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DEGRADATION OF ENDOSPORES FROM PASTEURIZED MILK WITH THE HELP OF NATURAL COMPOUNDS

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ABSTRACT

The objective of present study was to degrade the bacterial endospores by using different potential compounds (as like primary and secondary metabolites). For this two milk samples were collected and total eight endospore forming bacterial cultures were isolated. On the basis of identification seven out of eight were bacillus (endospore forming) and one culture was cocci (non-endospore forming). After identification, cultures were tested whether they are aerobic or anaerobic by glucose fermentation test. Further in-vitro analysis of cultures was done against different potential compounds by performing eighteen different tests. In secondary metabolites test against culture no.1&3, in primary metabolites use pectinase enzyme test against culture no.5, protease & pectinase enzymes mixture test against culture no.3 & 6, in secondary metabolites methanol extract of Tamala leaves test against culture no.2 & 5, in water extract of Tamala leaves test against culture no.4 & 7, in ethyl acetate extract of Tamala leaves test against all seven cultures, in ethanol and ethyl acetate extract of *Cajanus* leaves all seven cultures and in acetone extract of *Cajanus* leaves test against culture no. 2, 3, 4, 5, 6 & 7 were showing positive results. And rests of the cultures in different tests were showing negative results.

Key words: Endospore, *Cajanus,* Tamala, *Clostridium,* Dipicolinic acid.

INTRODUCTION

Ferdinand cohn first proposed thermo-resistant endospores after observing the growth of Bacillus subtilis on cheese after the cheese was boiled. Endospores are commonly found in soil and water, where they can live even centuries for long periods of time [1]. Gram-positive bacteria, especially of the genera Bacillus and Clostridium, have a mechanism of sporulation by that they can protect their DNA and become dormant under harsh conditions that would otherwise destroy them [2].This survival mechanism allows for the bacteria to survive in a metabolically inactive state for long periods of time until conditions, such as temperature or nutrient content, allow for germination and outgrowth. Some spores have been recovered and cultured after storage in amber for millions of years **[3]**. The structure of the spore is well-understood, separated into distinct regions **[4]**; an outer spore coat which is laminar and serves as a protective barrier, the cortex, composed of cross-linked peptidoglycan distinct from that found in the germinated bacterial cell wall and implicated in the heat resistance of spores; and the core, containing the bacterial DNA as well as protective molecules such as small acid soluble proteins (SASPs) and an aquo- coordination complex between Ca^{2+} and dipicolinic acid (pyridine-2,6-dicarboxylic acid) **[5,6]**. The spore center has a smaller moisture content relative to vegetative cells that is believed to be a key factor in the sensitivity to spore fire. Dipicolinic acid forms a complex inside the endospore nucleus, with calcium ions [7]. Within the endospore, calcium-dipicolinate abundant, can stabilize and secure the DNA of endospores. Heat resistant but lacking dipicolinic acid mutants have also been isolated. Small acid soluble proteins (SASPs) saturate the DNA of the endospore and protect it against heat, drying, chemical and radiation. They also serve as a source of carbon and energy during germination to form a vegetative bacterium [8]. The cortex may osmotically remove water from the endospore interior and the dehydration resulting in the endospores' heat and radiation resistance is considered to be very important. Endospores have 5 times more sulphur than vegetative cells [9]. The excess sulfur is stored as an amino acid, cystine, in spore coats.

The macromolecule responsible for maintaining the dormant state is believed to have a cystine-rich protein coat, which is stabilized by S-S bonds.

A decrease in such links has the potential to transform the binding site which causes the protein to grow. This protein conformation shift is believed to be responsible for the exposure of the effective enzymatic sites needed for endospore germination [10]. Ezio Ricca and Simon M Cutting (2003) suggested that the bacterial spore protective coat would offer two nano-biotechnology-relevant areas of interest for long-term exploitation. First, as a substratum for the transmission of biomolecules and second as a basis for understanding molecules which are self-assembled. This analysis provides an account of recent progress explaining the use of spore and, in particular, the spore coat as a medium for heterologous antigen presentation and safe immunization (vaccination). When interest in the spore coat grows, it seems likely they will be further exploited for the delivery of drugs and enzymes as well as a source of novel self-assembling proteins. Many spore-coat proteins are likely to be used for surface expression and distribution and spore-forming organisms other than B.sutilis may also be developed [11].Peter Setlow and Lei Li (2015) reported that the combination of low water content, the presence of CaDPA and DNA binding by SASPs in spores ensures the SP photo-chemistry is the dominant photochemical event in dormant spores. However, SP photobiology beyond dormant spores, such as its repair and mutagenic impacts in germinating spores is still an area where questions remain. It is now known that in dry or desiccated microorganisms exposed to UVC or UVB photons, SP is formed as a major DNA photo-lesion. Due to the presence of SP, cells appear to be more sensitive to uv irradiation. Since spore forming bacteria are responsible for a number of serious human diseases, including botulism (*Clostridium botulinum)* and anthrax (*Bacillus anthracis)* understanding SP photobiology may allow one to selectively target this photochemical process in spore forming bacteria to design better strategies to combat these deadly diseases, improving human as well as national security **[12]**. Anthony Friedline (2015) indicated that Bacillus pumilus SAFR-032 spores collected from a clean room setting are reported to show increased resistance to peroxide, dessiccation, uv radiation and chemical disinfection relative to other bacteria that form spores.

B PumilusSAFR 032 spores survival allow the production of more stringent disinfection agents than normal cleanroom sterilization practices. **[13]**.

MATERIALS AND METHODS

Sample collection:

Samples were collected from the milk stores of the Gomtinagar, Lucknow **[14]**.

Isolation of endospore forming bacteria:

The bacterial cultures were obtained from the milk samples after serial dilution. Further these cultures were streaked in sterilized nutrient agar plates to make pure cultures. The endospore forming cultures were separated from the other bacterial cultures by performing endospore staining **[15,16]**.

Biochemical tests for strain identification: Biochemical tests such as gram's staining, glucose fermentation test etc. tests were performed by following the bergey's manual for the identification **[17]**.

In vitro analysis of natural compounds against the endospores:

In this analysis different antibiotics, enzymes and phytochemicals were added to the endospore forming bacterial isolates to facilitate the endospore degradation **[19,20]**.

Antibiotics- 100µl of antibiotic was added to the 2-2ml isolated cultures and results were observed after 2 hours and 24 hours by doing endospore staining.

Kadamba plant fruit extract- 500µl of kadamba plant extract was added to the 500µl of isolated cultures and results were observed after 2 hours and 24 hours by doing endospore staining.

Chemical treatment- 500µl of chemical composition was added to the 500µl of isolated cultures and results were observed after 2 hours and 24 hours by doing endospore staining.

Protease enzyme- 500µl of protease enzyme was added to the 500µl of isolated cultures and results were observed after 2 hours and 24 hours by doing endospore staining.

Antibiotic and protease enzyme mixture- Antibiotic and protease was added to the 500µl of isolated cultures in 1:1 ratio and results were observed after 2 hours and 24 hoursby doing endospore staining.

Pectinase enzyme- 500µl of pectinase enzyme was added to the 500µl of isolated cultures and results were observed after 2 hours and 24 hours by doing endospore staining.

Protease and pectinase enzyme mixture- 500µl of protease and pectinase enzymes mixture of 1:1 ratio was added to the 500µl of isolated cultures and results were observed after 2 hours and 24 hours by doing endospore staining.

Methanol extract of tamala- 500µl of methanol extract was added to the 500µl of isolated cultures and results were observed after 2 hours and 24 hours by doing endospore staining.

Acetone extract of tamala- 500µl of acetone extract was added to the 500µl of isolated cultures and results were observed after 2 hours and 24 hours by doing endospore staining.

Ethyl acetate extract of tamala- 500µl of ethyl acetate extract was added to the 500µl of isolated cultures and results were observed after 2 hours and 24 hours by doing endospore staining.

Water extract of tamala- 500µl of water extract of tamala was added to the 500µl of isolated cultures and results were observed after 2 hours and 24 hours by doing endospore staining.

Ethanol extract of cajanus leaves- 500µl of ethanol extract of cajanus leaves was added to the 500µl of isolated cultures and results were observed after 24 hours by doing endospore staining.

Ethyl acetate extract of cajanus leaves- 500µl of ethyl acetate extract of cajanus leaves was added to the 500µl of isolated cultures and results were observed after 24 hours by doing endospore staining.

Benzene extract of cajanus leaves- 500µl of benzene extract of cajanus leaves was added to the 500µl of isolated cultures and results were observed after 24 hours by doing endospore staining.

Acetone extract of cajanus leaves- 500µl of acetone extract of cajanus leaves was added to the 500µl of isolated cultures and results were observed after 24 hours by doing endospore staining.

RESULTS

Sample collection:

The milk packets were purchased from the shops in Gomtinagar, Lucknow.

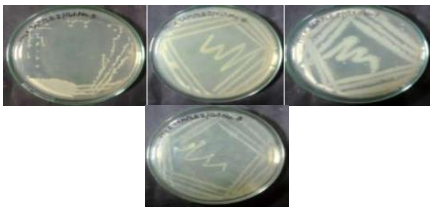
Table 1: The milk products with the brand name.

S no	Milk Brand	Product
1	Amul	
2	Namaste India	

Isolation and screening of endospore forming bacteria:

The bacterial cultures isolated from the sample and then the colonies were selected on the basis of morphological parameters described in table 2. The selected bacterial colonies were streaked in the separate nutrient agar plated to form pure culture plates. Further screening and analysis of the cultures were carried out from this pure culture plates.

a: Bacterial colonies observation after serial dilution and spreading



b: Pure culture plates after streaking.

Figure 1: isolation of pure bacterial cultures after spreading and streaking.

Table 2: Colony morphology of cultures

Colony morphology	C 1	C 2	C 3	C 4
Shape	Circular	Circular	Irregular	Irregular
Margin	Entire	Discrete	Discrete	Discrete
Elevation	Flat	Flat	Flat	Raised
Pigmentation	Off-white	Off-white	Off-white	Off-white
Opacity	Translucent	Opaque	Opaque	Opaque

Colony morphology	C 5	C 6	C 7
Shape	Irregular	Circular	Circular
Margin	Entire	Entire	Discrete
Elevation	Flat	Raised	Flat
Pigmentation	Off-white	Bluish	Off-white
Opacity	Opaque	Translucent	Opaque

Biochemical analysis of cultures:

Table 3: Biochemical tests of the cultures

Gram Staining	Endospore staining	Glucose fermentation
Gram negative *Bacillus*	Positive	Negative
Gram positive *Bacillus*	Positive	Positive
Gram positive *Bacillus*	Positive	Positive
Gram positive *Bacillus*	Positive	Positive
Gram negative	Positive	Positive
Bacillus		
Gram negative *Bacillus*	Positive	Positive
Gram positive *Bacillus*	Positive	Positive

Table 4: Observation of antibiotic testing for 1-7 cultures

After incubation of 2hrs	After incubation of 24hrs
Many endospores & some dead cells seen	90% dead cells and some endospores observed
Many endospores & some dead cells seen	Vegetative cells seen
Many endospores & some dead cells seen	All were dead cells
Many endospores seen	All were endospores positive
All were endospores positive	All were endospores positive
Endospores seen	Vegetative and endospores both seen
Endospores seen	All were endospores positive

Table 5: showing kdamba plant (fruit) extract for 1-7 cultures.

After incubation of 2hrs	After incubation of 24hrs
Endospores seen	Endospores seen
Endospores seen	Endospores seen
Endospores seen	Endospores seen
Endospores seen	Endospores seen
Endospores seen	Endospores seen
Endospores seen	Endospores seen
Endospores seen	Endospores seen

Note: effective result were not obtained.

Table 6: showing protease enzyme for 1-7 cultures.

After incubation of 2hrs	After incubation of 24hrs
Endospores seen	Vegetative cells, no endospores
Endospores seen	Vegetative cells, no endospores
Endospores seen	Vegetative cells, no endospores
Endospores seen	Endospores seen
Endospores seen	Endospores seen
Endospores seen	Endospores seen
Endospores seen	Endospores seen

Table 7: showing chemical treatment for 1-7 cultures.

After incubation of 2hrs	After incubation of 24hrs
All cells were dead, few endospores were seen	Vegetative cells, no endospores seen
Vegetative cells & new endospore formation Seen	Vegetative cells and endospores seen
Most cells died, no endospore formation seen	Mostly endospores seen
Almost cells were dead, some endospores seen	Mostly endospores seen
Almost cells were dead, some endospores seen	Dead cells and more endospores seen
All were endospores	All were endospores positive
Vegetative cells and endospores both seen	Vegetative cells & endospores seen

Table 8: showing antibiotic and protease enzyme mixture for 1-7 cultures.

After incubation of 2hrs	After incubation of 24hrs
Few endospores seen	Many endospores seen
All were dead cells, very few endospores seen	Many endospores seen
Many endospores seen	Few endospores seen
Vegetative cells seen, many endospores seen	Few vegetative cells, no endospores seen
Endospores seen	Many vegetative cells, no endospores seen
Endospores seen	Many endospores seen
Endospores seen	Many vegetative cells, many endospores seen

Table 9: showing pectinase enzyme for 1-7 cultures.

After incubation of 2hrs	After incubation of 24hrs
No vegetative cells, endospores seen	Vegetative cells, very less no of endospores
Many vegetative cells and many endospores seen	Many vegetative cells seen, no endospores
Many vegetative cells, endospores seen	All were vegetative cells.
No vegetative cells, many endospores seen	Many vegetative cells, few endospores seen
No vegetative cells, less endospores seen	No vegetative cells, no endospores
Both vegetative cells & endospores seen	Very less no. vegetative cells, no endospore
No vegetative cells, endospores seen	Vegetative cells, no endospore

Table 10: showing results of protease and pectinase enzyme mixture for 1-7 cultures.

After incubation of 2hrs	After incubation of 24hrs
Few endospores seen	Vegetative cells, very few endospores seen
Few vegetative cells, few endospores seen	Vegetative cells, very few endospores seen
Few vegetative cells, no endospores seen	Less vegetative cells, less endospores seen
Endospores seen	Few vegetative cells, more endospores seen
Endospores seen	Few vegetative cells seen
Endospores seen	No vegetative cells , no endospores
Both vegetative cells and endospores seen	Few vegetative cells only

Table 11: showing methanol extract of Tamala for 1-7 cultures.

After incubation of 2hrs	After incubation of 24hrs
No vegetative cells, no endospores	Few vegetative cells, endospores seen
No vegetative cells, no endospores	No vegetative cells , one endospore seen
Endospore seen	No vegetative cells, few endospores seen
Vegetative cells, few endospores	No vegetative cells, few endospores seen
No vegetative cells, no endospores	No vegetative cells and no endospores seen
Few endospores only	No vegetative cells, endospores seen
No vegetative cells, no endospores	No vegetative cells, very few endospores seen

Table 12: showing acetone extract of Tamala for 1-7 cultures.

After incubation of 2hrs	After incubation of 24hrs
Endospores were present	Endospores only
All were endospores positive	Endospores only
All were endospores positive	Endospores only

Many vegetative cells, many endospores seen	Endospores only
Only endospores seen	Many endospores seen
Both vegetative cells and endospores seen	Endospore seen
Few vegetative cells, many endospores seen	Endospore seen

Table 13: showing water extract of Tamala for 1-7 cultures.

After incubation of 2hrs	After incubation of 24hrs
Endospores seen	Dead vegetative cells, few endospores seen
Endospores seen	Both vegetative cells and endospores seen
Endospores seen	No vegetative cells, many endospores seen
Endospores seen	No vegetative cells, few endospores seen
Endospores seen	No vegetative cells, few endospores seen
Endospores seen	All were endospores
Endospores seen	Few endospores.

Table 14: showing ethyl acetate extract of *Tamala* results.

Culture no.	After incubation of 24hrs
1.	Very few endospores, dead cells seen
2.	Very few endospores, dead cells seen
3.	Very few endospores, dead cells seen
4.	Very few endospores, dead cells seen
5.	Very few endospores, dead cells seen
6.	Very few endospores, dead cells seen
7.	Very few endospores, dead cells seen

Table 15: showing ethyl acetate extract of *Cajanus* leaves results.

Culture no.	After incubation of 24hrs
1.	Few endospores, dead cells seen
2.	Few endospores, dead cells seen
3.	Few endospores, dead cells seen
4.	Few endospores, dead cells seen
5.	Few endospores, dead cells seen
6.	Few endospores, dead cells seen
7.	Few endospores, dead cells seen

Table 16: showing benzene extract of Cajanus leaves results.

Culture no.	After incubation of 24hrs
1.	Vegetative cells, very few endospores
2.	No vegetative cells, endospores seen
3.	No vegetative cells, endospores seen
4.	Few vegetative cells, endospores seen
5.	Few endospores seen
6.	Few endospores seen
7.	Many vegetative cells, few endospores seen

Table 17: showing acetone extract of Cajanus leaves results.

Culture no.	After incubation of 24hrs
1.	Many endospores seen
2.	Few endospores seen
3.	Few endospores seen
4.	Few endospores seen
5.	Few endospores seen
6.	Few endospores seen
7.	Few endospores seen

DISCUSSION

The objective of the study of "endospore degradation by using natural compounds" was to degrade bacterial endospores. In this two company‟s sample were collected from the stores. Sample1 was of Amul and sample2 was of Namaste India. In pasteurized milk of Namaste India we got four different bacterial colonies and also four bacterial colonies from the pasteurized milk of Amul.

but according to different literatures in milk there is mainly presence of two bacteria or bacterial spore after the pasteurization that are *Bacillus sp.* and *lacto bacillus sp*. Isolation of bacterial cultures was done by using serial dilution method. Bacterial culture of Namaste India was named as culture no.1, 2, 3 & 4 and bacterial cultures of Amul was named as culture no. 5, 6,7 & 8. Through Gram‟s staining, identification of bacterial cultures was done, in which culture no.1, 2, 3, 4 & 7 were Gram-positive *Bacillus* and culture no.5 & 6 were Gram-negative *Bacillus* and culture no.8 was Gram-negative cocci. Further endospore staining was done to differentiate between endospore forming and endospore not forming bacterial cultures, and we got culture no.8 as non-endospore forming and rest of the cultures were endospore forming. In general, only *Bacillus* bacteria form endospores. For in-vitro analysis of bacterial cultures against different potential bioproducts we involved only endospore forming cultures, as our goal was to degrade endospores.

In antibiotic test culture no.1&3, in pectinase enzyme test culture no.5, in protease & pectinase enzymes mixture test culture no.3 & 6, in methanol extract of Tamala leaves test culture no.2 & 5, in water extract of Tamala leaves test culture no.4 & 7, in ethyl acetate extract of Tamala leaves test all seven cultures, in ethanol and ethyl acetate extract of *Cajanus* leaves all seven cultures and in acetone extract of *Cajanus* leaves test culture no. 2, 3, 4, 5, 6 & 7 were showing positive results. And rest of the cultures in different tests were showing negative results.

CONCLUSION AND FUTURE PROSPECT

This little piece of investigation is only a preliminary step to degrade the endospores. The findings indicate that different natural compounds used against endospores have potential to degrade the endospores which has the application for dairy and food industry where endospores persist for long and creates risk for health by developing food borne diseases. In industries such endospore degrading compound would facilitate great help in sustaining long shelf-life of their products.

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