceutical industries is essential. In this study, bio indicators are used to validate accurate sterility. Bacillus stearothermophilus is used as a reference standard heat stable bio indicator. A 16S ribosomal RNA ribotyping sequence of Bacillus stearothermophilus is compared with different bacterial bioindicators including Bacillus pumilus, Bacillus atrophaeus, Clostridium sporogenes, and Brevundimonas diminuta. Results showed that Bacillus pumilus has the highest percentage (90.88%) similarity with the Geobacillus stearothermophilus followed by the Bacillus atrophaeus (90.84%), and Clostridium sporogenes (85.35%). The Brevundimonas diminuta was the least similar organism having a percentage similarity of 81.71% with the Geobacillus stearothermophilus. Bacterial spores are used as biological indicators for sterilization because they exhibit resistance to chemical and physical sterilization techniques. The protein common in all bacteria under study is Spo0A that make the bacteria heat resistant. The eradication of all these kind of heat resistant spores needs a high temperature which will also kill other viable cells.

Keywords: Bacillus stearothermophilus; Bacillus pumilus; Bacillus atrophaeus; Clostridium sporogenes and Brevundimonas diminuta; Spo0A protein.

1. Introduction

Bioindicators encompass biological processes, bacterial species, or communities and are used to assess the quality of the environment and how it changes over time [1]. Changes in the environment are usually caused by anthropogenic disturbances e.g., pollution, land use changes or environmental pressures e.g., drought, late spring cold, although anthropogenic pressures form the main focus of bio indicator research [2]. The widespread development and use of bioindicator indicators have taken place mainly since the 1960s. Different types of bacteria exist in different places such as soil, water etc [3]. Germs have a very high level of space and the average volume of any living thing, and
only a thin layer forms a boundary between them and their environment. Therefore, bacteria are susceptible to the slightest mutations and, as a result, should be very good early indicators [4]. The diversity of soil microbes and associated activities is diverse and complicated, for example Clostridium, Coliforms, Bacilli, and other ammonia oxidizing bacteria, sulfur oxidizing bacteria, and anti-biotic resistant [5]. Today, as indicated by the most recent information, the quantity of harmful toxins is developing at a normal pace of 4% each year [6, 7]. To take care of this natural issue, it is important to propose basic and safe ways of testing for poisons in the climate and diminish the expense of substance testing that laborers need [8].

Organic observing makes it conceivable to precisely evaluate and foresee deviations from natural frameworks from a typical reaction to openness to anthropogenic and technogenic factors. Diversity of bacterial communities in soil is an indicator of soil health [9]. In addition, 20 species of bacteria per area, which can grow in a small area supplemented with 2,4-dichloroaniline as the only carbon source, were selected and identified. Vaporized hydrogen peroxide (VHP) is increasingly being used to remove impurities from hospital-segregated cells. Commercially available bio indicators, usually of the genus Geobacillus stearothermophilus, are used to test the efficacy of the antiseptic phase [10].

Radiant microorganisms like Vibrio and its catalysts are at present being utilized in harmfulness testing to screen the climate. In these bioluminescent trials the force of light relies upon the material and the centralization of contaminations in the water tests. The advantages of bioluminescent testing are around 10-60 seconds, they are exceptionally delicate to 0.1 nM, and they are precise (RSD isn’t over 10%) and have a wide scope of direct reaction. The bioassay recognizes significant normal poisons and can be trailed by substance tests. Assessment of brilliant microbes was portrayed in the present manner by glowing microorganisms put away as soon as 1969. In the last part of the 1980s testing was done similarly in Germany as a method for identifying contamination. Then glowing minute tests were performed by different specialists [11].

Bacterial contamination such as Bacillus subtilis in the area is an ongoing problem within the health, pharmaceutical and food industries. Therefore, the removal of waste is an important step in ensuring the infertility of food processing equipment, reducing the spread of germs, and preventing the transmission of nosocomial infections. Conventional disinfection and disinfection techniques that are widely used in the disinfection process include high temperatures, chemicals, or ionizing radiation. Bacterial grains are often used as a biological indicator (BI) for infertility, especially since bacterial traits show high resistance to chemical and physical methods of sterilization. Therefore, the process that achieves the total spore inactivity ensures complete elimination of other polluting microorganisms. Clostridium was used as a biological indicator of sterilization for wet temperatures. In addition to research related to the prevention and control of disease caused by this organism, studies on the clinical use of botulinum toxin for beneficial purposes are ongoing. Benefits include pain relief, improved spasticity control and the use of cosmetic enhancements [12].

Sporulation means formation of spores from vegetative cells under unfavorable conditions. Bacterial spores are used as biological indicators for sterilization because they exhibit resistance to chemical and physical sterilization techniques. The protein common in all bacteria under study is Spo0A stage [13]. This is stage of sporulation and a master regulator but many of its effects on the global pattern of gene transcription are likely to be mediated indirectly by regulatory genes under its control. In response to nutritional stress, gram positive bacteria Differentiate into endospore that is initiation of sporulation can be
controlled by spo0A which is a master regulator and is activated by phosphorylation. Its phosphorylation is done by multi component phosphorelay system. This phosphorylation on histidine and aspartate residues are fluctuating, allow a temporal control of sporulation initiation. Although the phosphorylation takes place on histidine because it is basic amino acid while aspartate is absent in this protein structure. Phosphorylation can be ATP dependent or can be on substrate level in which phosphate is present in inorganic form [12, 13].

The main objective of this study is to compare different bacterial bioindicators for Bacillus pumilus, Bacillus atrophaeus, Clostridium sporogenes, and Brevundimonas diminuta with Geobacillus stearothermophilus (Reference standard) using 16S RNA sequences. Another goal is to find out the protein common in all bacteria that is Spo0A stage and its importance in making these bacteria as bioindicators.

2. Materials and Methods

FASTA format of 16S rRNA of the selected strains was retrieved from NCBI database. BLAST tool was used to compare similarities and dissimilarities among the query sequence and the subject sequence. The concerned organism strain was searched out and then RefSeq was used represented by NR_accession number for each strain.

Inclusion criteria:

The query sequence of 16S rRNA from Geobacillus stearothermophilus under the accession number >NR_115284 was compared with the subject sequences of Clostridium sporogenes (>NR_029231.1), Bacillus atrophaeus (>NR_024689.1), Bacillus pumilus (>NR_043242.1), and Brevundimonas diminuta (ACCESSION CP035093.1).

Exclusion criteria:

All other bacteria which are not used as bioindicators were excluded from the study.

The biological indicators used to evaluate the effectiveness of antimicrobial processes include a carrier of germs to which the test material attaches. Carriers are made of very hot paper filter when in contact with a full steam, while carriers made of fiberglass glass and wet filter paper do not. It was made from a raw material to produce soybean processing and contrasted to a standard BIS. Sand, recovery media, and dry-fermented spores were used to make it. The BIS was created and upgraded in stages [14]. The recuperation medium included SOD (1.0g/L), SOM (30.0g/L), tryptone (40.0 percent g/L), and bromothymol blue (0.02 percent g/L). The surrounding dampness and strong state aging settings of the bioreactor meaningfully affected spore creation or dry hotness opposition. Just Mn2+ affected the definition emphatically, permitting Mg2+, K+, and Ca2+ to be disposed of. The medium required 6.8 minutes longer to ideal than the base, and the spore creation was 2.3 CFU/g dry sand (10,000 starting qualities). For BIS, D160°C = 6.6 0.1 min. The sporulation segment used to be once lessened to three days, and the enchantment of hotness harmed spores used to be adequate to arise as mindful of non-sterile BIS in 21 hours. Moreover, the methodology process duration used to be decreased from 29 to 15 days. No turn upward has of course approved BIS from soybean molasses [15]. For phylogenetic relationship among the strains, MEGA (version 5.2) software was used to construct evolutionary phylogenetic tree for homology observation. Then, the 3D structure of Spo0A protein was retrieved from PDB-NCBI (https://www.rcsb.org/structure/1FC3) and visualized by using 3D modeler software PyMol (version 2.5.2).

2.1 Bacillus atrophaeus
Bacillus atrophaeus spores are employed to divulge disinfection and sterilization operations, in precise dry warmth and ethylene oxide sterilization. This bacterium's natural sterilization warning signs are produced via sporulation, which penalties in a dormant structure resistant to warmth stress. The effectiveness of the utilization of nano materials as carriers as a replacement of paper strips for spore recuperation and bioindicator practice was studied in this work. The consequences of nanomaterial shape sorts on D values and spore absorption have been investigated and compared. The findings component to the nanomaterial's possible as a bioindicator support. Among the examined nanomaterial adsorbents, silica and gamma alumina had the most spore absorption effectively (107 CFU/mL) and spore resistance (6.5 min). The study's findings show off that nanoparticle can also exchange general strips owing to their longer endurance [16]. The discovery of novel glycerol functions is of world magnitude due to via environmental and financial troubles springing up from surplus glycerol produced at some stage in biodiesel production. To create a less high-priced sterilizing natural indicator system (BIS) (spores service restoration medium), glycerol was as quickly as used as the main substrate. Product improvement and optimization using sequential experimental design. D160 °C rate of 6.60.1 min is carried out by using capability of the use of the precise restoration medium. Solid-state fermentation produced 2.31.2108 CFU/g dry be aware of Bacillus atrophaeus spores. Sporulation kinetics findings confined this gadget to forty-eight hours. Germination kinetics recognized nonsterile BIS in 24 hours. The encouraged BIS carried out nicely towards dry-heat and ethylene oxide sterilization.

Cost breakdowns ranged from 41.8 to 72.8 percent (feedstock). As a fully carbon source, glycerol is used in this study, ensuing in huge fee monetary financial savings and earnings from a biodiesel byproduct [17].

2.2 Geobacillus stearothermophilus

Geobacillus comprises Gram positive bacteria, thermophilic spore-formers, found in a wide range of sources from tropical springs, cool soil, to food-producing plants, including dairy plants. Geobacillus stearothermophilus formerly Bacillus stearothermophilus is a thermophilic, aerobic bacterium, which produces heat-resistant compounds. Create biofilms that adhere to the surface of stainless steel in dairy plants. G. stearothermophilus contains endospores which is a type of bacterium that is very resistant. Bacteria produce seeds under difficult conditions where nutrient levels are low. The spore core is water-free (10-25% water content) which makes it very resistant to heat and chemicals. To protect the DNA Ca+2 dipicolinic acid complex and low fat-soluble proteins (SASP) bind to spore DNA and increase the resistance of the molecules to shrinkage. The outer spore contains a loose peptidoglycan that blocks the flow of water and acts as a barrier to entry into chemicals such as lysozyme [16]. G. stearothermophilus also contains thermo stable lipase enzyme that can withstand up to 90° C. This heat stability may be due to its specific protein chains length and interaction between the chains.

2.3 Clostridium sporogenes alternative to Bacillus stearothermophilus

Clostridium sporogenes is a rod-shaped, gram-positive bacterium that it produces spores and has flagellar motility. It is an anaerobe bacterium. It causes food spoilage. It is used in food industries for low temperature steam or pasteurization applications. It is used as a substitute for pathogens such as C. botulinum and C. perfringens for testing the efficacy of commercial sterilization. C. sporogenes spores have also been proposed as a vector for treatment of cancer by drugs. C. sporogenes bioindicator spores are more resistant than Bacillus stearothermophilus and they need temperature of at least 110° C for their process. It also uses in the wet heat sterilization method in place of B. stearothermophilus [17].

2.4 Bacillus pumilus
The antifungal metabolites are created by B. pumilus. The antifungal metabolites repressed mycelial development of numerous types of Aspergilli, Penicillium and Fusarium. They likewise restrained creation of aflatoxins, cyclopiazonic corrosive, ochratoxin An and patulin. Physical cold barometrical surface miniature release (SMD) plasma working in surrounding air has promising properties for the disinfection of delicate clinical gadgets where regular strategies are not appropriate. The antimicrobial consequences for Gram-negative and Gram-positive microbes of clinical importance, as well as the growth Candida albicans, were tried. The disinfecting impact on standard bioindicators (bacterial endospores) was assessed on dry test examples that were enveloped by Tyvek coupons. The trial D 23 °C qualities for Bacillus pumilus was 0.3 min [18].

3. Results

Bacillus pumilus has the highest percentage similarity with Geobacillus stearothermophilus which is 90.88% and the percentage dissimilarity between respective organisms is 9.12%. Bacillus atrophaeus has the second highest percentage similarity with Geobacillus stearothermophilus which is 90.84% and the percentage dissimilarity between the respective organisms is 9.16%. Clostridium sporogenes is ranking third having a percentage similarity of 85.35% with Geobacillus stearothermophilus and percentage dissimilarity of 14.65% between the respective organisms. Brevundimonas diminuta is the least similar organism having a percentage similarity of 81.71% with Geobacillus stearothermophilus and percentage dissimilarity between the respective organisms is 18.29% (Table-1).

3.1 Geobacillus stearothermophilus comparison with Clostridium sporogenes

Thermal obstruction of Clostridium sporogenes PA3679 and the thermo physical properties as warm conductivity, thickness, warm diffusivity, and explicit hotness are set for the foie gras delivered in Quebec and other subsidiary items. A numerical model was fostered that can animate the temperature at the focal point of the adversary grass jam. The outcomes for warm opposition and warm properties are shown overall with the distributed information; these qualities were utilized with the model to anticipate the temperature at the coldest zone. The anticipated temperatures contrasted with the test temperature and are of low blunder.

3.2 Geobacillus stearothermophilus comparison with Brevundimonas diminuta

Brevundimonas diminuta ATCC 19146, chose as a natural pointer, was ready to endure channel expulsion. The channel hardware was intended to consider efficient investigation of test factors. Test techniques have been created to consider the greatest convergence of microbes and to make sense of the impacts of a few boundaries (e.g., bacterial numbers, filtration tension, time, and liquid science) away. These techniques were additionally used to quantify the expectation of maintenance by actual assessment of layers. The effect of the test factors tried was a component of the size of the channel openings. Bacterial maintenance by film channels regularly utilized for cleansing was autonomous of bacterial numbers, channel tensions and liquid synthetic compounds and was time-subordinate. Cleansing of the channel is accomplished reliably with a solitary layer channel of 0.22 (μm) through consistent sifting if 16 hours long. The air pocket point of the layer channels was a strong indicator of bacterial maintenance. No bacterial excess has been seen over few air pocket focuses. A tiny trial of bacterial cell entrance as a component of profundity inside the channel raised the bacterial maintenance model in view of the consecutive separating of cells by a three-layered channel structure. The channel ability data is joined with the volume and microbial substance of the fluid to compute the
possibilities affirming barrenness. Treatment by channel has been viewed as a solid and unsurprising strategy when utilized under controlled conditions.

3.3 Comparison of *Geobacillus stearothermophilus* with *Bacillus pumilus*

Blast results of query *Geobacillus stearothermophilus* strain BGSC 9A20 16S ribosomal RNA, complete and *Bacillus pumilus* strain ATCC 7061 16S ribosomal RNA, partial sequence 1434 have been given in Figure 1.

3.4 Comparison of *Geobacillus stearothermophilus* with *Bacillus atrophaeus*

*Bacillus atrophaeus* has been used as a subject microorganism in this article because its characters are widely used to study character resistance and morphology. Understanding the mechanisms involved in cell cycle and seed survival is important in developing seed techniques; producing products that can withstand biodefense, food and drug use; and the development of new bioactive molecules and grain-marking methods. Blast results of query *Geobacillus stearothermophilus* strain BGSC 9A20 16S ribosomal RNA, complete and *Bacillus atrophaeus* strain JCM 9070 16S ribosomal RNA, partial sequence has been shown in Figure 1.

Table 1: BLAST results of Query *Geobacillus stearothermophilus* with other bacterial strains

<table>
<thead>
<tr>
<th>Query</th>
<th>Subject</th>
<th>Max Score</th>
<th>Total Score</th>
<th>Query Cover</th>
<th>E Value</th>
<th>Per Ident.</th>
<th>Acc. Len</th>
<th>Accession</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Geobacillus stearothermophilus</em></td>
<td><em>Clostridium sporogenes</em></td>
<td>1092</td>
<td>1418</td>
<td>97%</td>
<td>0.0</td>
<td>85.35%</td>
<td>1503</td>
<td>&gt;NR_029231.1</td>
</tr>
<tr>
<td></td>
<td><em>Brevundimonas diminuta</em></td>
<td>854</td>
<td>1112</td>
<td>81%</td>
<td>0.0</td>
<td>81.71%</td>
<td>1452</td>
<td>CP035093.1</td>
</tr>
<tr>
<td></td>
<td><em>Bacillus pumilus</em></td>
<td>1869</td>
<td>1869</td>
<td>89%</td>
<td>0.0</td>
<td>90.88%</td>
<td>1434</td>
<td>&gt;NR_043242.1</td>
</tr>
<tr>
<td></td>
<td><em>Bacillus atrophaeus</em></td>
<td>2030</td>
<td>2030</td>
<td>97%</td>
<td>0.0</td>
<td>90.84%</td>
<td>1515</td>
<td>&gt;NR_024689.1</td>
</tr>
</tbody>
</table>
Evolutionary history was inferred using the Neighbor-Joining method [19]. The optimal tree is shown. The tree is drawn to scale, with branch lengths (next to the branches) in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method [20] and are in the units of the number of base substitutions per site. The proportion of sites where at least 1 unambiguous base is present in at least 1 sequence for each descendent clade is shown next to each internal node in the tree. This analysis involved 5 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1563 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Figure 2).

Figure 2: Original phylogenetic tree conducted in MEGA11

Spo0A has three chains A, B and C and each chain has number of molecules which are described (Figure 3). Role of disulphide bridges in Spo0A is in stabilization of the folding of a single polypeptide chain. Role of nitrogen oxygen sulphur bonding in Spo0A is again in stabilizing the protein structure. Amino acids that are made up of nitrogen performs its role by acting as the building blocks of all proteins and enzymes (Figure 4). Role of sulphur in Spo0A helps in the formation of intracellular antioxidants such as glutathione and N-acetyl cysteine Sulphur amino acids are used. Similarly, oxygen is present in water molecule that surrounds the protein and is linked with protein through weak electrostatic forces.

Figure 1. Graphical and Dot Plot representation of BLAST results of Query and Subject Strains

**Query:** Geobacillus stearothermophilus strain BGSC 9A20 16S ribosomal RNA

**Subjects:**
1. Clostridium sporogenes strain McClung 2004 16S ribosomal RNA, partial sequence
2. Brevundimonas diminuta ATCC 11568 16S ribosomal RNA, partial sequence
3. Bacillus pumilus strain ATCC 7061 16S ribosomal RNA, partial sequence
4. Bacillus atrophaeus strain JCM 9070 16S ribosomal RNA, partial sequence
3DSPo0A

Chain A
(Molecule 1: Atoms C, O, N, S)
(Molecule 2: Atom O)

Chain B
(Molecule 1: Atoms C, O, N, S)
(Molecule 2: Atom O)

Chain C
(Molecule 1: Atoms C, O, N, S)
(Molecule 2: Atom O)

Figure 3: Spo0A has three chains A, B and C and each chain has number of molecules

Conjugation
Aromatic Ring

Sulphur

Nitrogen

NOS Binding

Oxygen

Figure 4: Atoms, disulphide bridges and NOS bonding in Spo0A

4. Discussion:

In this study, bioindicators were used to validate accurate sterility. Bacillus stearothermophilus is used as a reference standard heat stable bioindicator. 16S ribosomal
RNA ribotyping sequence of Bacillus stearothermophilus was compared with different bacterial bioindicator strains like Bacillus pumilus, Bacillus atrophaeus, Clostridium sporogenes and Brevundimonas diminuta. Bacillus pumilus is a vigorous, pervasive Gram-positive, bar molded endospore-framing microscopic organisms. It tends to be disconnected from a wide assortment of soils, natural surfaces, and plants, even from the inside of Sonoran Desert basalt. B. pumilus were additionally present in shuttle get together offices as metabolically torpid spores. Bacillus spores are famously impervious to troublesome circumstances, for example, low or no supplement accessibility, outrageous drying up, H2O2, UV, gamma-radiation, or compound sterilizations [24, 25].

In above study, 16s rRNA sequence of all test bacteria was taken and blasted with 16s rRNA sequence of G. stearothermophilus. Blast gives us the percentage similarity between test organisms and the control organism, and this percentage homology indirectly guides us about percentage dissimilarity between two organisms. The more percentage similarity means that the test bacteria is more capable of acting as a bioindicator in sterilization.

Spo0A protein is the initial phase of sporulation and acts as regulatory protein. It is the beginning stage for the production of mature spores. If it’s absent in bacteria, then that bacterium is incapable to produce spores [26].

Conclusion:

After a lot of research, a single common protein Spo0A was obtained in all the above said bacteria (Geobacillus stearothermophilus, Bacillus atropheus, Brevundimonas diminuta, Bacillus pulmulis, Sporulation based on the property that make these bacteria to act as bioindicator in sterilization. All of these bacteria are capable for the production of spores under stressful condition to act as a bioindicator in sterilization. The production of specific spores indicates the presence of specific bacteria at a place representing them as a bioindicator. Although these bacteria have different mechanisms and different modes of actions but all of them are common in producing spores.

Author Contributions:

Conceptualization, H, N. and K, B. N.; methodology, H.N.; software, H, N. and K, B. N.; validation, A, A.; B, A.; formal analysis, M, A.; investigation, A, S.; resources, A, R. M.; data curation, H, N. & M, A.; writing—original draft preparation, H, N.; K, B. N.; writing—review and editing, A, A.; B, A.; visualization, M, A; supervision, H, N. & S.; A.; project administration, H, N.; B. N. & B, A.; . All authors have read and agreed to the published version of the manuscript.” Please turn to the CRediT taxonomy for the term explanation. Authorship must be limited to those who have contributed substantially to the work reported.

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