Dissertation Report-I (Aug-Dec 2017)

"Optimization of formulation parameters to achieve controlled release of Probiotics"

Submitted in partial fulfilment of the requirements for the degree of

M.Sc. (hons.) Biotechnology

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DECLARATION

I hereby declare that the project entitled "**Optimization and formulation parameters to achieve control release of Probiotics**" is an authentic work proposed to be carried out at School of Bioengineering and Biosciences, Lovely Professional University, Phagwara, for the partial fulfilment of the award of **Master of Science (hons.)** in **Biotechnology** under the guidance of **Dr. Jibanananda Mishra**, Assistant Professor, LPU, Punjab.

This is our original work and has not been submitted for any degree/diploma in this or any other University. The information furnished in this report is genuine to the best of my knowledge and belief.

Place: PHAGWARA, PUNJAB

Date:

Manisha Sharma
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CERTIFICATE

This is to certify that **Miss. Manisha Sharma**, **11613986**, have completed the dissertation-II work for the project entitled "**Optimization of formulation parameters to achieve controlled release of probiotics**" under my guidance and supervision. To the best of my knowledge, the present work is the result of her original investigation and study.

No part of the report has ever been submitted for any other degree at any university.

The Dissertation-II report is fit for submission and the partial fulfilment of the conditions for the award of **M.Sc. (hons.) Biotechnology**.

Place: PHAGWARA, PUNJAB

Date: **Supervisor Signature**

ACKNOWLEDGEMENTS

History of all great works into witness that no great work was ever done without either active or passive support. All the praises and thanks are for the most omnipotent, gracious, ubiquitous, merciful GOD, who blessed me with his grace and mercy to complete my dissertation project.

I would like to express my gratitude to all those who gave me the opportunity to complete the dissertation report entitled "**Optimization of formulation parameters to achieve controlled release of probiotics**". I want to thank the School of Biosciences and Bio-engineering of Lovely Professional University for giving me the permission to commence the dissertation, to do the necessary research work and to use departmental facilities. I furthermore want to thank **Dr. Jibanananda Mishra**, who gave and confirmed the permission and encouraged me to go ahead with my project.

I am deeply indebted to my supervisor, **Dr. Jibanananda Mishra,** stimulating suggestions and motivation helped me in research and writing of the dissertation report.

Thank you!

Manisha Sharma

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INTRODUCTION

Probiotics are live microorganisms which when administered in adequate amount provide health benefits to the host (WHO, 2003). Some people think that microorganisms are "germs" that are harmful, but this is not true in every case. Microorganisms are good friends of the human and helps in the daily functioning of the body such as digestion of food, destruction of harmful pathogens, etc. Probiotics were identified by Elie Metchnikoff referred to as

"Father of probiotics". Probiotics contain various types of microorganisms as bacteria such as *Bifidobacterium* and *Lactobacillus,* and yeast (*Saccharomyces boulardii*). Usually, Lactic acid bacteria strains are broadly utilized as probiotics. There are diverse sources for isolation of probiotics such as mammary areola, feces of infants, oral swabs, plants, pickles and traditional dairy or starter cultures (**Kailasapathy, 2009**).

Probiotics have been associated with humans since a long time. It is observed that breast feeding healthy infants have healthy gut microbiota compared to that in infants with diarrhea. Human gut micro flora has enormous contribution in maintenance of general health. This healthy micro flora is prerequisite for protection of host from the infectious diseases. Use of probiotics not only improves immunity but also reduces cholesterol problem, and is expected to be beneficial for cancer prevention. There are many other health benefits due to administration of probiotics. Feeding of probiotics reduces the antibiotic associated diarrhea. Rotavirus is an acute diarrhea all over world. Replication of this virus is very rapid and normal gut flora is very helpful in protection from infection from rotavirus. Lactose intolerance is also widely present in the present population. It is due to deficiency of beta – galactosidases enzyme in such individuals. This results in their inability to metabolize lactose and results in diarrhea, constipation and abdominal pain, when they intake milk or milk products. But these patients responded well upon administration of probiotics. Traditional dairy products such as dahi, cheese, yoghurt, etc. also contain probiotics.

Probiotics are not complex substances, they are just living microorganisms. As per general convention, only viable, live, and specific strain of microbes are considered as probiotics. These strains have specific properties such as tolerance to gastrointestinal conditions, stimulation of gut immune system, secretion of specific nutrients, etc. Recently, need for probiotics are increasing in a large scale that companies are trying introduce probiotics in maximum food supplements.

Mainly probiotics are present in dairy products such as fermented milk, yogurts, ice-creams, cheese and many others. Non-dairy products also contain probiotics such as cereals, soy products and juices, etc. Apart from the uses of probiotics in human, these are also helpful in maintaining microbial balance in aquacultures. In aquatic organisms, apart from internal GI tract conditions, there should be proper maintenance of surrounding water for the aquatic animals. Stress conditions are avoided due to these favorable conditions (Bruno *et al.,* 2000).

Isolation of probiotic microorganisms: As stated earlier, dairy products such as milk, cheese, curd, buttermilk, can also be used to isolate probiotic microbes. Probiotic bacteria such as LAB have been found to grow selectively on DeMan, Rogosa and Sharpe (MRS) broth. Spread plating has been commonly used for samples which have been serially diluted at appropriate levels. These petriplates are incubated at 37ºC for 48h after that microbial colonies are observed. (Mishra and Sharma, 2014). These colonies are then picked under sterile conditions and then propagated for molecular identification.

Identification is done by characterization by the help of gram staining, catalase test, and PCR using *Lactobacillus*-specific primers. Probiotics properties of that particular strain is determined using gastric fluid, fastitious intestinal fluid, bile salt mixture, production of specific substances. Antibiotic resistance in probiotics is also determined to check antibiotic susceptibility and sensitivity.

To be classified as ‘Probiotics’, microorganisms need to fulfil these criteria:

1. Survival of microbes at low pH while passing to GI tract, tolerance to bile.

2. Adherence of microbiota to the epithelial cells of intestine.

3. Gut micro flora stabilization.

4.Inhibition of pathogens.

5. Growth with rapid multiplication rate in the GI tract.

6. Suitable gut residence time.

Production of a probiotic formulation is not an easy task. It includes a lot of care of the probiotic microbe during the process, till its delivery to consumers and intake. Further, many safety parameters are checked *in vitro* as well as *in vivo*, leading to the development of a probiotic formulation. These formulations can be modified to achieve higher probiotic survival and efficacy by many strategies, one of which is encapsulation.

Need for encapsulation:

Survival of microorganisms in the GI tract has always remained a persistent question. Oral administration of probiotics is the easiest method for the delivery of microorganisms to the intestine. But, it is important to check the viability of the microorganisms throughout the oral delivery from mouth to the intestine. While oral feeding, acidic condition in stomach due to gastric fluids and other enzymes adversely affect the viability of probiotics. They can be affected by bile juices present in the stomach which might also affect their survival and show their proper benefits. Because of this reason, free delivery of probiotics is not an efficient method.

Encapsulation is said to be physical, mechanical or chemical method to enclose substances around a material to produce spheres or capsules. Encapsulation is very important to keep the microorganisms in the active form and adequate amount till the target. Encapsulation is done in such way that whatever time the probiotics remains in the stomach or harsh environment, its survival rate should be maintained properly. Microorganisms should not get released in stomach pH. Coating material or membrane should be little permeable, so that microbes inside can get their nutrients to survive. Cells can be immobilized within a coating which can be 1-1000µm in size. Microencapsulation is better to be done than macroencapsulation, as the latter has less viability of cells. Mild conditions can also be used to ensure microencapsulation.

Polymer for encapsulation:

Varieties of polymers have been developed in recent times. Polymer selection is the main target for encapsulation so that the growth and viability of cells will be maintained for longer duration. Mostly water soluble polymers are used which allows low molecular weight nutrients uptake for the cells within. Polymers used for encapsulation can be synthetic and natural.

1.Synthetic polymer: PLGA, ethylene glycol, etc.

2.Natural polymer: Chitosan, alginate, k-carrageenan, gelatin, etc.

In this report “Chitosan” is used as the polymer for encapsulation of probiotics.

Chitosan is a natural linear polysaccharide, extracted from shells of shrimps. Chitosan is a product formed from deacetylation of chitin. It forms crosslinks with opposite ions and get polymerized in the presence of polyions (anions). This polymer is also known as weak polymer since it is not soluble in water and require a dilute acidic environment. Many researches has shown that chitosan as a good encapsulation agent (Shu and Zhu, 2000).

Dispersion method:

There are various types of techniques available for encapsulation based on the type of polymer, required size, ease of production, final aim of formulation, etc. Mostly used techniques are:

1. Spray drying: Spray drying is mild and cost-effective method. Probiotic cells and polymer matrix are dissolved to form a solution. . A hot steam gas is passed to the drying chamber and evaporates the solvent. The resulting microspheres are taken out by transporting to a cyclone separator. The polymer matrices are generally gum Arabic and starches because they form microspheres during the drying process.

2. Spray and Freeze Drying: it is quite clear from its name that low or freeze temperature is used in this with vacuum. This is done evade oxidation process and water phase transitions. At last the got dried matter must be grounded. By this less surface area and large size particles are generated. This technology is beneath frequently acclimated compared with added encapsulation techniques. Cryoprotectants are added increase the shelf life of cells.

3. Emulsification: Emulsions are regularly utilized to process encapsulates. Furthermore is an arrival frameworks to oils like essential oils, w-3, flavours, etc. In aqueous solution. A chance to be dried should be more with more stability. In this method, oil of interest is added to the aqueous solution by mechanical agitations and thin layer of polymer will be formed which when get dried will be having encapsulated oil in polymer matrix. Various encapsulate materials can be used such as maltodextrin, gum arabic, modified celluloses, etc. For encapsulating probiotic

cells by emulsification a discontinuous phase of polymer -cell in small proportion is added to the vegetable oil having large volume. Then this is homogenized and there would be emulsions of water in oil. To produce stable emulsion an emulsifier is used such as Tween 80 and calcium chloride as a solidifier. According to the size of bead needed variant agitation speeds should be given. (Kailasapathy, 2009)

4. Extrusion method: Emulsion process of encapsulation was tedious method. Extrusion method or encapsulating probiotic cells is simple and old method. In this method microspheres or macrospheres are produced by giving external force (vibration, electrostatic, jet cutter, coaxial air flow, and atomization). Desired size of the particle can be produced by selecting the needle diameter and creating difference between polymerizing solution and dropper.

5. Coacervation: It is used to say that Coacervation method lead to the development of encapsulating technology. In this method, hydrocolloids solution initially is phase separated i.e. coacervate phase which is then having active substance is emulsified in the same or different reaction media.

6. I**onotropic gelation method:**

Ionotropic gelation technique, is one of the most widely method used for encapsulation due to their and biodegradability and biocompatibility .This method is also known as Polyelectrolyte complexation. In this method, simple interaction occurs between ionic polymer and oppositely charged ions, forms crosslinking. By this method hydrogel beads are formed. Polyelectrolyte complex membrane is used to be formed by addition of polycations. Polyvalent cationic solution is used in which solution having cells or polymeric solution is dropped gradually, this lead to formation of hydrogel beads. Three dimensional structure is formed by crosslinking cells and cationic solutions. (Patil *et al*., 2012).

Polyelectrolyte solution (chitosan + probiotics) in syringe

↓

Added drop wise by syringe under continuous stirring

↓

Counter ion solution (TPP)

↓

Microspheres / beads

Fig.1: Formation of microspheres by Ionotropic gelation method.

Here, we have used the Ionotropic gelation method because of ease of its operation during optimization and scale up of the whole process at industrial scale up.

Scope of the Study

All the probiotics containing preparations suffer loss of cells during storage and due to stomach acids after ingestion. Our approach would help the probiotics bypass stomach acids and slow release in intestine would allow the probiotics with more time to show beneficial effects. This would also help lower down the concentration of probiotics in the products and would cut production costs.

Objectives of the study

1. Selection of a particular probiotic strain.

2. Optimization of formulation parameters for high encapsulation of selected probiotic.

3. Estimation of the extent of controlled release from the optimized formulation.

Review of Literature

Probiotics came into existence industrially after the noble laureate “Elie Metchnikoff” shown that fermented milk consisting viable cells helps in long living life of Bulgarians. After his work many experiments are being on microbes to be used as probiotics and their health benefits. Below there are list of some investigations and experiments done on probiotics and references.

SI. NO.	WORK DONE	REFERENCES
1	Survival of free probiotics and encapsulated probiotics are checked in this research. Apple and orange juice were the substrate for investigation. Eight different strained bacteria were used to study this. At the end it is observed that microencapsulated probiotics in both juices were more stable than free probiotics.	Ding and Shah, 2008
2	Experiments were done to demonstrate for detection of probiotics in human mammary glands. There were observed presence of rod-shaped LAB in the mammary	Martin *et al., 2003*
3	They investigated that probiotics and prebiotics provides protective role in colon cancer. They have shown that viable probiotics and prebiotics act as anticarcinogen. Experiment is done on Rat colon carcinogen 1, 2-dimethylhydrazine. Ingestion of pre and probiotics shown on the	Wollowski *et al.,* 2001

		formation of short chain fatty acids. Probiotics prevented or deactivate the genotaxic carcinogen.	
	4	They investigated the feasibility of vegetable oil encapsulation. They used three methods for this: spray drying, emulsification, fluid bed based agglomeration.	Turchiuli et al., 2005
	5	This paper review the different methods or technologies to encapsulate cells in their viable form. Dispersion methods such as: Atomization, emulsification, extrusion methods were used. Encapsulation is done by coating material like alginate, chitosan, milk proteins, k-carrageenan etc	Burgain.et.al.,2011
	6	The probiotic characteristics of selected indigenous lactobacillus strains such as ability to adhere to epithelial cells and level of their hydrophobicity under *in vitro* conditions was studied. They demonstrated high adhesion ability and higher percentage of hydrophobicity in *L. plantarum* lp9 and 91.	Duary et al., 2011
	7	They performed a quantitative examine with bacterial cells labelled with [3H] thymidine to explore factors associated with the adherence of human disengages. L. acidophilus BG2FO4 and NCFM/N2 and L. gasseri ADH human Caco-2 intestinal cells. They demonstrated that their adherence to human intestinal cells is through systems which include distinctive blends of starch and	Greene and Klaenhammer, 1994

		protein factors on the bacterial cell surface.	
	8	In this study, probiotics for aquaculture is studied Probionts bacterial strains were tested in aquatic animals. Experiments were done on larvae of fishes. In result mortality rate reduction is observed. This investigation resulted in the microbial balance inn aquacultures	Gomez.et.al.,2000
	9	In this discussion various hypothesis were studied. Regarding food and health. Need and safety of probiotics and prebiotics were mentioned and their various aspects related to health. Food versus drugs and health vs. disease is seen.	Sunders.et.al.,2011
	10	They demonstrated that L. rhamnosus GG MBF, as a dynamic bodily fluid particular surface grip with an assumed subordinate association in pilus-intervened mucosal attachment, has an influence in the follower components amid intestinal colonization by this probiotic strain	Ossowski et al., 2011
	11	In this research, safety measurements, design, target sites, regulation and outcome of probiotics were studied. With these clinical trials were also mentioned.	Shane et al., 2010
	12	In this study, in vitro methods for isolation and selection of Lactobacilli probiotics were shown. This aims to produce better probiotics than before. Positive results were seen with	Morelli.,2000

		scientific conditions of current selection.	
	13	They described the adhesion capabilities of a recombinant L. lactis strain producing an extracellular protein from L. plantarum. The results show that this protein may offer the bacterium a mechanism to bind to N-acetyl glucosamine-containing polymers, such as human mucins, present in different environments.	Sanchez.et.al.,2011
	14	In this experiment, ionic gelation method is used for producing chitosan nanoparticles.TPP is used crosslinking agent. Chitosan with drug is mixed and gel beads were being formed with the cross linker TPP. Formulation factors and parameters were studied. Stability of chitosan-TPP nanoparticle is also studied.	Sediqa. et al., 2015
	15	In this review, probiotics role in cholesterol lowering is evaluated. And there recommendation as potential bio therapeutics for metabolic diseases. Bile, functions of BSH, and microbial BSH activity on host is tested. They also discussed effects of probiotics on plasma lipids.	Kumar et al.,2012
	16	In this study, use of symbiotic is done in order to protect encapsulated probiotics. Alginate-inulin coencapsulation is done to target the colon. Different concentration of alginate and inulin is tested at the conditions of GI tract. Also aim to check their molecular and biopharmaceutical properties. Results shows that use of inulin	Atia et al.,2017

		provides more protection to the probiotics and colonic controlled release systems were also provided.	
	17	They experimented on fecal samples of human volunteers consuming pearls and found that probiotics strains were improved y encapsulation. Colony counts were compared with two encapsulation methods. Randomized double-blinded, two arm trial with six healthy people were done. Colony morphology or strain specific PCR techniques used to check the colonization. In results, more efficient and improved viable cells were produced.	Mai.V et al.,2017
	18	In this study, use of probiotics against Rotavirus is studied. Bifidobacterim adolescentis and Lactobacillus casei strains were tested with MA104 infected cells. In results it is expected that antiviral effect may be coming from viral particles instead of blocking effects that were needed for entrance of viral particles.	Fernandez-Duarte et al.,2017
	19	Microencapsulation of a probiotic and prebiotics in alginate chitosan capsules improve survival in simulated gastrointestinal conditions. Chitosan was used as coating material Lactobacillus gasseri (L) and Bifidobacterim bifidum (B) as probiotic and prebiotic quercetin (Q) used. This encapsulation results in low yield of (L+Q) and (B+Q). Storage rate at 4°C show zero survival rate with quercetin. Separately encapsulation was done. B.bfidum was	Maria et.*al. 2010.*

	resistant to pH 2 upto 2h, improved survival rate	
20	Survival of L. acidophilus as probiotic bacteria using chitosan nanoparticles. Results demonstrated that the size of chitosan particles increases by increasing chitosan concentrations from 0.005 to 0.5 gL. No. of cells reduced were only 3.27 to 3.23 log CFU/ml in gastric condition with respect to free cells.	Ebranhinegad et.al.,2017
21	Microencapsulation of probiotic bacteria. Thisarticleshowsresearcharound microencapsulation and its application in preparation of stable consortia of probiotic bacteria. It predominantly focus on agro food, nutraceutical and pharma industry.	Rocha.et.al.,2016
22	An enhanced strategy for microencapsulation of probiotic microscopic organisms for the security in acidic and bile condition amid capacity. This examination gave the method for applying covering of palm oil and poly-L-lysine (POPL) to alginate. They utilized 8 unique strains of probiotics. Electron microscopy used to quantify size of microspheres and viability of cells. Results shows increase in the size of capsule.	W.K Ding.et.al.,2009
23	The influence of coating materials on some properties of alginate beads and survivability of microencapsulated probiotic bacteria. In	W Krasaekoopt.et.al.,2004

		this research, L. acidophilus547, B. bifidum ATCC, L. casei were encapsulated into uncoated Ca-alginate and separately with chitosan sodium alginate poly-L-lysine with alginate. In comes about, survivability of three probiotics in uncoated dots and covered dabs and free cells utilized condition 0.6% bile. Chitosan covered alginate dabs gave the best insurance to L. acidophilus, casei.	
	24	Cross-connected |Chitosan microspheres for embodiment of diclofenac sodium: impact of crosslinking specialist. Epitome of diclofenac sodium was finished with chitosan cross linkage, microspheres are further describes by FTIR, X-RD and SEM. Invitro discharge were learned at various pH cushions. Polymer crystallinity increments in the wake of crosslinking show by X-RD.	S.G Kumbar.et.al.,2000
	25	Probiotics contained foods not containing milk or milk constituents with unique reference to L. plantarum 299. The product is a lactic acid fermented oatmeal combined with fruit drink. Approx.5X CFU of L. plantarum 299 were found. This microbe can increase the conversion of carboxylic acids in feces and reductions stomach swelling. It likewise diminishes fibrinogen focus in blood.	American Society Of Clinical Nutrition, 2001

26	Characterization of L. plantarum PH04, a potential probiotic bacterium with cholesterol lowering effects.Stationary phase of L plantarum was secluded from defecation of newborn children and tried for acids and bile resilience movement. Bile salt hydrolase action was nine times more prominent in stationary phase.	T D T Nguyen.et.al.,2007
27	L. plantarum - survival, capacities and potential probiotic properties in the human intestinal tract. It has a demonstrated capacity to survive gastric travel thinks about are developing to decide the movement of L. plantarum in the human intestinal tract. This audit indicates impacts of bacterium on host	M C De Vries.et.al.,2006
28	Properties of potential L. plantarum strain. In this examination, fifteen Lactobacillus strains were portrayed for probiotic properties, among them 13 of the strains were of L. plantarum, L. acidophilus, L. pentosus. Resistance to corrosive and bile salts, capacity to mature fructooligosaccharides, galactosidases movement, and anti-microbial were contemplated. Acid tolerant were L. plantarum, acidophilus. Eight strains were having capacity to matured FOS. From all the testing L. plantarum were observed to be the most encouraging strain as probiotics.	A Cebeci.et.al.,2003

29	Modified alginate and chitosan for lactic acid bacteria immobilization. Viable cell count were generated by this type of encapsulation, alginate and chitosan with succinylation to increase anionic charges. At pH 1.5 free cells died and there were 22-26% viable cell count of immobilized cells without succinylation. With succinylated alginate and chitosan survival rate was 66%.	Canh Le-Tien.et.al.,2004
30	Securing probiotic microorganisms by microencapsulation: challenges formodern applications. This study shows that microencapsulation is very important to maintain the viability of cells. Gastric conditions are also reviewed. Stability of beads are improved by using different coating materials.	Susanna.et.al.,2010

MATERIALS AND METHODS

Strain used:

The strain of bacteria used in this research is Lactobacillus. plantarum.

L. plantarum is a gram negative bacteria comes under rod shape category. It can be isolated from humans, mammalian gastrointestinal tract, saliva and food product. They are tolerant to acid or bile juices and can survive in harsh conditions.

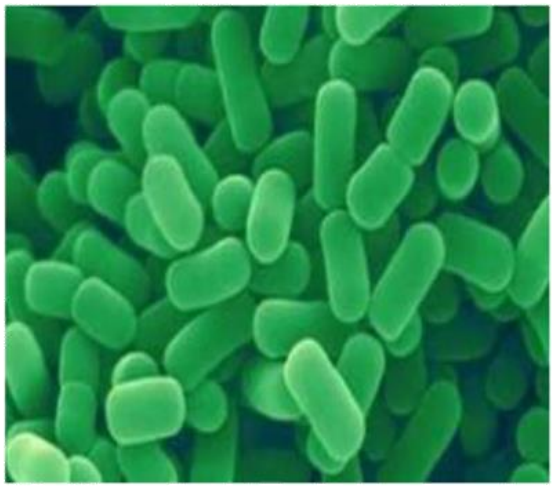

Fig 2 . Electron micrograph of L. plantarum.

The optimum temperature for L. plantarum is in between 15-45°C and it can live under 3-2 pH range. L. plantarum is a heterofermentative that produce lactic acid, acetic acid and ethanol.etc. from sugar under controlled conditions. This bacterium can change itself to homofermentative from heterofermentative. L. plantarum has large genome consist of 3.3Mb circular chromosomes. It is one of the biggest genome arrangement among LAB. They can grow in the absence or presence of oxygen, so they are considered as facultative anaerobes, heterofermentative. It follows the Embden – Meyerhoff-Parnas (EMP) pathway. So it can behave like heterlactic and homolactic simultaneously.

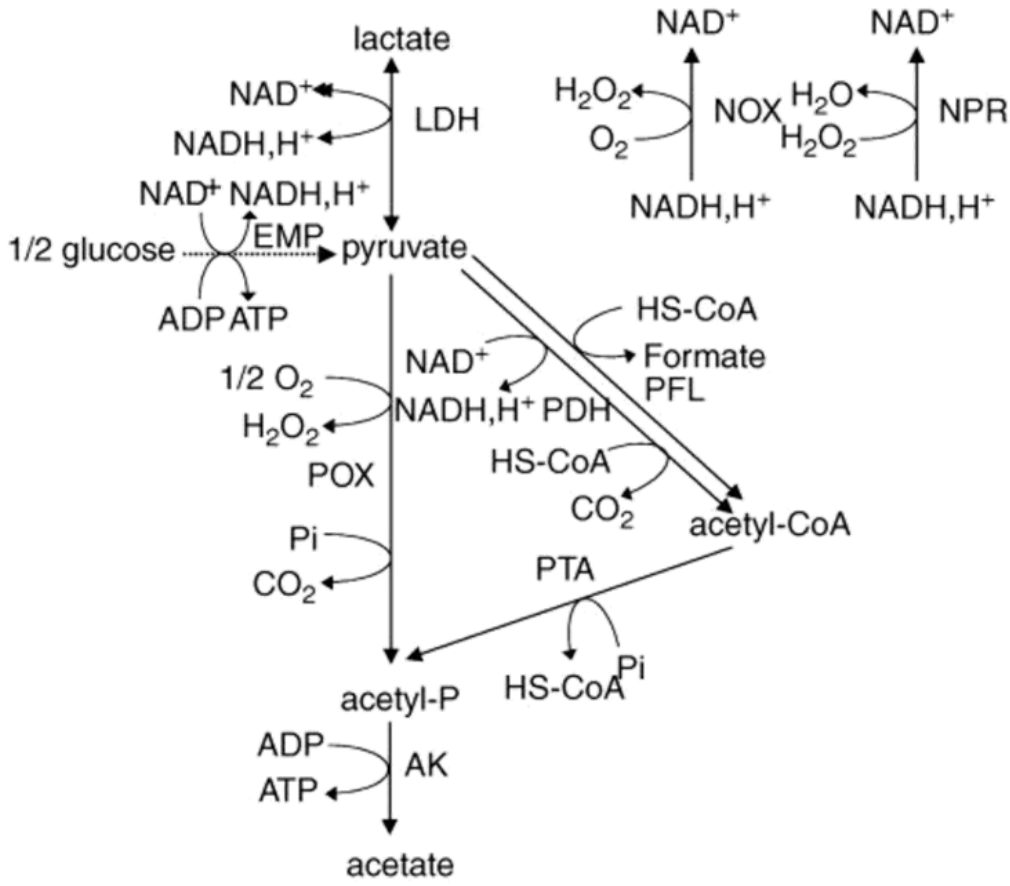

Fig.3 EMP pathway.

Majorly L. plantarum is isolated from environments which are rich in proteins like yogurt. It has peptide uptake system in it, peptidase enzyme degrade these peptides when they are ingested in the bacterium. . L. plantarum efficiently under stress environment like low pH, gastrointestinal tract, high salt conc.

. L. plantarum is considered as safe probiotic and is of major interest in research field. They reduces the negative impact of pathogen and diseases at a certain level in humans. Also it release some antimicrobial compounds such as Bacteriocin, which does not allow pathogenic bacteria to form colonies. In future this spp. Can be used to work as Vaccine Vehicle due its properties.

Chitosan is a natural linear polysaccharide, extracted from shells of shrimps. Chitosan is a product formed from deacetylation of chitin. It forms crosslinks with opposite ions and get polymerized in the presence of polyions (anions). This polymer is also known as weak polymer since it is not soluble in water and require a dilute acidic environment. Many researches has shown that chitosan as a good encapsulation agent (Shu and Zhu, 2000).

Sodium Tripolyphosphate: Sodium triphosphate (STP), likewise sodium Tripolyphosphate (STPP), or Tripolyphosphate (TPP) is an inorganic compound with recipe Na5P3O10. It is the sodium salt of the polyphosphate penta-anion, which is the conjugate base of triphosphoric corrosive. It is delivered on an expansive scale as a part of numerous household and modern items, particularly cleansers. Natural issues related with eutrophication are ascribed to it's across the board utilize**.**

RESEARCH METHODOLOGY

In this research, Ionotropic gelation method for encapsulation of probiotic is demonstrated. This method is discussed in detail in dispersion methods.

Formulation of probiotic: L. plantarum 2621 strain of bacteria is used for the probiotic formation. Initial culture of the strain is formulated in MRS broth. It is DeMan, Rogosa and Sharpe broth. This broth supports the growth of Lactobacillus from any source. MRS is creamish brown color granules which on dissolution give clear yellow color solution. Use of Biosafety cabinet is must for inoculation process. Here, three test tubes was taken and washed properly. Added 5-5ml distilled water in each test tube. MRS broth was weighed according to the molecular weight for 5ml. Added 0.27g of broth in each test tube separately. Autoclaving must be done at 121˚C for 20 min. After proper autoclaving do not open the tubes in open environment, keep them in laminar hood. After 1 hr. inoculate the strain of bacteria into each test tube inside laminar air flow/Biosafety cabinet. Cap up the tubes immediately after inoculation is done and wrap with silver foil. Incubation of the cultures is being done in incubator at 37˚C for 1-2 days. Transfer the cultures to freezing temperature at 4˚C after 2 days of incubation for the slow growth of cells. Otherwise, cell death will be there. Another option is to revive the culture after 4-5 days to have healthy cells. This solution formed is a probiotic formed from L. plantarum strain.

Preparation of glycerol stock for probiotics: Firstly autoclaving of cryo tube should be done which is a crucial step. Cryotubes should be filled in a beaker or flask. After sterilizing, 150µl of pure glycerol is added into the cryo tubes inside laminar air flow. Again put all the cryotubes having glycerol in beaker and cover the beaker properly and autoclave for 20min. After this these glycerol tubes should be preserved for one day at least. After 1 day probiotic freshly made was added into glycerol. Maintain the laminar air flow properly, 850µl of probiotics is added into 150µl of glycerol, which will be 15% glycerol stock. Mix them well 3-4 times. This stock can be preserved for 2 months at -20˚C without any fear of cell death.

ENCAPSULATION

Chitosan and probiotics beads: Chitosan is crosslinking agent. Chitosan used here is of Hi-Media having fiber like structure. Chitosan do not dissolves in water or alkaline solutions. It needs slightly acidic environment for its dissolution. Acetic acid is good for this, 2ml of 99% pure

glacial acetic acid is mixed with 1.2g of chitosan, makeup volume upto 50ml by adding distilled water. Place the beaker in magnetic stirrer for 1 hr. continuously. It majorly depends on the conc.of acetic acid used. After given time, there would be formation of white gel of chitosan. Place it in freezer for 1 day for proper mixing.

After a day take out chitosan gel from freezer 15min before using, 10ml of the gel is added to a new beaker. To this 10ml add glycerol stock with probiotics which was preserved earlier. Mix them well. Make Sodium Tri Poly Phosphate solution 50ml in a clean beaker. And put that solution in ice for 30 min.

Prepare the LAF, take out new disposable injection or syringe and suck chitosan+ probiotic sol.in it. Place the chilled beaker of TPP sol just below it with continuous stirring manually. Gradually, added drop wise chitosan + probiotic gel into TPP solution from a height 5-10cm. keep stirring the TPP solution so that no clumps will form. As soon as it falls on the TPP solution there was formation of microsphere. These microspheres were then filtered out from TPP sol. in whattman filter paper. Keep it for 2-4 min and change the filter paper 3-4 times to remove the traces of TPP sol. clean petriplate is taken having filter paper or brown paper and place the microspheres in it. Air dry them for 1-2 day and if needed dry in oven for 3hr at 37°C. There is formation of brownish yellow color beads, reduced in size. Store them in plastic zipper bag and further characterization is being done.

Project outcomes:

- A pH-responsive controlled release formulation containing probiotics.
- A formulation assuring safe delivery of probiotics directly into intestine.
- A formulation ensuring increased gut residence time and consequent enhanced benefits.
- Lowering of production costs of fermented probiotic products.
- This would lead to patents in which companies like Yakult, Chrysan-Hansen and Nestle would be very much interested.
-

Proposed work plan

Probiotics release in the target host should be in proper viable form. Initially probiotic formulation have been performed by taking L. plantarum. Prepared glycerol stocks the cells cultured and used as probiotic in further research. Encapsulation is done to protect it from harsh and unsuited environment in the stomach. The polymer chitosan could be used for the preparation of polyelectrolyte complex and to encapsulate probiotics. Tri polyphosphate (TPP) is used as cross linker having opposite charge than chitosan.

Freshly prepared 10 ml of 1.5% chitosan was gently mixed into glacial acetic acid (1-2% Acetic acid is sufficient to provide acidity to the polymer). This polymer solution is mixed with probiotics. The polymer solution has to be dropped by a sterilized syringe with or without needle. TPP solution contained in the glass beaker is placed just below the syringe. Polymer solution taken in syringe has to be dropped from a height into the TPP cross linker, with continuous stirring. As the polymeric solution with polycations comes in contact with each other, hydrogel beads begin to form. These are the encapsulated probiotics inside chitosan coating.

After the formation of microspheres, it is washed with distilled water and filtered to remove the cross-linking agent. Filtrate obtained then air dried for 6-8hours and if needed oven dried for 3 hours at 37ºC, remove all the moisture. Fully dried microspheres are then sealed and can be stored in plastic pouch.

Further experiments can be done to check the viability of microencapsulate probiotics in different pH conditions, temperature and salt conditions.

RESULTS AND DISCUSSIONS

Formulation of probiotic was the first priority. Because it's better to use fresh probiotic to study their properties. As we can check every aspect from day one .probiotic formation from L. plantarum was done in aseptic conditions. Results came was very much desirable and ready to use. MRS broth (autoclaved) was inoculated with L. plantarum in Biosafety cabinet. On the time of inoculation the color of inoculated broth was plain yellow. Incubation was done for 2 days. After 2 days, probiotic cells presence was clearly shown by the change of plain yellow color culture to turbid yellow after one day incubation.

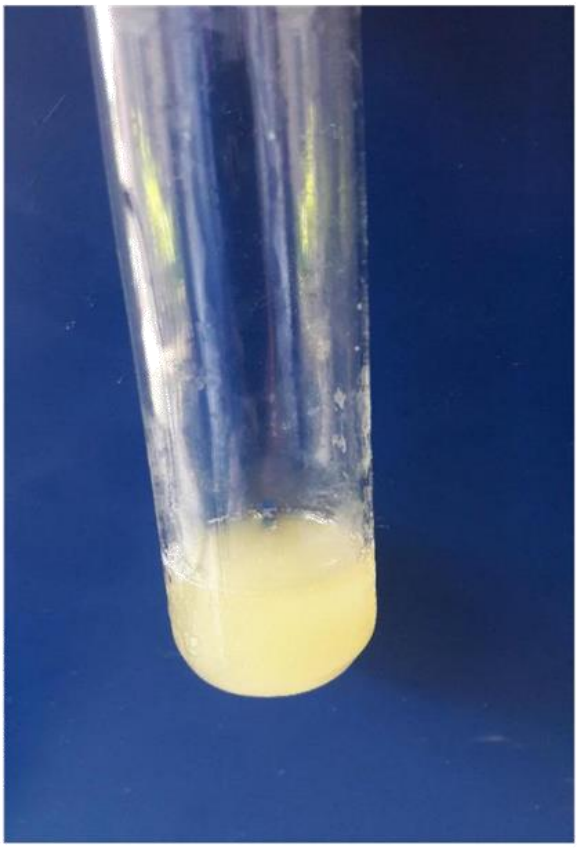

Fig4 Turbidity due to proliferation of cells.

Turbidity also indicated that now the raw probiotic is formed. From this grown and proliferating MRS broth with agar is also made in petriplates. Total 50 ml of mixture (autoclaved) is made of

MRS broth and Agar-Agar and poured into two petriplates. After they fully solidified, streaking was done on the petriplates with freshly grown probiotic cells. Incubation was done for 2 days at 37℃. After 2 days there were white colonies formation in the petriplates (fig.4). Keep them in -20℃, there would be overgrowth of bacteria which will lead to mutations in them.

This is our preserved culture for future if there will be need for it.

Figure 5. L. plantarum colonies on MRS with agar petriplate..

Glycerol stocks were made from probiotics live cells in MRS broth cultures. Glycerol stocks of the cells are made to preserve cells for longer duration. There is water in the cells which is very smartly replaced by glycerol. This causes very less ice crystals formation as compared to water. These cells can remain viable and there was no or least cell death after 20 day in -20°C, due to inactive metabolism of cells

Chitosan microspheres/ beads were made by encapsulating probiotic cells inside. This was very successful, there were formation of proper beads having cells inside it. Beads or microspheres

formed were initially white in color and soft. After air dried in next day white colored beads get changed into brownish yellow in color. Size of the beads also get reduced into microns. This was done by injecting chitosan mixed with probiotics into chilled anhydrous sodium Tri Polyphosphate solution. Web like crosslinking of chitosan and TPP sol. does not allow probiotics to come out and it remain encapsulated within. Thus it can be checked for controlled release in gastrointestinal tract and acidic environments.

(A)

(B)

Fig.6 (A) shows freshly made beads of encapsulated probiotics before drying. (B) beads after dried.

Gram staining:

Gram staining is normally done to distinguish or analyze between different types of bacteria. It shows different color intakes for gram positive and negative type of bacteria. Gram positive cells will purple color due to crystal violet and gram negative shows pink color due to safranin. Here, gram staining is being to observe the viability and type of bacteria. There was clear observation of pink colored micro-organisms under light microscope at 100x. By this it is clearly detected that the cells are **gram negative** type with **rod shape** structure. And the Viability of cells was approx... 90% after 2 days of culture.

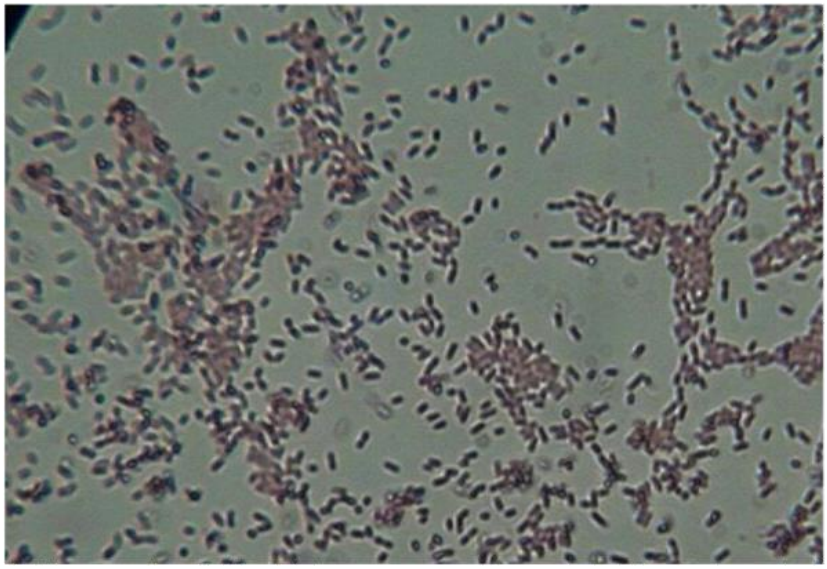

Fig.7. Shows presence of healthy gram negative bacteria using light microscope..

CONCLUSION

From all studies, this is clear that probiotics provides several health benefits to organisms. Humans have natural flora to get rid of diseases. Sometimes these flora get destroyed by some external effects such as low pH etc., so the probiotics plays the role of natural flora to fight against diseases. Every probiotic has its own mechanism of action and rate of survivability. It is essential to protect these probiotics from harsh conditions. In this research, there is formulation of probiotic and its encapsulation till now. Results obtained are reliable for future work. Another part will be its optimization and viability of cells at every harsh condition. This will ensure the effect of probiotic if it should ready to use for humans or need more variations.

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APPENDIX

EQUIPMENTS USED:

- Autoclave
- Laminar Air Flow
- Weighing balance
- Magnetic stirrer
- BOD incubator
- Low temp. Freezer
- Hot air oven
- Microwave
- Microscope
- Syringe

CHEMICALS USED

- Chitosan
- Sodium Tripolyphosphate
- Glacial Acetic acid

MEDIA USED

- MRS broth

STRAIN USED

- *Lactobacillus plantarum*

TOPIC APPROVAL PERFORMA

School of Bio Engineering and Bio Sciences
Program :P260-H::M.Sc. (Hons.) (Biotechnology)

COURSE CODE : BTY797 **REGULAR/BACKLOG :** Regular **GROUP NUMBER :** BSRGD0180

Supervisor Name : Dr. Jibanananda Mishra **UID :** 21039 **Designation :** Associate Professor

Qualification : _____ **Research Experience :** _____

SR.NO.	NAME OF STUDENT	REGISTRATION NO	BATCH	SECTION	CONTACT NUMBER
1	Manisha sharma	11613986	2016	B1617	9779577175

SPECIALIZATION AREA : Zoology **Supervisor Signature:** _____

PROPOSED TOPIC : Optimization of formulation parameters to achieve controlled release of probiotics

Qualitative Assessment of Proposed Topic by PAC		
Sr.No.	**Parameter**	**Rating (out of 1**
1	Project Novelty: Potential of the project to create new knowledge	6.50
2	Project Feasibility: Project can be timely carried out in-house with low-cost and available resources in the University by the students.	7.00
3	Project Academic Inputs: Project topic is relevant and makes extensive use of academic inputs in UG program and serves as a culminating effort for core study area of the degree program.	6.50
4	Project Supervision: Project supervisor's is technically competent to guide students, resolve any issues, and impart necessary skills.	7.00
5	Social Applicability: Project work intends to solve a practical problem.	7.00
6	Future Scope: Project has potential to become basis of future research work, publication or patent.	6.50

PAC Co mmittee Members		
PAC Member 1 Name: Dr. Ashish Vyas	UID: 12386	Recommended (Y/N): Yes
PAC Member 2 Name: Himanshu Singh	UID: 11691	Recommended (Y/N): NA
PAC Member 3 Name: Dr. Joydeep Dutta	UID: 14336	Recommended (Y/N): NA
PAC Member 4 Name: Dr. Umesh Goutam	UID: 14691	Recommended (Y/N): NA
DAA Nominee Name: Mamta Sharma	UID: 18431	Recommended (Y/N): NA

Final Topic Approved by PAC: **Optimization of formulation parameters to achieve controlled release of probiotics**

Overall Remarks: Approved

PAC CHAIRPERSON Name: 11840::Dr. Neeta Raj Sharma **Approval Date:** 08 Apr 2017

11/29/2017 5:17:26 PM