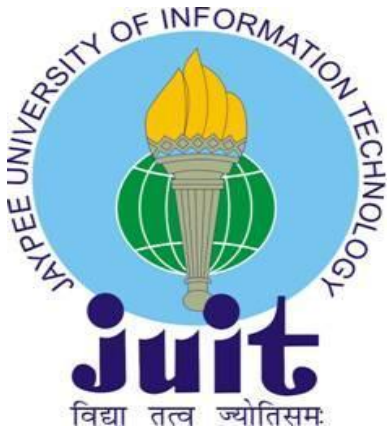
PHYSICAL AND BIOLOGICAL TREATMENTS TO INACTIVATE FOOD SPOILING *Bacillus subtilis*

Enrollment No. - 101711, 101718

Name of Students - Ankita Thakur , Enesh Vashisht

Name of Supervisor - Dr. Gunjan Goel



2013-2014

Submitted in partial fulfillment of the Degree of

Bachelor of Technology

DEPARTMENT OF BIOTECHNOLOGY,

JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY,WAKNAGHAT SOLAN (H.P.)

TABLE OF CONTENTS

Chapter No. Topics

3.3.1 Temperature treatment to *Bacillus subtilis*

3.3.2 PH treatment to *Bacillus subtilis*

3.4 Evaluation of natural barriers (microbial products/ spices) against *B. subtilis*

3.4.1 Effects of spices

3.4.2 Preparation of extracts

3.4.3 Agar assay

3.5. Effect of lactic acids culture

3.5.1. Different lactic acid culture was obtained from different sources

Chapter 4 Results

4.1 Growth curve of *Bacillus subtilis*

4.1.1Inactivation kinetics of *B.subtilis* spores at 80-100˚C

4.1.2. Calculation

4.2.2 Effect of pH

4.3. Effect of spices

4.4 Effect of Lactic Acid Cultures

Conclusion

References

CERTIFICATE

This is to certify that the work titled "**Physical and Biological Treatments to Inactivate Food Spoiling *Bacillus subtilis***" submitted by Ankita Thakur and Enesh Vashisht to the Department of Biotechnology and Bioinformatics, Jaypee University of Information Technology, Waknaghat in the partial fulfillment of the requirements for the award of the degree of **Bachelor of Technology in Biotechnology** is a record of bonafide research work carried out by them under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

Dr. Gunjan Goel

Assistant Professor (Grade-II)

Department of Biotechnology & Bioinformatics

Jaypee University of Information Technology

Waknaghat-173234

Solan, Himachal Pradesh

Date

ACKNOWLEDGEMENTS

We are very thankful and express our deepest gratitude to Dr. Gunjan Goel for providing us such a great opportunity of working on this project.

We would like to pay our most sincere thanks to Dr. R.S.Chauhan, Head of Department, Department of Biotechnology and Bioinformatics, for providing us with opportunities and facilities to carry out the project.

We also thank Mrs. Deepika Sharma and Ms. Niharika Singh (PhD scholars) for providing her full cooperation with keen interest. We are indebted to other faculty members and our friends and all those who provided their reviews and suggestions for improvising our project.

Signature of Students

Name of Students

Ankita Thakur, Enesh Vashisht

Date

SUMMARY

Bacillus subtilis, also known as the **hay bacillus** or **grass bacillus**, is a Gram-positive, a member of the genus *Bacillus*. It is rod shaped and has the ability to form tough protective endospores allowing the organism to tolerate extreme environmental conditions, it is an obligate aerobe. Its ability to produce endospores has made the organism popular in the field of thermo bacteriology also. In commercial food processing protocols, heat inactivation studies play a vital role in the optimization of thermal processes, which are critical control points, often striking a balance between safety, organoleptic and nutritional acceptability of foods, therefore spoilage by this bacterium in food processing units in still a major concern. While *B. subtilis* is not a major agent of food spoilage or food-borne disease, this organism is a model spore former and one that is genetically tractable, with many strains available with mutations and reporter genes that can facilitate analysis of its resistance to the food production environment. The organism has the similar characteristics with *B.cereus* which is reported to cause outbreaks due to its ability to produce toxins resulting in types of illness: one type characterized by diarrhea and the other, called emetic toxin, by nausea and vomiting. Therefore to control the intrinsic or extrinsic entry of pathogens to the food processing units, there is a need to check the efficacy of various biological/ physical methods which can limit the growth of the *B.subtilis*. For this, various physical treatments (pH, temperature, UV radiation and microwave rations) and biological (lactic acid bacteria (LAB) and plant herbs) were evaluated for their anti-bacillus action so that a combination of technique could be developed against the proliferation of this bacteria. Among the factors listed above, the biological treatments seemed to be most economic and promising as antibacterial action was observed in case of application of LAB and clove, fennel.

List of Figures and Tables

Figures:

Sr. No. Figure Description

Tables :

Sr. No. Table Description

Chapter 1

INTRODUCTION

Bacillus subtilis, also known as the hay *Bacillus* or grass *Bacillus*, is a Gram-positive, a member of the genus *Bacillus*. It is rod-shaped, and has the ability to form a tough, protective endospore, allowing the organism to tolerate extreme environmental conditions. When there is nutrient limitation, some bacteria respond to it by forming an endospore, a morphologically distinct cell type. This process is called sporulation and has been the subject of continuous microbiological investigation since the seminal 19th century reports [1].

The endospore is metabolically dormant which is environmentally resistant cell and has the capability to survive extremes of temperature, desiccation and ionizing radiation. The endospore can survive from thousands to millions of years. A number of factors are responsible for this robustness including dehydration of the spore core and compaction of chromosomal DNA [2, 3].

The aerobic spore-forming Gram Positive *Bacilli* belonging to the genus *Bacillus* and other closely related genera are playing an increasingly important role in the food and beverage industries. However, the significance of these organisms is poorly understood. The extent to which *Bacillus* and closely related genera may be responsible for outbreaks of food associated illness is most likely understated in many countries due to the major emphasis on other gram negative pathogens such as *Salmonella* spp. *E.coli*. In Asian countries, there are only 42% cases reported on the spoilage and diseases by *Bacillus sp.*. Food like Meat (pies, stews, curries, sandwiches), chinese food, poultry and poultry products, seafood and seafood products, bakery products , cereal products , rice , Asian boxed meals , dairy products (milk, UHT milk, yoghurt, cream) have been reported to associated with occurrence of *Bacillus* sp. A lots of literature is available on *B.cereus* as a food borne pathogen however, the investigations should be extended to include all species including those in the closely related groups.

The major spoilage caused by *B.subtilis* is the ropiness in bread caused by bacteria and it is generally caused by *Bacillus subtilis*, but *B. licheniformis*, *B. megaterium*, *B. pumilus* and *B.*

cereus can also be the causative agents [4]. Bacterial spores cause major problems in the food industry because of their ubiquitous occurrence and their intrinsic high stress resistance features [5]. The spores which retain their viablility after baking can germinate and cause spoilage of bread by rope formation, if they are exposed to a warm and moist environment. Rope in bread is initially characterized by a sweet fruity odour, similar to that of over-ripe melons or pineapples. It is followed by enzymatic degradation resulting in discoloration and the crumb eventually becomes soft and sticky to the touch because of the breakdown of starch and proteins by microbial amylases and proteases, and by the production of extracellular polysaccharides [6, 7]. *B. licheniformis*, *B. subtilis* and *B. pumilus*, which comprise the subtilis group, have been associated with several human infections that cause a range of diseases and incidents of foodborne gastroenteritis [7, 8].

Infections that can be caused by *B. subtilis* include bacteremia, endocarditis, pneumonia and septicaemia. *B. subtilis* contaminated foods have been the cause of various studies of food contamination that have been reported [9]. *B. subtilis* is considered an opportunist with no pathogenic potential. It produces different exo-enzymes contributing to the decay of organic matter. There is an extracellular enzyme known as subtilisin which licit allergic or hypersensitive reactions in individuals who are repeatedly exposed to it. But, the toxigenic properties are very low. *B. subtilis* does not produce significant quantities of extracellular enzymes or toxins and is generally considered to have a low degree of virulence to humans [10].

In the dairy industry, *Bacillus* species cause a continuous hygiene problem due to the formation of biofilms within milking pipelines and on surfaces of equipment. It leads serious economic losses due to food spoilage and equipment impairment. There have been investigations in which the mechanism by which the model organism *Bacillus subtilis* forms biofilms in laboratory mediums in vitro is studied but there is a little knowledge of how these biofilms are formed in the natural environments (eg. in milk).

Given the ubiquitous in nature and the environmental conditions and with outbreaks, the exact nature of its involvement has not been established. *B. subtilis*, like other closely related

species in the genus, *B.licheniformis, B. pumulis,* and *B. megaterium*, have been shown to be capable of producing lecithinase, an enzyme which disrupts membranes of mammalian cells.

While *B. subtilis* is not a major agent of food spoilage or food-borne disease, this organism is a model spore former and one that is genetically tractable, with many strains available with mutations and reporter genes that can facilitate analysis of its resistance to the food production environment. The organism has the similar characteristics with *B.cereus* which is reported to cause outbreaks associated with foods. The formation of spore by these *Bacillus sp.* often pose a challenge to the thermal efficacy of heat processes resulting in reduced shelf life of many processed foods. Higher heat treatments alter the organoleptic properties of food and hence the need of the hour is to use of a combination of antibacterial agents along with mild heat treatment to inhibit the microbial growth individually which is the basis of "hurdle technology" and in the last decade there has been an explosion of research interest in realizing the potential benefits of this technology. However, a thorough understanding of the physiological responses of micro-organisms to stresses imposed during food preservation is essential if novel combination systems based on milder food processing procedures are to be developed effectively and therefore, this study investigates the effect of physical as well as biological methods as preferred method of ensuring safety and quality of foods.

Chapter 2

REVIEW OF LITERATURE

2.1 Food Preservation and Hurdle Technology

Hurdle technology is the deliberate combination of novel preservation techniques that are already existing so that the microorganisms present should not be able to overcome these preservative factors or hurdles [11]. There are various types of hurdles that can be used against the microorganisms. These hurdles can be temperature, pH, microwave and UV radiations, water activity, preservatives, herbal extracts, etc. When a hurdle is provided to the microorganism, it becomes a stressed condition for the microorganism. The microorganism, in order to overcome this hurdle, has to do some effort. When the higher hurdle is given then the effort is also higher, i.e. larger number organisms needed to overcome it.

It has been known from the centuries that a combination of preservation factors influences the stability of microorganisms and the safety of foods. In many traditional foods, this concept is more or less unconsciously used, especially in the developing countries. The concept was re-invented in the meat industry where there was a conscious employment of hurdles and it was found to be highly favorable for the shelf-stable sausages production. This concept is now used for the preservation of a wide range of food products including dairy products, fruits and vegetables, bakery products, beverages, etc. Several novel preservation factors that facilitate the development of such hurdles have been assessed in bioconservation, bacteriocins, ultrahigh pressure treatment, edible coatings and gas packaging [12].

Hurdle technology is a very crucial concept in the food preservation. When we apply hurdles to a stable food product then these hurdles concertedly control the spoilage by microbes and food poisioning, but the desired fermentation fermentation process is not affected by these hurdles. Since it is a concerted effect, the individual hurdles may be applied at a lower intensity than it would be required if only a single hurdle were to be used as a preservation technique. This concept of applying concerted hurdles has proven successful. The proper combination of hurdles leads to the microbial stability and safety. It also stabilizes the nutritive, sensory and economic properties of a food [11].

2.1.1 The behavior of microorganisms

Microorganisms react to the stress factors homeostatically. When a stress factor disturbs their environment, they usually react in ways that maintain some key element of their physiology constant. There are many homeostatic reactions. Varying results are produced due to the stress. In some of the cases, the microorganisms become metabolically exhausted and then die, as they are trying to overcome the hostile conditions so the microorganisms strain every possible repair mechanism to overcome a hostile environment. In such cases, microorganisms may die more quickly in apparently favourable conditions. for example, *Salmonellae* generally survive the ripening process in the fermented sausages, but will vanish more quickly if the products are stored at ambient temperature. If the products are stored under refrigeration, they will survive for long time and will possibly cause food-borne illness [13]. As compared to the ambient temperature, salmonellae survive in mayonnaise at chill temperatures. But in some cases, some bacteria generate heat shock proteins and thus become more resistant or even more virulent under the stresses. Sometimes the microorganisms acquire cross-tolerance as microorganisms can become more tolerant to other stresses due to a particular stress. An example to this is the reaction to changes in pH. In many foods, the pH of the cytoplasm of the microorganisms will normally be one or two units higher than that of their environment. Microorganisms can grow at low pH values. It depends on their ability to prevent protons from crossing the cell membrane and entering the cytoplasm. It also depends to a large degree on their ability to export any protons that do gain access, and thus a satisfactorily high intracellular pH is maintained. The pH of the cytoplasm will fall if the net influx of the proton cannot be prevented. It will lead to cessation of growth and death of the cell.

However, there are many bacteria, yeasts and moulds that can react to the acid conditions for enhancing their survival. The stress response due to the exposure to the acid conditions gives rise to adaptation such that the efficacy of proton export increases. This acid tolerance response results in microorganisms that have been exposed to even mildly acidic conditions to become able to grow or survive at low pH values that would otherwise have been lethal. Acid-adapted cells may also acquire tolerance to other environmental stresses, e.g. in *S. typhimurium*. *L. monocytogenes* became more acid tolerant when acid-shocked, but also it exhibited a rise in

heat resistance. Also. it has been shown that the heat shock response that follows mild heating can result in cells becoming more acid tolerant.

Metabolic exhaustion is a factor that is of important consideration in hurdle technology. The stressed cells require the expenditure of energy for homeostatic stress responses. Then, the appropriate target to pursue is the restriction of the availability of the energy. As an example, if a food can be preserved by lowering the pH, then the addition of a weak acid preservative can also be done because this will amplify the effect of the protons or to allow a milder, higher pH to be employed [14].

Table 1: Homeostatic responses to stress by microorganisms [15]

Stress factor	Homeostatic response
Low levels of nutrients	Nutrient scavenging; oligotrophy; stationary-phase response; generation of viable non-culturable forms
Lowered pH	Extrusion of protons across the cell membrane; maintenance of cytoplasmic pH; maintenance of transmembrane pH gradient
Lowered water activity	Osmoregulation; accumulation of compatible solutes; avoidance of water loss; maintenance of membrane turgor
Lowered temperature for growth	Cold shock response; changes in membrane lipids to maintain satisfactory fluidity
Raised temperature for growth	Heat shock response; membrane lipid changes
Raised levels of oxygen	Enzymatic protection from hydrogen peroxide and oxygen-derived free radicals
Presence of biocides	Phenotypic adaptation; reduction in cell wall/membrane permeability
Ionizing radiation	Repair of single-strand breaks in DNA
High hydrostatic pressure	Uncertain; possibly low spore water content
High voltage electric discharge	Low electrical conductivity of the spore protoplast

Competition from other microorganisms	Formation of interacting communities; aggregates of cells showing some degree of symbiosis; biofilms
Presence of weak organic acid preservatives	As lowered pH, and sometimes extrusion of the organic acid

2.1.2 The hurdle technology Basic Aspects

Microorganisms are put in hostile environment so as to inhibit their growth or shorten their chances of survival or cause their death. The microorganisms respond to their hostile conditions determines whether the microorganisms would grow or die. Homeostasis, metabolic exhaustion and stress reactions of the microorganisms are the important factors in relation to the hurdle technology. Hurdle technology foods are preserved by considering the concept of multi target preservation which is gentle but also most effective [16].

2.1.3 Homeostasis

Homeostasis refers to the tendency of the microorganisms by which they maintain uniformity and stability in their internal environment. For example, the higher organisms as well as the microorganisms have a feature that they maintain a defined pH. Homeostasis is well studied in the higher organisms at molecular, subcellular, cellular and systemic levels, and this knowledge of higher organisms should also be employed to the microorganisms that cause food poisoning and spoilage. When developing a hurdle technology, homeostasis is a major factor to consider. When we apply some preservative factors or hurdles, the homeostasis of these microorganisms is disturbed. They will not multiply and remain in their lag phase or may die before the homeostasis is re-established. Therefore, by disturbing the homeostasis of microorganisms in a food temporarily or permanently, food preservation can be achieved.

2.1.4 Metabolic exhaustion

Metabolic exhaustion is an another phenomenon which is of practical importance. It can cause the autosterilization of the food. This was first observed in the experiments where the liver sausages that were mildly heated were adjusted to different water activities by adding salt and

fat and then this product was stored at 37° C after the inoculation with Clostridium sporogenes [17].

2.1.5 Stress reactions

On the application of various stresses like pH, temperature, water activity, etc., the bacteria may produce heat shock proteins in response and develop resistance for these stresses or become more virulent. In some cases, the microorganisms acquire cross tolerance, thus the stress reactions might have a non-specific effect. The microorganisms may produce various responses to these stresses and it may be problematic for the application of hurdle technology. When different stresses are applied at the same time then in order to manufacture or synthesize the heat shock proteins the microorganisms need to spend huge amount of energy, and in some cases they become metabolically exhausted and die. So, when the different stresses are received by the organism at the same time then the survival of the microorganisms in the stressed conditions would be difficult as the activation of the genes which lead to the synthesis of heat shock proteins is difficult. So therefore, multi-target preservation of food products is helpful in preventing the synthesis of heat shock proteins, which ultimately could alter the microbial stability and safety of hurdle technology foods [16].

Table 2 : Major hurdles used to preserve foods [15]

Type of hurdle	Examples
Physical hurdles	Aseptic packaging EM energy High temperatures Ionizing radiations Low temperatures Modified atmospheres Packaging films Photodynamic inactivation Ultra-high pressures

	Ultrasonication UV radiation
Physic-chemical hurdles	Carbon dioxide Lactic acid Ethanol Lactoperoxidase Low pH Low redox potential Low water activity Organic acids Oxygen Ozone Phenols Phosphates Salt Smoking Sodium nitrite/nitrate Sodium or potassium sulphite Spices and herbs Surface treatment agents
Microbially derived hurdles	Antibiotics Bacteriocins Competitive flora Protective cultures

2.1.6 Biological methods for food preservation

Bacteriocins of lactic acid bacteria (LAB) : LAB and their antimicrobial metabolites control the spoilage and pathogenic bacteria in foods as they have the potential as natural preservatives.

Nisin is a bacteriocin which has found practical applications in some industrially processed foods.

Bacteriocins are the anti- microbial substances of lactic acid bacteria. They serve as biopreservatives having bacteriocidal or bacteriostatic activity. These bacteriocins are synthesized ribosomally and released extracellularly as bioactive peptides or peptide complexes. Some potential food borne pathogens are inhibited by the bacteriocins are *Clostridium botulinum, Enterococcus faecalis, Listeria monocytogenes, Staphylococcus aureus* and *Bacillus species*. These bacteriocins can be degraded by the proteases in the gastrointestinal tract, thus, considered as safe biopreservatives [17]. There are four classes of antimicrobial peptides from lactic acid bacteria: lantibiotics; small (<13kDa) hydrophobic heat stable peptides; large (~30kDa) heat-labile proteins; complex proteins requiring additional carbohydrates or lipid moieties to attain antimicrobial activity.

Most of the bacteriocins are bacteriocidal, Nisin acts on the cytoplasmic membranes of the Gram-positive bacteria and cause lesions. After the treatment of nisin the whole or the intact cells and the membrane vesicles exhibit efflux of amino acids and cations. Proton motive force is depleted due to the loss of these substances, which further interferes with cellular biosynthesis. All this leads to death of the cell as there is a collapse of membrane potential.

2.2 Effect of Heat

When bacterial spores are suspended in an aqueous environment, they have the unique property of heat resistance. It is termed wet heat resistance. The spores in water are generally resistant to approx. 40°C higher temperatures than that of growing cells of the same strain [18]. The core water content is the main factor determining the spore wet heat resistance. The spores of various *Bacillus species* contain 27–55% of core wet weight as water. If the core water content is lower, the spore resistance to wet heat is higher. The spores which are formed at higher temperatures have lower water contents than the spores formed at lower temperatures. The spores formed at lower temperatures have lower wet heat resistance than those formed at higher temperatures.

Spores are also significantly more resistant to dry heat than the corresponding growing cells [18]. The killing of spores by dry heat is due to the DNA damage. The actual reason behind the damage is not clear but the damage is mutagenic [19].

For the elimination of foodborne pathogens there is a wide use of application of high temperatures. It is because of the effectiveness of the heat and the ability of heat to cause damage to the various structures and components of the microbial cell. These inclide RNA, DNA and cytoplasmic membranes. It also leads to the denaturation of the proteins which leads to the destruction of the activity of the enzymes and metabolism in the microorganisms that are controlled by enzymes.

2.3 Effect of pH

Bacillus subtilis can survive over many log units of environmental pH. It can maintain the pH of its cytoplasm which preserves the protein and nucleic acid stability. Environmental pH is important for the pathogenesis of related *Bacillus* species, such as the food-borne pathogen *Bacillus cereus*, which encounters acidic environments in the gastrointestinal tract and in food products where organic acids are used as preservatives. Cytoplasmic pH homeostasis has been studied extensively in *B. subtilis*, which maintains cytoplasmic pH within approximately pH 7.3 to pH 7.6 during vegetative growth over a range of environmental pH, from pH 6.0 to pH 9.0 [20].

2.4 Effect of microwave radiation

A microwave waveguide applicator was developed to generate a uniform and measurable distribution of the microwave electric-field amplitude. The applicator enabled the killing efficacy exerted by microwaves on *B. subtilis* spores to be evaluated in comparison with conventional heating at the same temperature value. Spores of *B. subtilis* ATCC 6633 were used throughout the study as they are reported to be optimal indicators for microwave sterilization assays [21]. Both microwave irradiation and boiling produced spore damage [22]. Spore cores appeared similarly degranulated, thus suggesting that material had been released from spores after both treatments. The grey and uniform areas corresponding to the nucleoids of untreated spores disappeared, while white areas and black spots were observed in spores subjected to both treatments. While spore cores appeared correspondingly modified by microwaves and boiling, the effects on the cortex layer were found to be different. The traditionally heated spores exhibited an extremely relaxed cortex, which was up to 10 times wider than that of the untreated spores. In contrast, the cortex layer of microwave-irradiated spores maintained its original width

although the exposure time and the sample temperature were the same for those spores treated with microwaves or those treated with conventional heat. The relaxation of the cortex was always observed, whatever was the plane of symmetry of the sample. Moreover, as spore processing for electron microscopy was identical.

2.5 UV radiation

UV radiations have a germicidal effect; UV light is a non-ionizing, short wavelength (4-400nm) form of radiation commonly used to sterilize hospital and laboratory work surfaces. It is harmful to humans and has ability to mutate or alter DNA within cells [23]. When DNA absorbs UV light, pyrimidine dimers are formed between adjacent thymine or cytosine molecules in the DNA strand. Unless repaired, mutations in essential genes can lead to cell death [24,25,26]. The germicidal effects of UV light depend on several factors including length of exposure and the type of cell being treated. Bacteria that form endospores, such as *Bacillus subtilis*, are more resistant to UV than vegetative cells, such as *Staphylococcus aureus*. The bactericidal effect of UV radiations was observed on *B. subtilis* and *E.coli* when placed 5 cm away from the UV source.

Chapter 3

MATERIALS AND METHODS

3.1 Revival of the bacterial culture

Non pathogenic strain- *Bacillus subtilis (NCDC-71)*

- Lyophilized culture of Bacillus *subtilis* was revived in Nutrient Broth tubes.
- The test tubes were incubated overnight in the shaker at 37°C.
- Turbidity in the tubes represented bacterial growth.
- The purity of the culture was checked on nutrient agar plate by appearance of single type of colony.

3.2.1 Nutrient agar Composition:

Ingredients gms / Litre

Peptic digest of animal tissue	5.0
Sodium chloride	5.0
Beef extract	1.5
Yeast extract	1.5
Final pH (at 25°C)	7.4±0.2

3.2.2 Malachite green Staining

The endospore stain is a differential stain used to visualize bacterial endospores. Because of their tough protein coats made of keratin, spores are highly resistant to normal staining procedures. The primary stain in the endospore stain procedure, malachite green, is driven into the cells with heat. Since malachite green is water-soluble and does not adhere well to the cell, and since the vegetative cells have been disrupted by heat, the malachite green rinses easily from the vegetative cells, allowing them to readily take up the counter stain.

3.2.3 Procedure:

With the help of a loop the bacteria was smeared on the slide. The slide was heat fixed. Then the smear was stained with malachite green and the slide was kept over water bath for 3-4

minutes .this step was performed so that the stain could get inside the cell wall .the stain was kept for few minutes till all the stain evaporates completely then the slide was allowed to cool down . the slide was washed with water and the excess stain was removed. The strain was treated with safranin and was kept for 2 minutes the slide was washed with water and excess stain was removed by blotting paper. And the slide was observed under microscope.

3.3 Physical stress

3.3.1 Temperature treatment to *Bacillus subtilis*

The bacterial growth from nutrient agar plates was scrapped off and suspended in 10ml of normal saline. Further this suspension was kept in water bath at different temperature 60- 90˚C. An aliquot of samples (1ml) were collected at different time intervals (0, 5, 10, 15 mins). Samples were stored in cold room until further use. The samples were plated on NA plate after serial dilution. The plates were incubated at 37°C for 24 h and cfu was counted to determine the effect of heat and to calculate the D-value.

3.3.2 pH treatment to *Bacillus subtilis*

Nutrient broth was prepared with different pH values (2.0- 9.0). Each flask was inoculated with 100µl of bacterial culture. Samples were collected from each flask at different time intervals (i.e. 0h, 4h, 8h, 12h, 16h, and 24h) for growth determination by optical density at 600nm.

3.4 Evaluation of natural barriers (microbial products/ spices) against *B. subtilis*

3.4.1 *Effects of spices*

- Fenugreek
- Clove
- Coriander
- Black pepper
- Cinnamon
- Fennel

- Cumin

3.4.2 <u>Preparation of extracts</u>

- The spices listed above were grounded and the powdered spices were extracted with methanol.
- It was kept at 40˚C overnight (15hr)
- The extract was filtered and evaporated on hot plate.
- The residue obtained was collected and used for further studies by dissolving in DMSO.

Fig4: Preparation of extracts (a) powdered spices, (b) Heating on hot plate to evaporate methanol (c) Residue of spices

3.4.3 Agar assay

The standard agar well assay was used to determine the antibacterial activity of the extracts. An overnight suspension of *B.subtilis* was spread over the plates. After 20 min, wells were punctured on the agar plates (4wells on each plate) and 50 µl different concentrations of different plant extracts (clove, coriander, black pepper, fenugreek, fennel, cumin, cinnamon) were added. The petriplates were incubated at 37°C for 24 h and observations were made for the anti-bacterial zone.

3.5. Effect of lactic acids culture

3.5.1. Lactic acid cultures used

- *St- Streptococcus thermophilus* (NCDC-218)
- *Lc- Lactobacillus casei* (MTCC-5462)
- *Lh- Lactobacillus helveticus* (MTCC-5463)
- *Lp- Lactobacillus plantarum* (MTCC-5422)
- *R- Lactobacillus rhamnosus* (MTCC-5462)
- *NIR- Lactobacillus rhamnosus GG*
- C1,C2,C3,C4- obtained from curd.
- D3,D4- from Dosa mix.
- *D-Lactobacillus delbrueckii* (MTCC-991)

3.5.2 Extracts from LAB

An overnight grown culture of all the LAB in MRS broth was centrifuged at 10000 rpm for 15 min. The supernatant was transferred in sterilized vials and used for antibacterial activities using agar well assay as described above. The petriplates were incubated at 37°C for 24 h and observations were made for the anti-bacterial zone.

Chapter 4:

RESULTS AND DISCUSSION

4.1 Growth curve of *Bacillus subtilis*

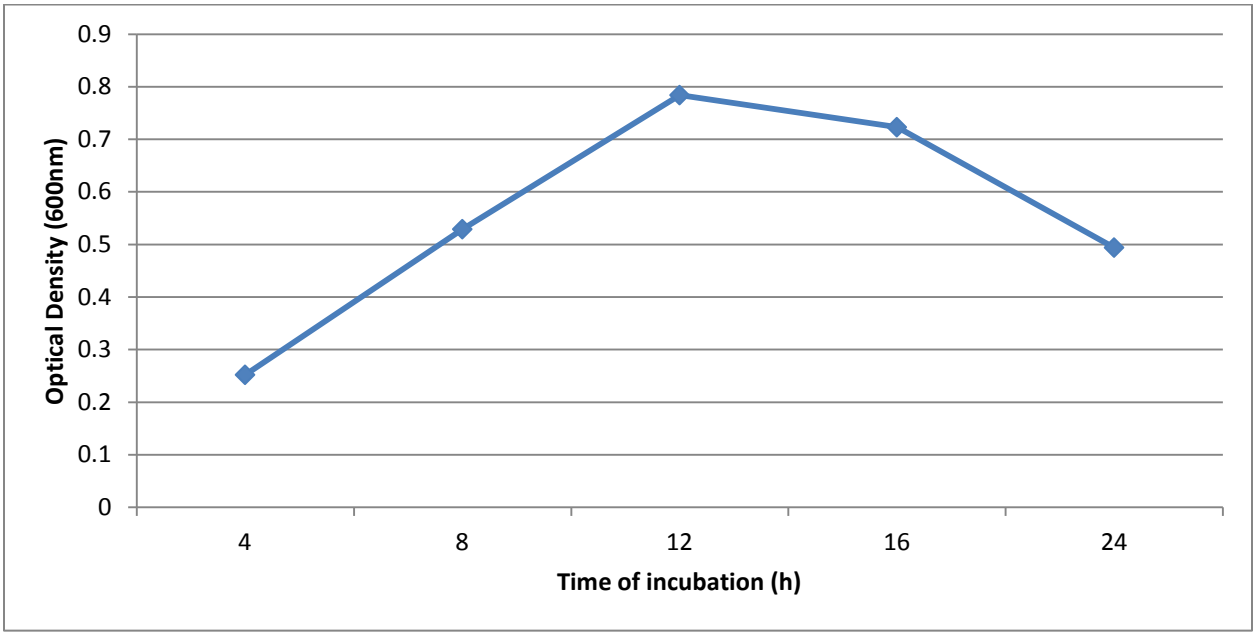


Fig.1: Standard growth curve of *Bacillus subtilis*

4.1.1Inactivation kinetics of *B.subtilis* spores at 80-100˚C

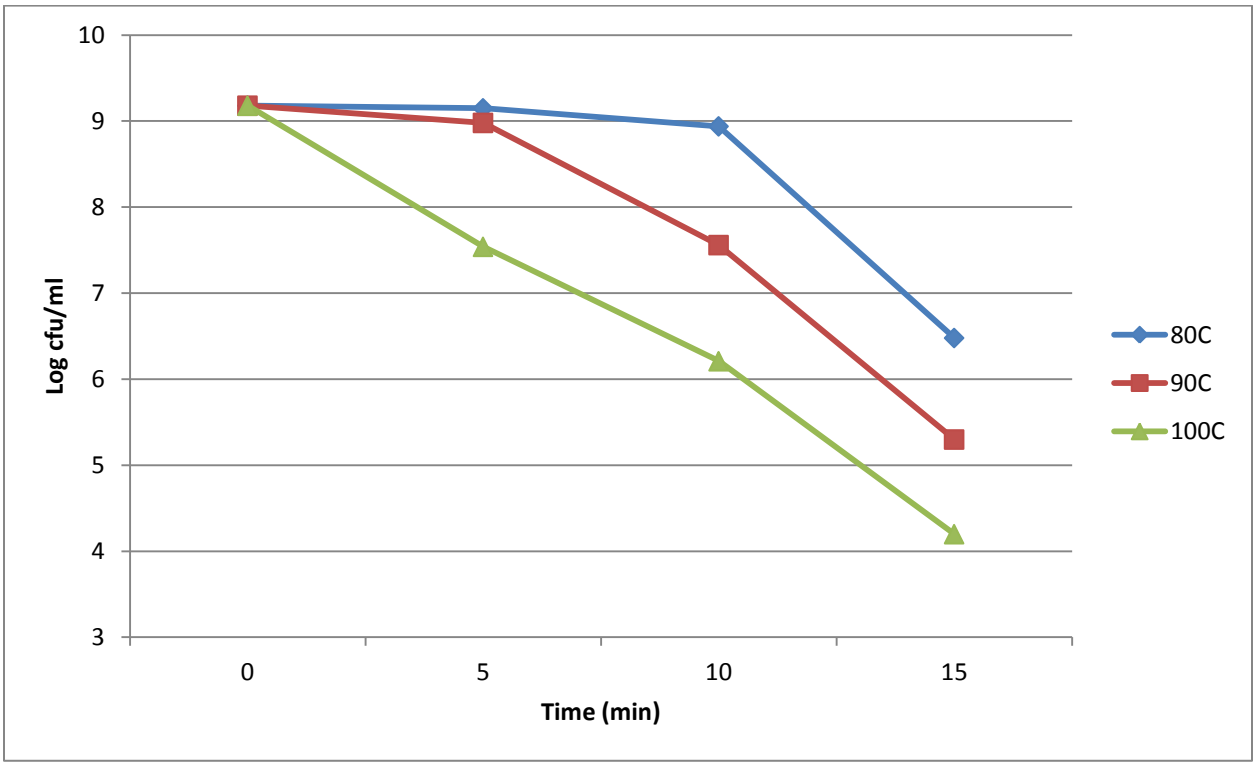


Fig.2: Grow curve obtained after heat stress at different temperatures

- D-value (microbiology) - the *decimal reduction time*, the time required at a certain temperature to kill 90% of the organisms being studied

4.1.2. Calculation

D-value at 100˚C:2 min

D-value at 90˚C: 3 min

D-value at 80˚C: 4 min

4.2.2 Effect of pH

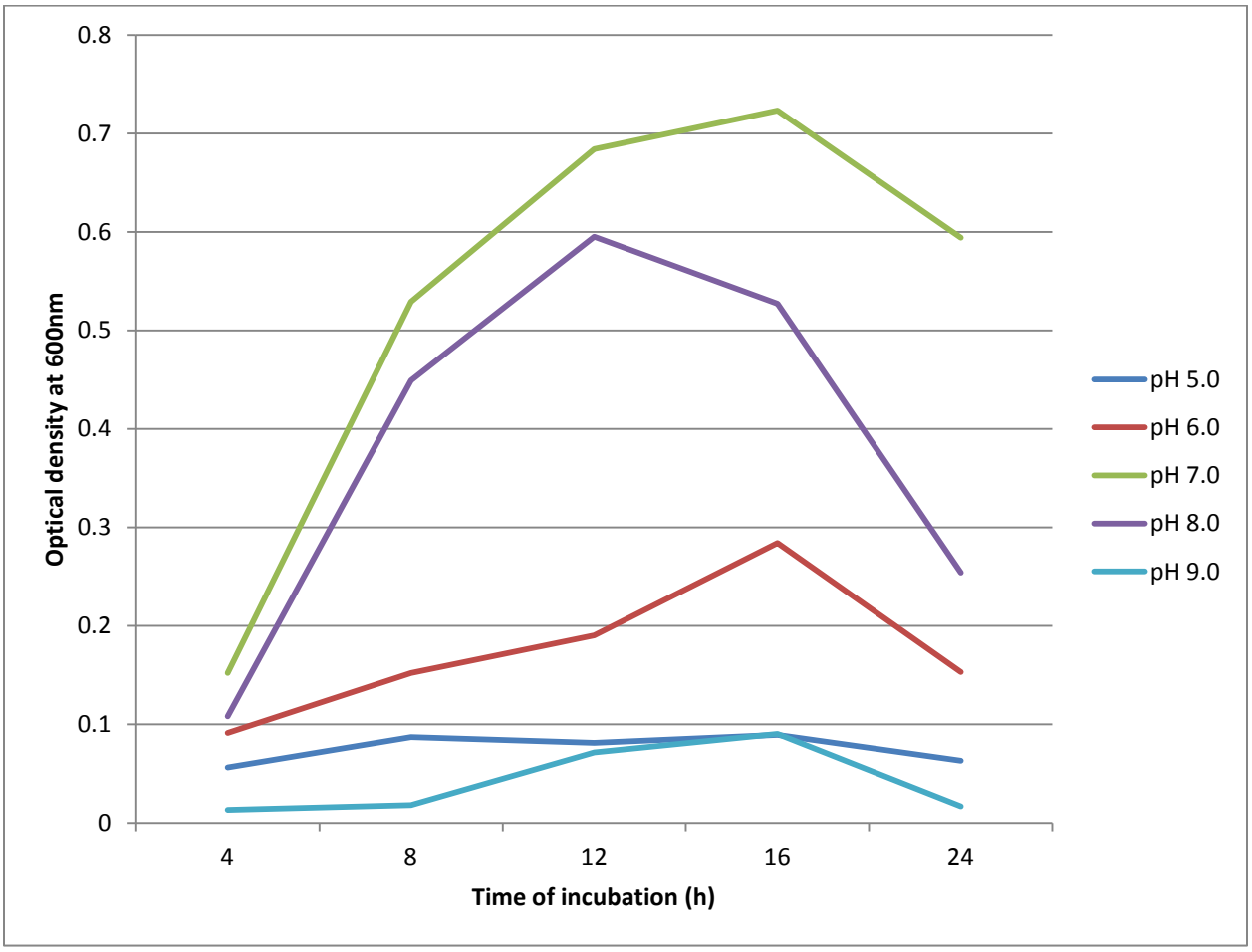


Fig.3 Growth curve of *Bacillus subtilis* growth at various pH

4.3.3 Effect of spices

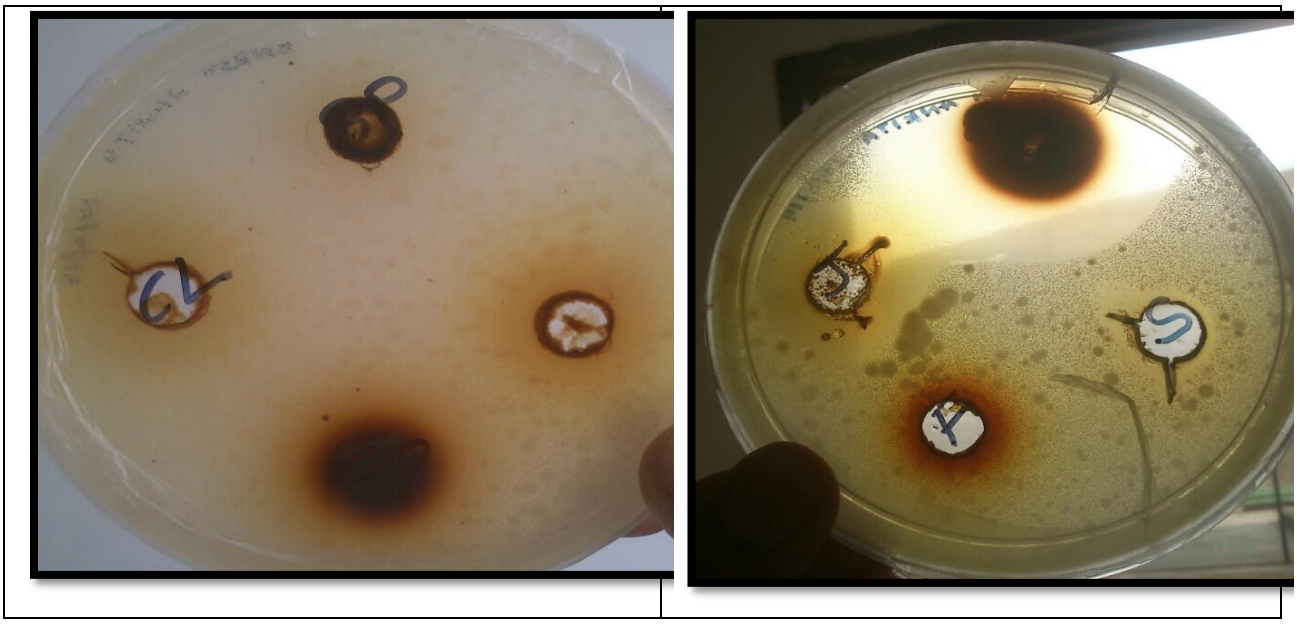


Fig.5 Effect of extracts on *B.subtilis*

Inhibition ring was observed in the following:

- Clove
- Fennel
- Cumin

4.4 Effect of Lactic Acid Cultures

The antibacterial activity was observed by following cultures:

LH- Lactobacillus helveticus, ST- Streptococcus thermophilus, D-*Lactobacillus delbrueckii* , R – *Lactobacillus rhamnosus* D3 and D4 – from Dosa mix, LP – *Lactobacillus plantarum,* C2, C4 – obtained from curd

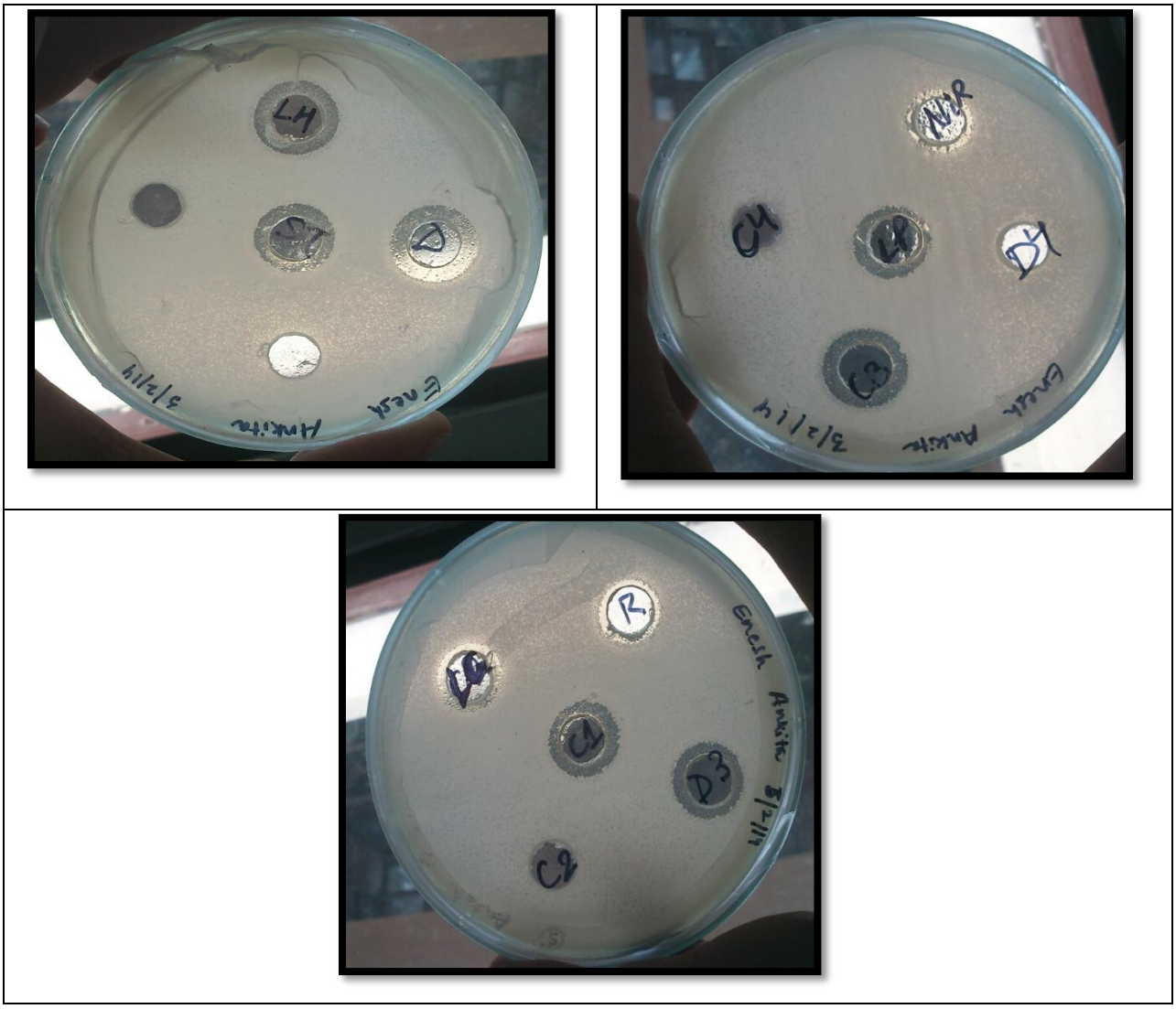


Fig.6 Effect of LAB on *B.subtilis*

CONCLUSION

Food spoilage has been a major problem in the food industry. Various measures have been taken to preserve the food and avoid its contamination by microorganisms. *Bacillus subtilis* has been shown to cause food spoilage, especially in the contamination of bread. It leads to the ropiness of bread. By studying the effect of various stresses against this microbe, we can make an effort to prevent its contamination. The determination of the temperature at which the growth of the *Bacillus subtilis* is inhibited can suggest us the temperature for the processing of the particular food matrix. Similarly, when there is knowledge about the acidic and the alkaline conditions in which the growth of the bacteria is affected, we can incorporate such conditions in the food matrix. There are a number of spices that are inhibitory for the growth of the Bacillus subtilis. Once the inhibitory effect is known then we can incorporate the extracts of these spices into the food matrix in order to prevent the food from spoilage against *Bacillus subtilis*. Some bacteria have the capacity to inhibit the growth of another species of bacteria. *Lactobacillus* is a species of foodgrade bacterium, some species of which have inhibitory effect against *Bacillus subtilis*. Since *Lactobacillus* is foodgrade bacterial species , these can be incorporated in the food matrix so as to prevant the food from spoilage by *Bacillus subtilis*. Thus we can see a number of parameters, both physical and biological, that are inhibitory for the growth of *Bacillus subtilis*. By combining one or more parameters, there can be the establishment of a process which is inhibitory for *Bacillus subtilis*. Such an intelligent is termed as hurdle technology. Hurdle technology has found many applications in the food industry. This technology is used against various microbes to inactivate them in the food matrix or prevent their growth. These inhibitory effects of various parameters against *Bacillus subtilis* can help in the development of a technique which can lead to the prevention of food spoilage by this microorganism.

References

1. Douglas Higgins and Jonathan Dworkin. Recent progress in Bacillus subtilis sporulation, FEMS Microbiol Rev.,2012; 131-148
2. Nicholson WL, Munakata N, Horneck G, Melosh HJ, Setlow P. Resistance of Bacillus endospores to extreme terrestrial and extraterrestrial environments. Microbiol Mol Biol Rev. 2000;64:548–572.
3. Veening JW, Stewart EJ, Berngruber TW, Taddei F, Kuipers OP, Hamoen LW. Bet-hedging and epigenetic inheritance in bacterial cell development. Proc Natl Acad Sci U S A. 2008;105:4393–4398
4. Thompson JM, Waites WM, and Dodd CER (1998). Detection of rope spoilage in bread caused by Bacillus species. J Appl Microbiol 85: 481-486.
5. Sorokulova IB, Reva ON, Simirnov VV, Pinchuk IV, Lapa SV, and Urdaci MC (2003). Genetic diversity and involvement in bread spoilage of Bacillus strains isolated from flour and ropy bread. Lett Appl Microbiol 37: 169-173.
6. Mentes Ö, Ercan R, and Akçelik M (2007). Inhibitor activities of two Lactobacillus strains, isolated from sourdough, against rope-forming Bacillus strains. Food Control 18: 359-363.
7. Rachna Pandey, Alex Ter Beek, [...], and Erik M. M. Manders.(2013) Live Cell Imaging of Germination and Outgrowth of Individual Bacillus subtilis Spores; the Effect of Heat Stress Quantitatively Analyzed with SporeTracker. PloS One 8(3): e58972
8. Artun Yibar, Figen Cetinkaya and Gul Ece Soyutemiz (2012). Detection of Rope-producing Bacillus in bread and identification of isolates to species level by Vitek 2 system. J.Biol. Environ. sci.,: 243-248.
9. Khusro A., Preetamraj JP and Panicker SG. (2013). A Comparative Assessment Of The Antibacterial Effects of *Allium sativum* And Antibiotics On New Strains Of Bacillus Species Isolated From Poultry Farm. International Journal of Biological & Pharmaceutical Research. 4(12): pp: 840-846.

10. EUROPEAN COMMISSION HEALTH & CONSUMER PROTECTION DIRECTORATE-GENERAL. Directorate E – Safety of the food chain Unit E.3 - Chemicals, contaminants, pesticides (2006)
11. Leistner L. (1995) in New Methods of Food Preserrvation (Gould, G.W., ed.), pp.1-21, Blackie.
12. LEISTNER, L. 1995. Principles and applications of hurdle technology. In: Gould,G.W. (ed.) New Methods for Food Preservation. London: Blackie Academic & Professional, pp. 1–21.
13. BOOTH, I.R. 1995. Regulation of cytoplasmic pH in bacteria. Microbial Rev., 49:359–78.
14. Ohlsson, T., and N. Bengtsson. 2002. The hurdle concept (Chapter 7).Minimal Processing Technologies in the Food Industry, CRC PressLLC, New York. pp. 175-195.
15. Sun-Young Lee. (2004). Microbial Safety of Pickled Fruits and Vegetables and Hurdle Technology. Internet Journal of Food Safety, Vol. 4, 2004, p. 21-32
16. Leistner. L., 2000. Basic aspects of food preservation by hurdle technology. International Journal of Food Microbiology (2000). 181-186.
17. K. Jeevaratnam, M Jamuna and A S Bawa, Indian Journal of Biotechnology, Biological preservation of foods- Bacteriocins of lactic acid bacteria, 2005, 446-454
18. P. Setlow (2006) Spores of Bacillus subtilis: their resistance to and killing by radiation, heat and chemicals. Journal of Applied Microbiology. Vol 101. pp.514-525.
19. Ray B. Methods to Detect Stressed Microorganisms. J. Food Prot. 1979;42:346–355.
20. F. Celandroni (10 SEP 2004) Effect of microwave radiation on Bacillus subtilis spores. Journal of Applied Microbiology. Vol 97 Issue 6.
21. E. Ghelardi (2006) Spores of Bacillus subtilis: their resistance to and killing by radiation, heat and chemicals. Journal of Applied Microbiology. Vol 101. Issue 5
22. Leuschner, G.K. and Lillford, P.J. (2001) Investigation of bacterial spore structure by high resolution solid-state nuclear magnetic resonance spectroscopy and transmission electron microscopy. International Journal of Food Microbiology 63, 35–50
23. Errington J. Bacillus subtilis sporulation: regulation of gene expression and control of morphogenesis.Microbiol Rev. 1993;57:1–33.
24. Daniel R A, Errington J. Cloning, DNA sequence, functional analysis and transcriptional regulation of the genes encoding dipcolinic acid synthetase required for sporulation in Bacillus subtilis. J Mol Biol.1993;232:468–483.

25. Aronson A I, Fitz-James P C. Structure and morphogenesis of the bacterial spore coat. Bacteriol Rev.1976;40:360–402.
26. Cutting S, Panzer S, Losick R. Regulatory studies on the promoter for a gene governing synthesis and assembly of the spore coat in Bacillus subtilis. J Mol Biol. 1989;207:393–404.