Effect of temperature and storage on free and encapsulated *Lactobacillus acidophilus* NCIM 2660 and *Lactobacillus bulgaricus* NCIM 2056 in different food matrix

For partial fulfilment for the award of the Degree of

Master of Science

IN

LIFE SCIENCE

Under the esteemed guidance of

Dr. R. Jayabalan

Submitted by

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CERTIFICATE

This is to certify that the thesis entitled "**EFFECT OF TEMPERATURE AND STORAGE ON FREE AND ENCAPSULATED *Lactobacillus acidophilus* NCIM 2660 AND *Lactobacillus bulgaricus* NCIM 2056 IN DIFFERENT FOOD MATRIX"** which is being submitted by **Ms. Savitri Kumari,** Roll No. **413ls2034**, for the degree of master of science in Life Science from National Institute of Technology, Rourkela, is a record of bonafide research work, carried out by her under my supervision. The results embodied in this thesis are new and have not been submitted to any other university or institute for the award of any degree or diploma.

R. Jayabalan.

ACKNOWLEDGMENT

It is by the love and blessings of God almighty and my parents that I am to complete my project successfully and present this piece of work for which I am eternally indebted.

It would not have been possible to finish my dissertation without the support of my friends, and staff of the department. I would like to extend my sincere thanks to all of them.

I am highly indebted to and wish to convey my deep sense of gratitude to **Dr. S.K Bhutia**, Assistant Professor & Head, Department of Life Sciences, National Institute of Technology, Rourkela for providing me with necessary support to complete this project successfully.

I am highly indebted to **Dr. Rasu Jayabalan** for his guidance and constant supervision as well as for providing necessary information regarding the project & also for their support in completing the project.

I would like to express my gratitude towards my parents & teaching and non-teaching staff of "Department of Life Science" for their kind co-operation and encouragement which help me in completion of this project.

My thanks and appreciations also go to my friends in developing the project and people who have willingly helped me out with their abilities.I am highly grateful towards the PhD scholars, for their immense help and support throughout the project work. I am highly grateful towards my labmates, Rina Yadav, Kumar Sagar Jaishwaal, and Shilpa Swagatika who constantly helped me during the work.

Date: **Savitri Kumari**

Place:

DECLARATION

I do hereby declare that the Project report entitled **"Effect of temperature and storage on free and encapsulated *Lactobacillus acidophilus* NCIM 2660 and *Lactobacillus bulgaricus* NCIM 2056 in different food matrix"** submitted to the Department of Life Science, National Institute of Technology, Rourkela for the partial fulfillment of the Master Degree in Life Science is a faithful record of bonafide and original research work carried out by me under the guidance and supervision of **Dr. Rasu Jayabalan**, Assistant Professor, Department of life Science, NIT, Rourkela.

Date: 11th May 2015 **Savitri Kumari**

Place: Rourkela **413LS2034**

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LIST OF ABBREVIATIONS

g	**Gram**
mg	**Milligram**
L	**Litre**
ml	**Millilitre**
º	**Degree**
C	**Centigrade**
h	**Hour**
Min	**Minute**
%	**Percentage**
M	**Molar**
mM	**Millimolar**
rpm	**Rotations per minute**
CFU	**Colony forming units**

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ABSTRACT

In today's world, the research into the health benefits of probiotics has rocketed sky high. The survivability of probiotics in foods depends on various factors during processing and storage. Heat is used in the process of a lot of foods. The fact was given that the amount of probiotics on the consumption of foods should be at least 10^7 CFU/g and that probiotic bacteria are sensitive to heat, so the survival of probiotics during thermal processing are the main challenges to food manufacturers. The main objective of the work is to study the effect of alginate encapsulation on temperature tolerance of *Lactobacillus acidophilus* NCIM 2660 and *Lactobacillus bulgaricus* NCIM 2056. Free cells of *L. acidophilus* NCIM 2660 and *L. bulgaricus* NCIM 2056 were exposed to different temperatures (60^0C, 70^0C, 80^0C , and 90^0C) at different intervals of time (1 min, 5 min, 10 min, 15 min, 20 min, 25 min, and 30 min). It was observed that *L. acidophilus* NCIM 2660 and *L. bulgaricus* NCIM 2056can tolerate upto 100^0C for 1 minute as free cells. In order to increase the temperature tolerance capacity, encapsulation was tried. Encapsulated *L. acidophilus* NCIM 2660 and *L. bulgaricus* NCIM 2056were exposed to different temperatures i.e. >100^0C for different time intervals. Results revealed that encapsulation could improve the temperature tolerance of *L. bulgaricus* NCIM 2056up to 120^0C for 1 minute. The present study revealed the advantage of encapsulation in protecting the bacterial cells from high temperature. These encapsulated cells can be utilized to formulate functional foods which will be heat processed before consumption. *L. acidophilus* NCIM 2660 and *L. bulgaricus* NCIM 2056was incorporated in orange peel jelly as free cells and *L. plantarum* NCIM 2083 and *L. bulgaricus* NCIM 2056 was incorporated in dried snacks and their survivability in food matrix was studied for 3 weeks.

Key words: Bacterial cells; encapsulation; probiotics

1. INTRODUCTION

Novel probiotics intended to control the gut microbiota for getting better health outcomes are in claim as the importance of the gut microbiota in human health is publicized. Probiotics have been reported to play a therapeutic role by lowering cholesterol, improving lactose tolerance, nutritional enhancement and preventing some cancers and antibiotic associated diarrhoea (7). The probiotic health benefits may be due to the production of acid and/or bacteriocins, competition with pathogens preventing their adhesion to the intestine and enhancement of the immune system (3).

Eli Metchnikoff, who won Nobel Prize, defined "Probiotics" as live microbes which when administered in adequate amounts, confer a health benefit. The term "probiotics" is derived from two Greek words, "pro" meaning "for" and "biotics" meaning "life" (12).

How might they work?

Probiotics are used for several different types of digestive trouble but since there are many different kinds of probiotics not all will have the profit that you are looking for as it relates to your health (9). Possible beneficial effects of probiotics consist of:

- Fascinating and/or destroying toxin released by certain "bad" germs that can make you ill.
- Immune system is boosting.
- Preventing injurious microbes from attaching to the gut wall and increasing there.
- Signals are transfer to the cells to for making the mucus in your intestine strong, which helps it act as a barrier beside infection.
- Production of B vitamin which is vital in maintaining healthy skin, a healthy nervous system and preventing anemia.
- Produces some substances that prevent infection.

Probiotics Encapsulation

Probiotics can only exert their function after they reach the intestines. But before reaching the intestines, they have to pass through a variety of harsh environment starting from the moth itself. The effect of probiotics depends on the number of viable cells that reach the intestines. First they have to tolerate enzymes present in the saliva. After reaching the stomach, probiotics are subjected to an acidic pH of 2-3. Next, the transit of probiotics from acidic to alkaline pH also decreases their viability (14).

Hence, to ensure that a good amount of probiotic cells reach the intestine, it is necessary to protect the cells from the harsh environment. This can be achieved by a number of methods. Encapsulation of cells in a hydrocolloid matrix is one such method. Suspension of cells in the matrix ensures its protection from enzymes, acidic and alkaline pH. The use of biopolymers such as alginate, k-carrageenan, xanthan gum, and gellan gum to trap cells is a common technique to protect cells. The presence of a barrier between the cells and the in vivo conditions ensures their survival (3).

2. LITERATURE REVIEW

Probiotic Bacteria

The human digestive tract contains a large population of bacteria, which may be helpful, benign or pathogenic. The microbial population of the gastrointestinal tract is first established immediately by following birth but changes throughout life in reaction to food, drugs and other environmental factors. Balance between these bacteria is essential for maintaining health while an imbalance may lead to disease (5). Intake of probiotic containing food is one way to tip the balance in the direction of improved gut health. The Food and Agriculture Organization/World Health Organization (FAO/WHO) has set forward recommendations for defining, labeling and validating the effectiveness of probiotics in food (10). According to FAO/WHO Probiotics are define as'Live microorganisms which when administered in adequate amounts confer a health benefit to the host'.

During Processing and Consumption destruction of Probiotic organisms

If any food processing or passage through the gastrointestinal tract on the way to the colon kills the probiotic organism, it will not be helpful (5). Various important stressors to the organism during consumption and processing are described in the following sections.

Digestion

Digestive factors such as enzymes, bile and pH are potentially caustic to probiotic bacteria. In particular, low pH, from stomach acid or food processing, can regulate the function of cell enzymes and change transport of nutrients into the cell. Different researchers have investigated that the low pH affects the survivabilityof probiotic bacteria(6, 7, 15 and 13). Specifically, Lee and Salminen (15) have found Lactobacillus can survive when pH is as low as 3.7 to 4.3 while Bifidobacteria generally cannot survive at pH values below 4. Additional investigation, Jia et al (13) studied effects of time and pH dependence on the inactivation of a range of species of *Bifidobacterium* by adapting a system typically used for indicating thermal destruction of bacteria: D- and z-values.

Food Processing

Thermal processing

Heat treatment reduces the viability of bacteria by denaturing important proteins and enzymes. The time for decimal reduction in a given microorganism at a given temperature is given by the D-value.

Mechanical stress

Mechanical force applied to a product is caused by shear stress, such as by mixing, and can be fatal to bacteria. On the other hand, *Lactobacilli* and *Bifidobacteria* are gram positive organisms with thick cell walls and can resist most shear forces encountered in food processing unless very high shear is introduced by high-speed blending or homogenization (15).

Environmental factors

Redox Potential (Eh)

Aerobic bacteria need positive Eh values (oxidized environment) whereas anaerobic bacteria need negative Eh values (12). The main genera of probiotic bacteria, *Bifidobacterium* and *Lactobacillus*, are anaerobic or microaerophilic in nature, which indicates a preference for reduced oxygen (negative Eh) environments.

Water Activity

It is defined as the partial vapour pressure of water in a food in comparison to the vapour pressure of pure water at a specific temperature; it is asymbol of the amount of free water in a system. Suitable water activity is needed to control the osmotic homeostasis of the organisms (12). Little water activity can generate an osmotic pressure gradient between the cell cytoplasm and the environment that can stress or destroy the organism (15). In foods, bacteria normally require aw values above 0.8 to grow, though that number may change depending on other conditions such as pH and temperature. Very little water activity may also be used to preserve the organisms (e.g., freeze-dried probiotics can survive as long as 12 months storage time, 15). Encapsulating the cells can decrease the effects of these stresses on probiotic organisms.

Encapsulation

Bacteria may be protected by encapsulation or immobilization (18), where encapsulation refers to the development of acontinuous outside layer around the bacteria such that it is fully entrapped within acapsule wall and immobilization is the entrapment of bacteria within orthroughout a matrix. A major difference is that in immobilization a small amountof bacteria may be present at the surface of the immobilization material where as in encapsulation the bacteria are completely covered. Encapsulation may also occur in nature when bacteria exude an exopolysaccharide.

Polymeric Encapsulation Materials

The perfect system for encapsulation of probiotics uses food grade, in expensive and simple material that is not detrimental to bacteria viability (15). The procedure should result in a high encapsulation efficiency (i.e., the majority of input cells are encapsulated and not lost during the process) as well as high loading capacity. There should be no unfavourable effect on taste or texture when added to food. The encapsulation system will be defensive against an appropriate range of environmental stress during processing and storage as well as protecting the bacteria through the stomach and releasing them in the colon.

Brinques and Ayub (2) utilized alginate, pectin and chitosan as encapsulation materials for *Lactobacillus plantarum* BL011. Cells were encapsulated by incorporation of concentrated culture with either sodium alginate orlow-methoxyl pectin solution. The cell-polymer solution was emulsified into liquidvegetable oil then gelled by addition of calcium chloride. The encapsulated cells were then enclosed with either chitosan or sodium alginate. All coatings were found to increase the viability of encapsulated cells

compared to control underrefrigerated storage over 38 days, with pectin or alginate-chitosanencapsulatedcells experiencing the most protection.

Ding and Shah (6) also utilized alginate to encapsulate 8 different probiotic organisms. The encapsulated and free bacteria were exposed to acidic environment (pH 2) for 2 h, bile salt (3% oxgall or taurocholic acid) and mild heat treatment (65°C for 1 h). Tolerance to acidic conditions was species- and strain dependent and encapsulation offered good protection. Bile tolerance was also species- strain-dependent but encapsulation again offered protection.
Taurocholic acid was slightly more fatal to microbesin comparison to oxgall. The microencapsulation offered protection to heat treatment for 30 min at 65°C but no protection was realized after 1 h at 65°C, possibly due to degradation of the encapsulation material. Ding and Shah (7) followed up on this work by comparing the effectiveness of guar gum, alginate, xanthan gum, locust bean gum and carrageenan gum as encapsulation materials for ten probiotic strains of *Lactobacillus* and *Bifidobacterium*. Among these Xanthan gum and carrageenan gum provide the highest level of protection to simulated gastric juice and intestinal (bile) fluid. Overall xanthan and carrageenan gum offered similar protection to alginate but guar and locust bean gum offered little protection. A model system using 6-carboxyfluorine dye release was utilized to verify their findings. In this model, guar and locust bean gum encapsulation resulted in higher levels of release than carrageenan, xanthan or alginate. This study demonstrates the ability of certain polysaccharides to offer protection to probiotic bacteria but does not go into discussion of how or why this happens. The use of an aqueous dye as a model for bacterial release offered a more rapid experimental protocol.

Encapsulation Methods

There are different chemical methods of encapsulation that can be applied to bacteria (18):

Chemical methods

Polymerization is the most universal chemical method for encapsulation. In polymerization a matrix is formed around the material to be encapsulated byeither rapid solvent evaporation (i.e., spray drying) or a chemical change. Matrix polymerization has widespread applications within the food industry.

Physical methods

Extrusion

Extrusion involves suspending probiotics within a hydrocolloid solution (e.g. alginate) and extruding the suspension for hardening or setting solution, for example calcium chloride (14). This gentle method is accepted as it is simple and economical. By the size of needle and distance from needle to hardening bath the size and shape of beads can be controlled.

Spray-drying

The bacteria are mixed in a polymer solution or melted lipid that will solidify or condense to form a wall around the bacteria after spraying. This method was used by Adachi et al (1) to encapsulate a hydrophilic dye (1, 3, 6, 8-pyrenetetrasulfonic acid tetrasodium salt, PTSA) as a replica for probiotic bacteria using maltodextrin and gum Arabic.

Emulsification

This involves dispersing a bacterial culture in the internal water phase of a water-in-oil (W/O) or a water-in-oil-in-water (W/O/W) emulsion.

In relation to efficiency and stability of enzyme activities encapsulation is more advantageous than the free cell system (18).

3. OBJECTIVES

3.1 To study the effect of temperature on *L. acidophilus* NCIM 2660 and *L. bulgaricus* NCIM 2056 as free cells

3.2 To study the effect of temperature on *L.acidophilus* NCIM2660 and *L.bulgaricus* NCIM 2056 as encapsulated cells

3.3 To study the effect of storage on viability of *Lactobacillus bulgaricus* NCIM 2056 in different food matrices

4. MATERIALS AND METHODS

Microorganisms

Lactobacillus acidophilus NCIM 2660 *and Lactobacillus bulgaricus* NCIM 2056 cultures were maintained and sub-cultured in 500 ml MRS broth by inoculation of 100 µL culture from glycerol stock maintained in laboratory and incubated at 37^{o}C for 24 hrs. Cells were harvested by centrifugation at 7000 rpm for 20 min at 25^{o}C. Supernatant was discarded and pellets were suspended in 20 ml saline. The final cell concentration was adjusted to 1.32 x 10^{10}CFU/ml and 2.57 x 10^{10}CFU/ml for *Lactobacillus acidophilus* NCIM 2660*and Lactobacillus bulgaricus* NCIM 2056, respectively. Colony forming units was calculated by using the following formula

CFU/ml = Number of colonies x dilution factor/volume of culture plate

Microencapsulation

Lactobacillus acidophilus NCIM 2660 *and Lactobacillus bulgaricus* NCIM 2056 were encapsulated in sodium alginate matrix. Sodium alginate solution (3 %) and calcium chloride solution (0.5 M) was prepared, sterilized by autoclaving (120^{o}C for 15 min) and cooled to 38-40^{o}C. Sodium alginate solution (20 ml) was added to the calcium chloride solution (40 ml) in a drop wise manner with the help of syringe in order to make the beads. The beads were separated by filtration using filter paper, transferred to sterile falcons and stored in refrigerator.

Effect of temperature of on free cells

1 ml culture of *L. acidophilus* NCIM 2660 and *L. bulgaricus* NCIM 2056 were transferred to each test tubes containing 9 ml distilled water. These test tubes were exposed to different temperatures (60°C, 70°C, 80°C, and 90°C) for different time intervals (1 min, 5 min, 10 min, 15 min, 20 min, 25 min, and 30 min) in water bath. The cells were spreaded on MRS agar by spread plating method and enumerated after incubation at 37°C for 48 hrs.

Effect of temperature on encapsulated bacterial cells

Effect of temperature on encapsulated *Lactobacillus acidophilus* NCIM 2660 *and Lactobacillus bulgaricus* NCIM 2056 from 90^{0}C to 130°C was studied using distilled water as a suspending medium in oil bath. 1 g of microcapsules (10^{10} cells per g) was transferred in test tube containing 9 ml distilled water. After the heat treatment the content was cooled to room temperature and viable cells were enumerated.

Effect of Storage on free bacterial cells in jelly food matrix

Survivability of free bacterial cells in Jelly food matrix

Jelly was prepared using water extract of orange peels collected from NIT Rourkela hostel during December 2014 and January 2015. 1 g of *L. acidophilus* NCIM 2660 and *L. bulgaricus* NCIM 2056 were added to 20 g of jelly and survivability of *L. acidophilus* NCIM 2660and *L. bulgaricus* NCIM 2056 in jelly food matrix were checked for about 3 weeks.

Survivability of encapsulated bacterial cells in traditional dry food snack

1 g of encapsulated *L. plantarum* NCIM 2083 and *L. bulgaricus* NCIM 2056 was added to dry snacks and studied for 3 weeks. Every week the stored beads were separated from the food matrix and dissolved in Tri-sodium citrate (pH 6). From the dissolved beads 200 µL was poured on MRS agar by pour plate method and incubated at 37^0 C for 48 hrs. The cells were enumerated.

5. RESULTS AND DISCUSSION

Encapsulation of bacterial cells using sodium alginate beads

By using 3% sodium alginate and 0.5 M $Cacl_2$ solution, beads were prepared for *L. bulgaricus* NCIM 2056 *and L. acidiplillus* NCIM 2660. The beads are of same size, same diameter and round in nature.

Figure 1: Encapsulated bacterial cells

Effect of temperature on viability of free cells

1 ml culture was transferred to each test tubes containing 9 ml distilled water. These test tubes were exposed to different temperatures ($60^{O}C$, $70^{O}C$, $80^{O}C$, and $90^{O}C$) for different time intervals (1 min, 5 min, 10 min, 15 min, 20 min, 25 min, and 30 min) in water bath. The cells were spreaded on MRS agar by spread plating method and their survivability was examined after incubation at $37^{O}C$ for 48 hrs. It was observed that *L. bulgaricus* NCIM 2056 can tolerate $100^{0}C$ for 1 min as free cells.

Table 1: Effect of temperature on survivability of free bacterial cells at 60°C

	Time in minutes						
Name	**1**	**5**	**10**	**15**	**20**	**25**	**30**
Lactobacillus acidophilus NCIM 2660	S	S	S	S	S	S	S
Lactobacillus bulgaricus NCIM 2056	S	S	S	S	S	S	S

S – Survive

Table 2: Effect of temperature on survivability of free bacterial cells at 70°C

	Time in minutes						
Name	**1**	**5**	**10**	**15**	**20**	**25**	**30**
Lactobacillus acidophilus NCIM 2660	S	S	S	S	S	S	S
Lactobacillus bulgaricus NCIM 2056	S	S	S	S	S	S	NS

S – Survive, NS- Not surviving

Table 3: Effect of temperature on survivability of free bacterial cells at 80°C

	Time in minutes		
Name	**1**	**5**	**10**
Lactobacillus acidophilus NCIM 2660	S	S	S
Lactobacillus bulgaricus NCIM 2056	S	S	S

S- Survive

Table 4: Effect of temperature on survivability of free bacterial cells 90^{o}C

	Time in minutes		
Name	**1**	**5**	**10**
Lactobacillus acidophilus NCIM 2660	S	S	NS
Lactobacillus bulgaricus NCIM 2056	S	S	S

S - Survive; NS - Not Surviving

Table 5: Effect of temperature on survivability of free bacterial cells at 100^{o}C

Name	**30s**	**1 min**	**5 min**	**10 min**
Lactobacillus acidophilus NCIM 2660	S	NS	NS	NS
Lactobacillus bulgaricus NCIM 2056	NS	NS	NS	NS

S - Survive; NS - Not Surviving

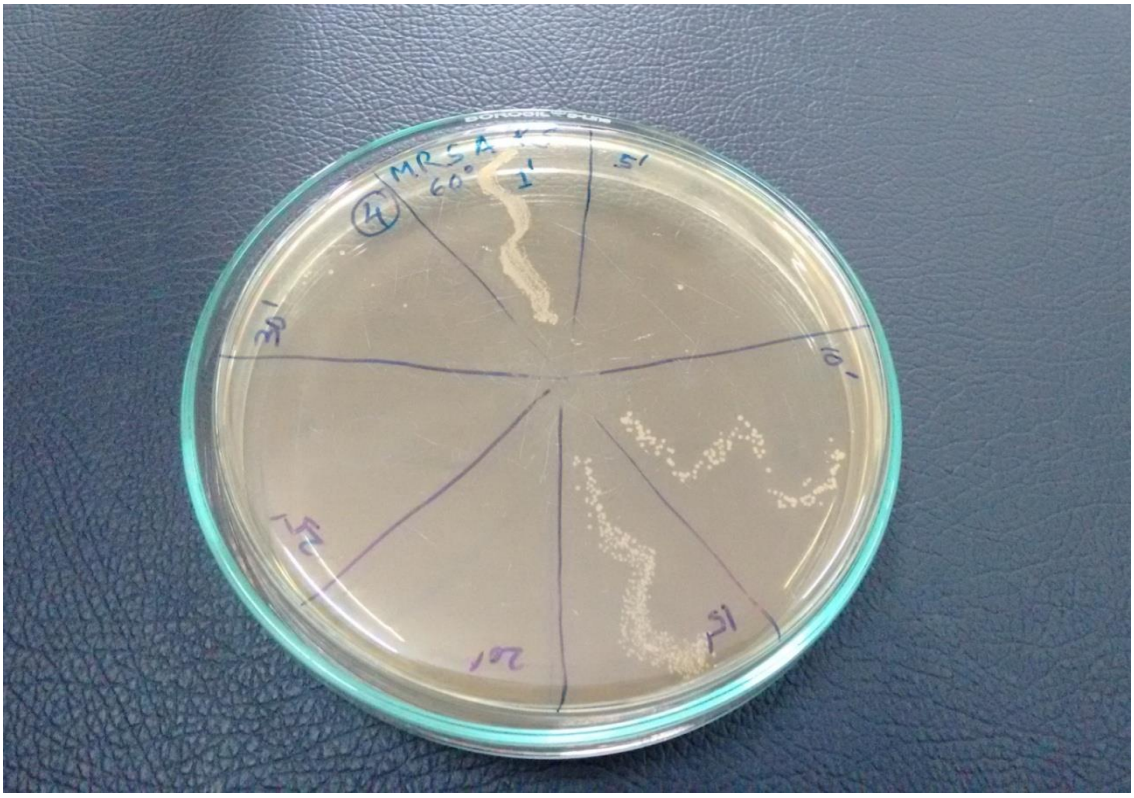

Figure 2: Plate showing surviving cells of *L. acidophillus* NCIM 2660 after exposing to 60°C for different time intervals

Effect of temperature on viability of encapsulated bacterial cells

Tolerance of encapsulated *Lactobacillus bulgaricus* NCIM 2056 *and Lactobacillus acidophillus* NCIM 2660 to heat treatment *(>100^{o}C)* was studiedusing distilled water as a suspending medium in oil bath. 1 g of microcapsules (10^{10} cells per g) were transferred in test tube containing 9 ml distilled water. After the heat treatment the content was cooled to room temperature and viable cells were enumerated. Results revealed that encapsulation could improve the temperature tolerance of *L. bulgaricus* NCIM 2056 up to 120°C for 1 min. It is may be due to release of some heat sock protein (HSP) by *L. bulgaricus* NCIM 2056.

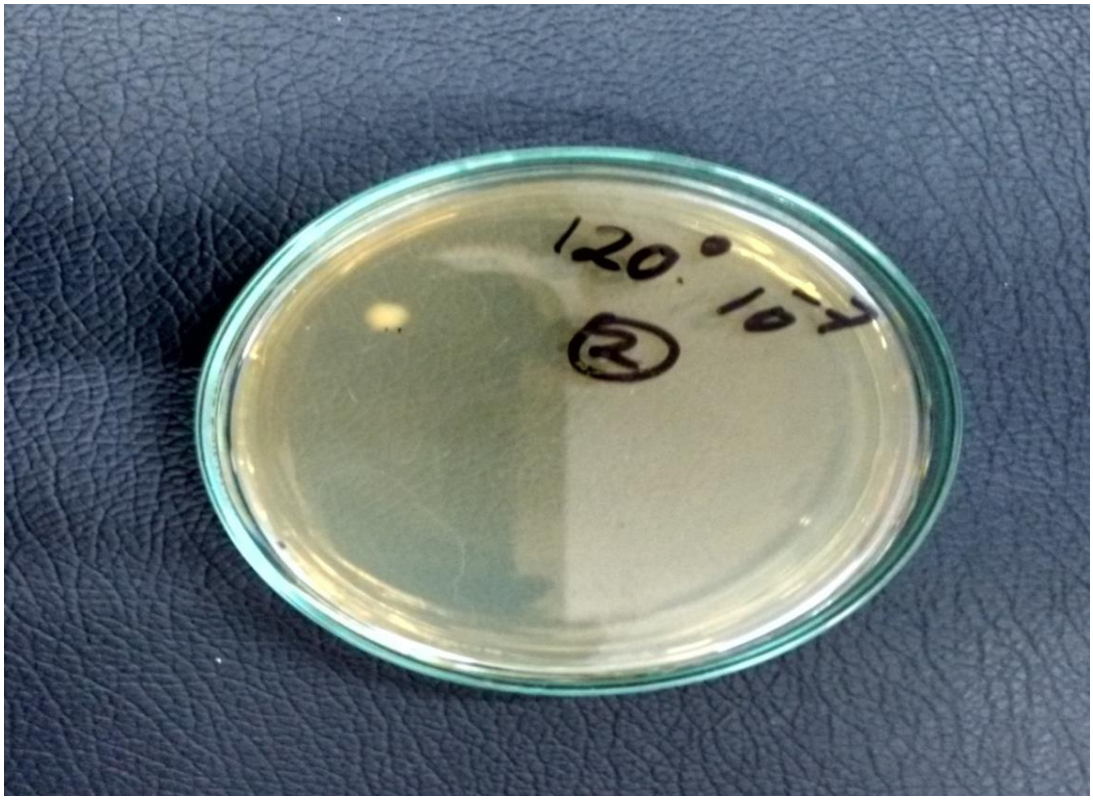

Figure 3: Plate showing surviving cells from encapsulated *L. Bulgaricus* NCIM 2660 after exposing to 120°C for one minute.

Table 6: Effect of encapsulation on temperature tolerance of bacteria at 100°C in different time periods

	Time in minutes (Log_{10} Number of viable cells)			
Name	**Initial**	**5**	**10**	**15**
Lactobacillus bulgaricus NCIM 2056	6.48	5	3	NS

S - Survive; NS - Not Surviving; Control - encapsulated bacteria without heat treatment

Table 7: Effect of encapsulation on temperature tolerance of bacteria at >100°C for 1min

	Temperature (°C) (Log_{10} Number of viable cells)					
Name	**Initial**	110°C	120°C	130°C	140°C	150°C
Lactobacillus bulgaricus NCIM 2056	4.89	3.47	3	NS	NS	NS

S - Survive; NS - Not Surviving; Control - encapsulated bacteria without heat treatment

Storage of free bacterial cells in jelly food matrix

Survivability of free bacterial cells in Jelly food matrix

Jelly was prepared using water extract of orange peels collected from NIT Rourkela hostel during December 2014 and January 2015. 1 g of *L. acidophilus* NCIM 2660 and *L. bulgaricus* NCIM 2056 was added to 20 g of jelly and survivability of *L. acidophilus* NCIM 2660 and *L. bulgaricus* NCIM 2056 in jelly food matrix was checked for about 3 weeks.

Table 8: Survivability of bacteria during storage period (3 Weeks)

	Log_{10} number of viable cells in 20 g of jelly	
NCIM strain	**Fresh**	**Stored**
L. bulgaricus	8.8	3.1
L. acidophilus	9.1	2.4

Survivability of encapsulated bacterial cells in traditional dry food snack

1 g of encapsulated *L. plantarum* NCIM 2083 and *L. bulgaricus*NCIM 2660 was added to dry snacks and studied for 3 weeks. Every week the stored beads were separated from the food matrix and dissolved in Tri-sodium citrate (pH 6). From the dissolved beads 200 µL was poured on MRS agar by pour plate method and incubated at 37° C for 48 hrs. The cells were enumerated.

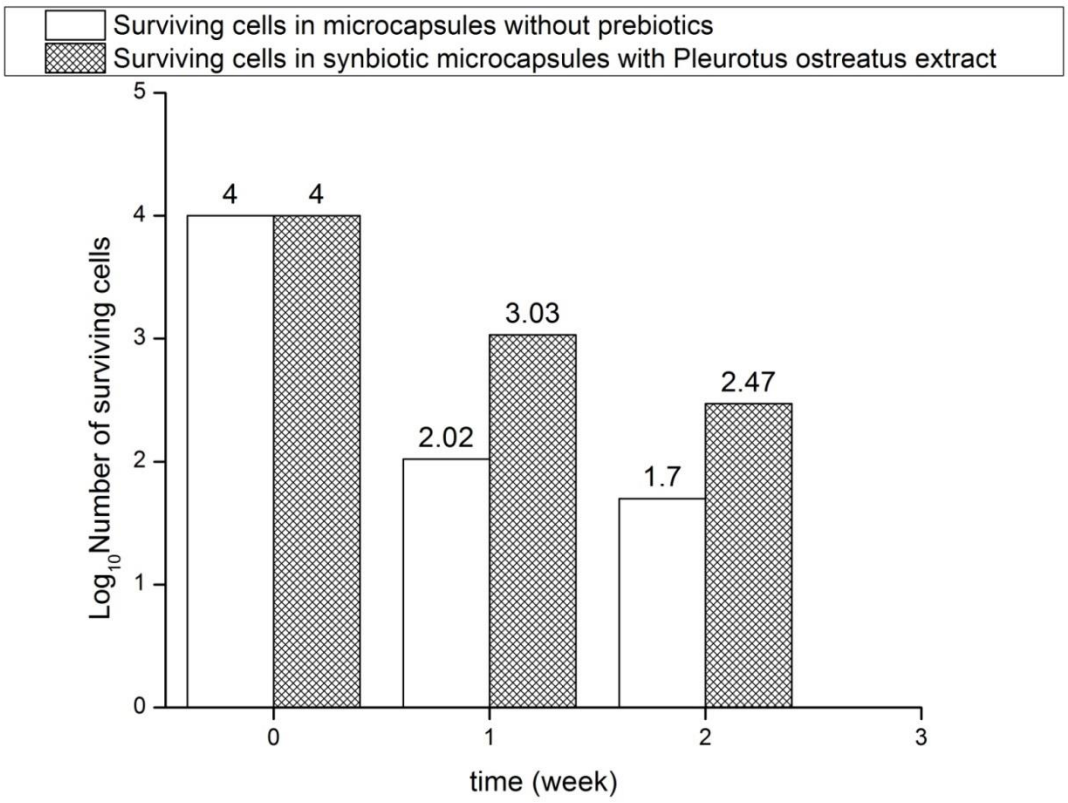

Figure 4: Number of surviving cells of *L. plantarum* NCIM 2083 in dry snack (3weeks).

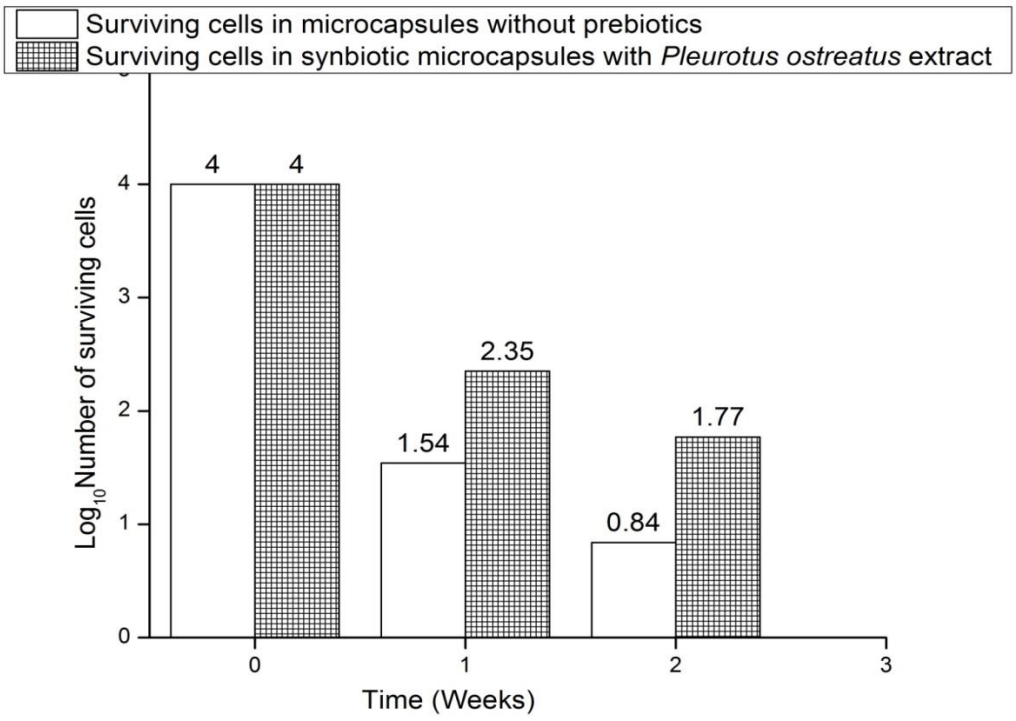

Figure 5: Number of surviving cells of *L. bulgaricus* NCIM 2056 in dry snacks (3weeks).

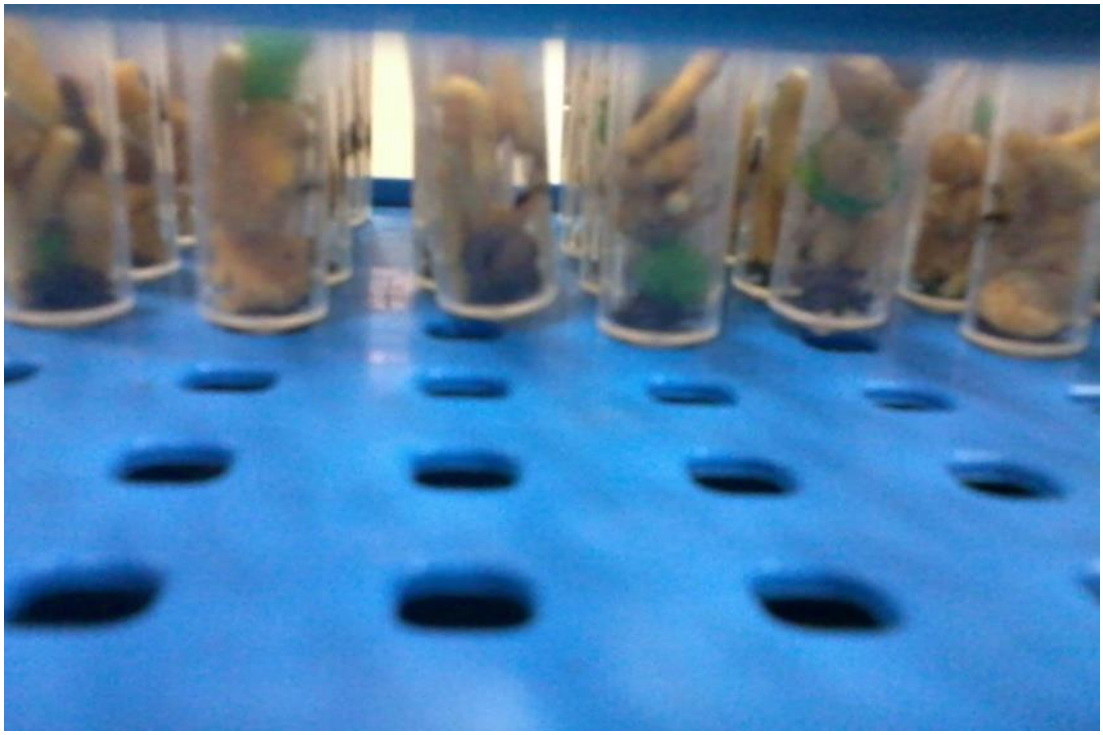

Figure 6: Stored alginate beads with encapsulated bacterial cells in mixture under room temperature

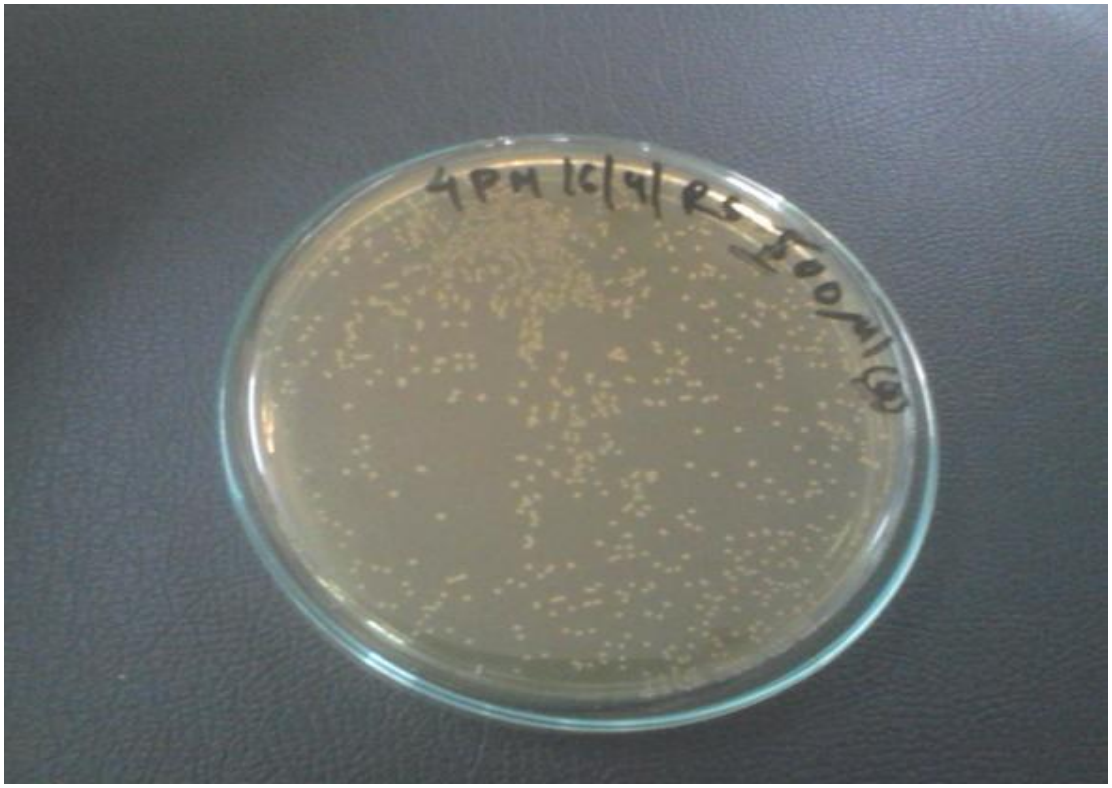

Figure 7: Plate showing surviving cells from encapsulated *L. acidophillus* NCIM 2660 after storage in dry snacks for 3 weeks.

6. CONCLUSION

Along with the various approaches, microencapsulation has emerged as the best alternative so as to overcome the problem of poor survivability of probiotic cultures in the food matrix. The above results of this study showed that the strain *L.bulgaricus* NCIM 2056 was found to be capable of better stress tolerance against temperature than *L. acidophilus* NCIM 2660. Hence *L. bulgaricus* is the better applicant to be used in dry functional food such as health mix and dry snack food. Inclusion of *Pleurotus osteratus* (mushroom) as prebiotic substance in synbiotic microencapsulation of *L. bulgaricus* enhanced the survivability than the control when it is stored in dry snack food. Prebiotics contains polysaccharide that retards the death of probiotic hence enhancing viability of cells.

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