

DEVELOPMENT OF PROBIOTIC CHOCOLATE FROM BAEI

Thesis submitted to Department of Life Science for the fulfilment of M.Sc Degree
in
Life Science

SWAPNA SONALI PANDA

ROLL NO: 412LS2051

UNDER THE GUIDANCE OF

Dr. RASU JAYABALAN



**DEPARTMENT OF LIFE SCIENCE
NATIONAL INSTITUTE OF TECHNOLOGY
ROURKELA 769008
ODISHA**

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Research is to see what everybody has seen but to think what nobody has thought. To go beyond the traditional way of thinking and utilizing age old techniques for creation of something new has been an amazing journey. I owe my gratitude to a number of people who have illuminated my path on this journey and helped me reach my destination.

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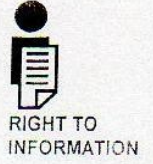
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राष्ट्रीय प्रौद्योगिकी संस्थान
NATIONAL INSTITUTE OF TECHNOLOGY
राउरकेला ROURKELA - 769008, ओडिशा ODISHA



Dr. R. Jayabalan
Assistant Professor
Department of Life Science

Date: 09.05.2014

CERTIFICATE

This is to certify that the thesis entitled “**DEVELOPMENT OF PROBIOTIC CHOCOLATE FROM BAEL**” which is being submitted by **Ms. Swapna Soanli Panda**, Roll No. **412LS2051**, for the degree of Masters 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 institution for the award of any degree or diploma.

R. Jayabalan
09/05/2014
R. Jayabalan.

Declaration

I hereby declare that the thesis entitled "**Development of probiotic chocolate from bael**", submitted to the Department of Life Science, National Institute of Technology, Rourkela for the partial fulfilment 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, Department of Life Science, NIT, Rourkela. No part of this thesis has been submitted by any other research persons or any students.

Date:

Place: Rourkela

Swapna Sonali Panda

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List of Abbreviations

Gm	Gram
Hr	Hour
L	Litre
MI	Micro litre
°	Degree
C	Centigrade
MI	Mili litre
Min	Minute
Ppm	Parts per million
%	Percentage
MRSA	de Man Rogosa Sharpe
<i>Spp</i>	Species
Psi	Pressure per sq. inch
Mm	Mili meter
No.	Number

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ABSTRACT

A functional food is a food that has been added with new ingredients or the present ingredients have be processed in a manner that it provides additional health benefits than the conventional form. *Aegle marmelos*, commonly called as bael is a plant of the Rutaceae family and is known since ancient times on account of its medicinal properties. The leaves, bark, fruits and flowers are edible and confers different health benefits. The fruits of the plant are widely consumed and its pulp has a sweet, aromatic flavor and imparts a characteristic taste when consumed. The parts of the plant are often found to be ingredients of many ayurvedic medicines and are also used locally in certain illness such as diarrhea, constipation, gastro intestinal disorders, irritable bowel syndrome etc. Most of the beneficial properties of bael are derived from the pulp of the fruit. Probiotics on the other hand refers to live microbes, which when consumed in adequate amounts confers some health benefits. A number of beneficial effects have been attributed to the use of probiotics. In order to provide the advantageous effects of both bael and probiotics, a product is formulated that contains both bael fruit extracts and probiotics, which forms a functional food and when consumed will provide the benefits of both.

1. INTRODUCTION

1.1 FUNCTIONAL FOODS

Due to the increase in population around the globe, the demand for food and its availability to satisfy the needs of human is increasing day by day. The United Nations Food and Agriculture Organization estimated that nearly 870 million people of the 7.1 billion people around the world are suffering from chronic undernourishment. Fifteen percent of these undernourished people are from developing countries (FAO, 2012). In such a scenario where the availability of quality food is becoming scarce each day, the need for food with improvised qualities seems to be a solution. Functional food fits the picture since functional foods are the kind of food that provides benefits beyond basic nutrition. Examples of functional foods include fortified foods, dairy supplements and whole grains. Due to developments in food industries, many traditional food items are now being modified to include beneficial components that will provide advantages beyond simple nourishment. The alteration of traditional foods also makes it easily acceptable by people than introduction of a new product.

1.2 BAEL

Aegle marmelos is commonly called as bael, or bel or Bengal quince or wood apple. The tree bears its origin in Western Ghats of India and is found in tropical and sub-tropical regions. The tree belongs to the Rutaceae family, the family to which citrus fruits belong. The tree holds a sacred value among Hindus and is often worshipped or its leaves are presented to the deities. The bael tree is also found in many South East Asian countries including Pakistan, Sri Lanka, Nepal, Myanmar, Bangladesh, Vietnam, Cambodia Thailand, Malaysia, Java, Philippines and Fiji. The trees are of great importance to the environment as they act as climatic purifier that is they

release greater percentage of oxygen in comparison to other trees. They also act as a sink for chemical pollutants as it absorbs toxic gases from the atmosphere and make them inert or neutral (Sharma et al., 2006). The plant bears sweet scented white flowers that are sometimes used in making perfumes (Encyclopedia Britannica, 2013). The tree is mostly valued for its fruit, which is pear shaped and the size ranges from 5-25 cm (2-10 inches) in diameter. The fruit has a hard, woody outer shell and inside is present a sweet, thick and aromatic pulp. In the pulp, the seeds are present in ridges and each seed is surrounded by thick slimy and transparent mucilage. The fruit is eaten as a delicacy when it's ripe, either by cutting into pieces or the pulp is mixed with milk, water and sugar and make into a sweet sherbet. Bael tree is known extensively for its medicinal values. Various parts of the tree such as leaves, fruit, bark and seeds are a constituent of many ayurvedic medicines. Apart from being used in medicines, they are also used traditionally in many ailments. Different parts of the plants contain hypoglycemic, hypolipidemic and blood pressure lowering factors (Lmbole et al., 2010). The pulp contains laxative properties and is even considered as the best laxatives known so far. The parts of plants are used in case of gastrointestinal related problems such as diarrhea, dysentery and diabetes. It has antibacterial and anti fungal properties. Bael is known to have anti cancer activity, pyretic and analgesic activities and also provides relief in constipation (Sharma et al., 2006). The pulp of the fruit is a natural source of natural antioxidants and bioactive compounds.

1.3 PROBIOTICS

The term “probiotics” is derived from two Greek words, “pro” means “for” and “bios” means “life” Probiotics refer to live microorganisms which when administered in adequate amounts, confer a health benefit on its host. The term Probiotics was coined in 1965 by Lilly and Stillwell, who defined them as “microbially derived factors that stimulate growth of other organisms”

(Guarner et al., 2008) Probiotics can be made available by formulating it into many different products such as food, drug and dietary supplements. The most commonly used probiotics include *Lactobacillus* and *Bifidobacterium.*, but microbes such as *Sacchaomyces cerevisiae* and some *E. coli* and *Bacillus* are also used as probiotics (Binns, 2013) Health benefits that have been attributed to probiotics include antimutagenic, anticarcinogenic, anti infection properties, immune system stimulation, serum cholesterol reduction, alleviation of lactose intolerance and nutritional enhancement (Mortazavian et al., 2007). Other health benefits of probiotics include improved balance of microbial flora of intestine, curing diarrhea and constipation and synthesis of vitamins (Mansouripour et al., 2013). The probiotics are non pathogenic and hence there is no fear of any sort of diseases from them.

1.4 PROBIOTIC PRODUCTS

Due to the numerous positive effects of probiotics on human and animal health, a number of probiotic products are now available in market. Probiotic products can either be available as dairy based products or non dairy based (Miller et al., 1998)

1. Dairy based probiotic products: Dairy based probiotic products includes those that are developed by fermenting milk with probiotics. These products include

- Yoghurt (spoonable, drinkable and shots)
- Dahi, kefir
- Cheese (long storage)
- Probiotic ice cream
- Probiotic margarine

2. Non-dairy based probiotic products: Non dairy based probiotic products include those that are derived from sources other than milk. They include

- Fruit and berry juices (non fermented)
- Fermented vegetable juices (tomato, carrot juice)
- Yoghurt (oat yoghurt)
- Probiotic olives
- Probiotic salami
- Probiotic bread (*Lactobacillus* used in traditional sour dough bread)
- Nutrition bars
- Probiotic chocolate

1.5 PROBIOTIC CHOCOLATE

As mentioned earlier, probiotics can be made available in the form of food, drug or dietary supplements. The concept of probiotics chocolate is not totally new. Many companies have claimed to produce probiotics chocolate with all the goodness of chocolate and benefits of probiotics. But development of a product with fruit extract that is added with probiotics is relatively new. Adding a layer of chocolate to the thickened fruit extract ensures its protection from spoilage creating organism since chocolate is not affected by spoilage creating microbes. Also the chocolate adds to the organoleptic property of the product.

2. REVIEW OF LITERATURE

2.1 BAEL

2.1.1 Systemic classification

Kingdom: Plantae

Subkingdom: Tracheobionta

Superdivision: Spermatophyta

Division: Magnoliophyta

Class: Magnoliopsida

Sub-class: Rosidae

Order: Sapnidales

Family: Rutaceae

Genus: *Aegle*

Species: *marmelos*

2.1.2 Bael tree

Bael tree is one of the most used medicinal trees that are found in India. Apart from being used for its medicinal properties, it also holds religious values for Hindus who consider this tree as sacred. Bael belongs to the family Rutaceae, the family to which citrus fruit belongs. Fig. 1 shows the photograph of a bael tree. The tree is slow growing and may reach a height of 40-50 feet. Tree has a short trunk and the bark is thick, soft and flaking. When wounded, the branches give out a clear gummy sap which hangs down like strands but eventually solidifies. The tree

bears alternate leaves that are in 2's and 3's. The branches sometime bear spines and the fruit is often drooping. The inflorescence of contains a bunch of fragrant flowers that vary from 4 to 7 in number (Chakraborty et al., 2012)



Fig.1: Bael tree

2.1.3 Bael fruit

Bael plant is mostly prized for its fruit. The fruit is a pyriform, may vary from oval to round, and size varies from 10-20 cm in diameter. The fruit has a hard, woody exocarp and inside it is a thick, fleshy and aromatic, slightly sweet pulp. The color of the pulp may vary from bright orange to sunset yellow and the pulp contains seeds that are present in grooves and is surrounded

by thick, clear mucilage. Both ripe and unripe fruits are used for their medicinal values. A number of phytochemicals are present in bael that makes it useful in many ailments. Fig.2 shows a bael fruit.



Fig.2: Bael fruit

2.1.4 Chemical constituents of Bael fruit

Bael gets its medicinal values on basis of the various chemicals present in it like alkaloids, coumarins, polysaccharides, essential oils etc.

- **Coumarins:** The coumarins present in Bael fruit includes marmelosin, marmesin, imperatorin, marmin, alloimperatorin, methyl ether, xanthotoxol, scoparone, scopoletin, umbeliferone, marmelide and marmenol (Sharma et al., 2006)
- **Alkaloids:** Aegelin, aegelinine, fragine, o-methylhalfordinine, o-isopentanylhalfordinol, N-2-[4-(3',3'-dimethylallyloxy)phenyl]ethyl cinnamide, o-(3,3-dimethylallyl) halofodinol, Ethyl cinnamide (Sharma et al., 2006)
- **Phenylpropenoids:** Hydroxylcoumarins, phenylpropenes, lignans (Chakraborty et al., 2012)
- **Polysaccharides:** Galactose, arabinose, uronic acid, L- rhamanose (Sharma et al., 2006)

- **Seed oils:** Palmitic acid, stearic acid, oleic acid, linoleic acid and linolenic acid (Sharma et al., 2006)
- **Tannis:** Highest percentage of tannins recorded in bael fruit is 9% and was recorded (Chakraborty et al., 2012)
- **Carotenoids:** Imparts color to the fruit pulp (Sharma et al., 2006)
- **Minor constituents:** Ascorbic acid, sitosterol, crude fibers, α -amyrin, crude proteins (Farooq S, 2005)
- **Proximate constituents:** Table 1 shows the proximate constituents of Green and Ripe Bael (Islam et al., 2011)

Table 1: Proximate analysis of Green and Ripe Bael powder (%)

Powder	Moisture	Ash	Vitamin C	Protein	Fat	Carbohydrate
Green Bael	2.64	0.64	56.99	1.75	0.67	93.73
Ripe Bael	4.04	1.10	57.09	3.75	1.24	90.33

- **Minerals:** Table 2 shows the mineral content of Green and Ripe Bael powder (Islam et al., 2011)

Table 2: Mineral content of Green and Ripe Bael powder (ppm)

Powder	Na	K	Ca	Mg	Zn	Cu	Fe
Green Bael	55.6	1356	78.9	142	0.66	0.67	19.3
Ripe Bael	119	4821	92.9	259	1.69	1.34	16.22

2.1.5: Therapeutic value of Bael

1. **Diarrhea and dysentery:** In case of chronic diarrhea and dysentery without fever, half ripe or unripe fruit acts as a remedy. Half ripe fruit is considered best for the purpose but fully ripe fruits or even fruit powder has shown effective results. When the fruit is still unripe, it is cut, dried and ground into powder. The unripe fruit can also be consumed by baking and then consumption with brown sugar or jaggery. After use of fruit, the amount of blood passed in the fecal matter reduces and the fecal matter gets a more solid form. (Sharma et al., 2006; Patel et al., 2012)
2. **Antiulcer activity:** Gastrouduoenal ulcer is a general disorder of the gastrointestinal tract. Many plants have shown effect in reducing ulcer like neem and turmeric. Bael is known to show gastroprotective activity. Unripe bael fruit extract serves the purpose. When used in rats, it produces a noteworthy inhibition of absolute ethanol induced gastric mucosal damage. This activity is shown due to the presence of a particular compound in the fruit, called, Luvanetin. Gastric ulcer is usually mediated by progress of oxidative stress. This compound, luvanetin might act by inhibition of oxidative stress producing compounds in the gastrointestinal tract thus preventing ulcer formation (Maity et al., 2009)
3. **Antidiabetic activity:** Diabetes has become a common disease around the world. When the body cannot produce ample of insulin, the blood glucose level increases. Antidiabetics aim at reducing the blood glucose level by inducing the production of a higher amount of insulin. Bael extract, when administered at a dose of 250 mg/kg of body weight, shows better result than glycenamide (antidiabetic drug). This antidiabetic effect may be due to the coumarins present in the fruit which induce the beta cells of

islet of Langerhans to produce insulin. Aqueous extract of bael seeds reduces blood glucose level in case of severe diabetic patients (Maity et al., 2009;Kamalakkannan and Prince., 2003)

4. **Antihyperlipidemic activity:** Increase in the concentration of cholesterol, triglycerides and fatty acids in blood causes atherosclerosis and thickening of walls of arteries which may eventually cause ischemic heart disease, coronary heart disease, myocardial infarction and cerebro vascular accidents. Although a number of drugs are available to reduce the lipid concentration of blood, but their use is limited due to significant side effects. Oral administration of aqueous extract of bael fruits and seeds separately at a dose of 250 mg/Kg of body weight to diabetes induced rats has shown significant decrease in the blood lipid level. The effect may be due to fat mobilization from deposits which is caused due to hydrolysis of triglycerides. The extract also increases glucose utilization. (Maity et al., 2009; Kamalakkannan and Prince, 2003; Kesari et al., 2006)
5. **Antioxidant activity:** Normal metabolic activities give rise to free radicals. These free radicals, mainly oxygen free radicals, referred as ROS (Reactive Oxygen Species) causes oxidative stress. ROS are harmful for the body as they damage macromolecules, DNA, proteins and lipids. Antioxidants are compounds that scavenge the free radicals and reduce oxidative stress. bael fruit has proven to show antioxidant activity. On administration of Bael fruit extract of 250 mg/kg of body weight, the activity of ROS scavengers such as glutathione peroxidase, glutathione reductase, superoxide dismutase (SOD) and catalase is shown to increase considerably. Use of above mentioned dose of Bael fruit extract shows better results than glibenclamide (36 µg/kg). The antioxidant

activity may be due to presence of flavonoids, alkaloids, sterols, tannins, phlobatannins and flavonoid glycosides (Kamalakkannan and Prince., 2003;Singh et al., 2000)

6. **Anticancer activity:** Cancer is one of the most dangerous diseases mainly because there is no complete treatment for it. Also the treatments that are available for cuing the symptoms have lots of side effects, and are also not cost effective. Hence search is going on to make available treatments of natural origin that will be cost effective and will show minimal side effects. Bael extract has been found successful in inhibition of *in vitro* proliferation of human tumor cell lines including Lecukenic K562, T-Lymphoid Jurat, Beta-Lymphoid Raji, Erythro Leukemic HEL (Lampronti et al, 2003)
7. **Antibacterial activity:** Bael extract has been found to have antibacterial properties. Its extract is effective against a number of pathogenic species such as *E.coli*, *Pseudomonas salanacearum*, *Aeromonas spp.* and *Xanthomonas vesicatoria*. Methanol extracts of Bael fruit is also effective against multidrug resistant *Salmonella typhi*. Seed extracts are effective against *Salmonella typhi*, *Salmonella paratyphi*, *Proteus vulgaris*, *Streptococcus fecalis*, *Vibro cholera*, *Pseudomonas aeuriginosa*, *Bacillus subtilis* and *Neisseria gonorrhoea*.(Rusia and Srivastava., 1988)
8. **Antifungal activity:** Extract of seeds of Bael is effective against fungus such as *Trichophyton rubrum*, *T. terrestrese*, *Epidermophyton floccosum*, *Aspergillus niger*, *A. flavus* and *Aspergillus fumigatus* (Pitre and Srivastava., 1987)
9. **Constipation:** Ripe fruit has been considered as the best of all known laxatives. In case of constipation, administration of ripe fruits cleans and tones up the intestines. Its regular use for 2-3 months has been effective in removal of even old and accumulated fecal matter from bowels. For best results, the pulp of ripe fruit is crushed and made into a

sherbet. Seeds are removed for reducing the bitterness and sugar and/or milk can be added to make it more palatable (Roy and Singh., 1980)

10. Radioprotective effect: Today cancer treatment includes radiation therapy that often results in many side effects and toxicity. Radiation causes a slow alteration in genome base pair, may cause esophagitis in lung cancer, acute mucositis and pharyngitis in case of neck and lung cancer. Due to many such profound effects of radiation therapy, its use is highly restricted unless utterly necessary. Hydroalcoholic extract of Bael fruits have been studied for their radioprotective effects. Its effects have been proven in mice that were exposed to varying degrees of gamma radiation. Use of 20 mg/kg of bodyweight of hydroalcoholic extract of bael fruit for 5 consecutive days before irradiation of gamma rays was seen to provide maximum protection. This action of bael may be due to lipid peroxidation along with elevation in GSH concentration in liver, kidneys, stomach and intestines of mice (Jagetia et al., 2004)

2.1.6. Products from Bael fruits

Bael fruit is available in many forms. These include raw and ripe whole fruits, fruit powder, fruit extract, fruit extract powder, bael seeds, dried unripe fruit slices, ripe fruit drink, bael fruit tea (either entirely made of bael or a blend of bael fruit and other ingredients such as ginger or lemon or others), bael fruit juice, fruit juice jelly and jam.

2.2 PROBIOTICS

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”. Though probiotics is a fancy term now a days, but humans have been using probiotics unknowingly since ages. For example, it is a well known fact that consumption of yoghurt or dahi provides a positive impact on the digestive system.

2.2.1 Mode of action of probiotics

The action of probiotics is mainly due to its colonizing and pH alteration activities in the gut. The activity of probiotics also depends on the strain and amount of probiotics consumed. Some mode of action of probiotics is listed below.

1. **Prevention of growth of pathogenic bacteria:** The normal microflora present in the intestine helps to keep an individual healthy by prevention of growth of pathogenic bacteria. But in case of some intestinal disorder, or after administration of high antibiotics, the normal microflora of intestines decreases in number. On administration of probiotics, these organisms go and colonize on the intestinal walls. Pathogenic microbes also need to colonize on the intestinal walls in order show their effect. Since they colonize on the surface of intestines, they compete with pathogenic microbes for adhesion. Along with this, they also help in restoration of normal microflora of the host (Guarner et al., 2008). Fig. 3 shows the effect of probiotics on the intestinal cells that prevent growth of pathogenic bacteria. This action might also be due to production of

acids (like lactic acid by *Lactobacillus* spp.) that creates acidic environment and prevents growth of harmful microbes.

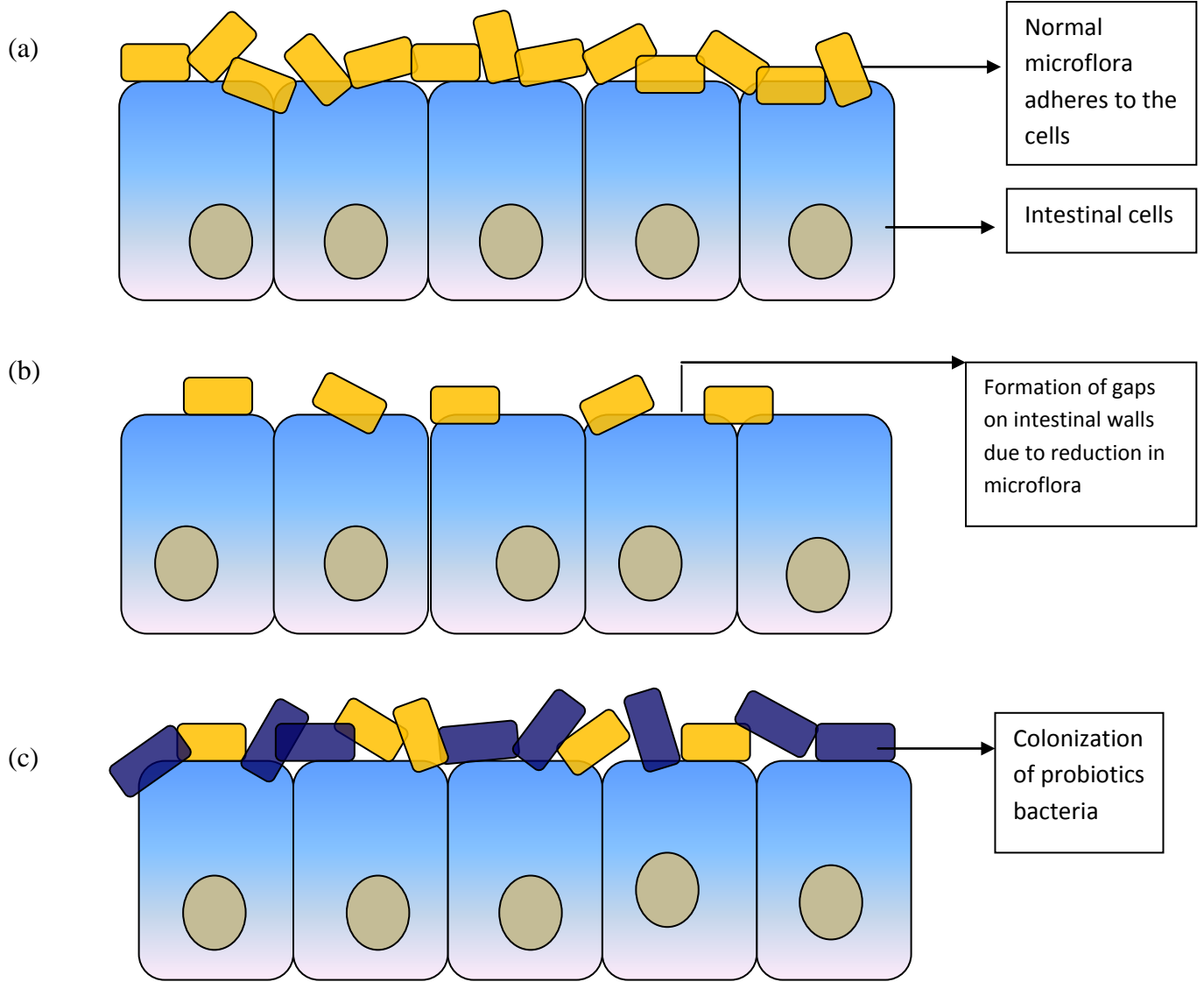


Fig.3: Role of probiotics bacteria in inhibition of growth of pathogenic bacteria. (a) Normal microflora (yellow) covers the surface of intestinal cells. (b) after gastrointestinal problem or administration of antibiotics, there is reduction in number of normal microflora and the intestinal walls have space for growth of pathogenic microbes (c) consumption of probiotics(blue) causes their colonization on intestinal walls leaving minimal space for pathogenic microbes.

2. **Immunologic effect:** The intestine is lined by lymphoid tissue. Allergy is initiated by interaction of food components with the intestinal mucosa, which identifies allergens and initiates an immunologic response. The immunologic benefits provided by probiotics such as prevention of allergies is due to activation of macrophages that increase the antigen presentation to B lymphocytes and increases secretion of Immunoglobulin A. Another reason may be that probiotics alter cytokine profiles and they induce hyposensitiveness to food allergens (Guarner et al., 2008; Falk et al., 1998)

3. **Alteration of colonic motility:** Gastrointestinal problems such as constipation are directly related to movement of intestinal digesta. If the movement of digested matter is not proper in the intestines, it leads to intestinal discomfort and constipation. Use of probiotics provides relief in such conditions, which might be due to stimulation of fermentation in large intestine (Ohashi and Ushida., 2009)

2.2.2 Proven beneficial effects of probiotics Fig.4 summarizes the clinically proven effects of probiotics

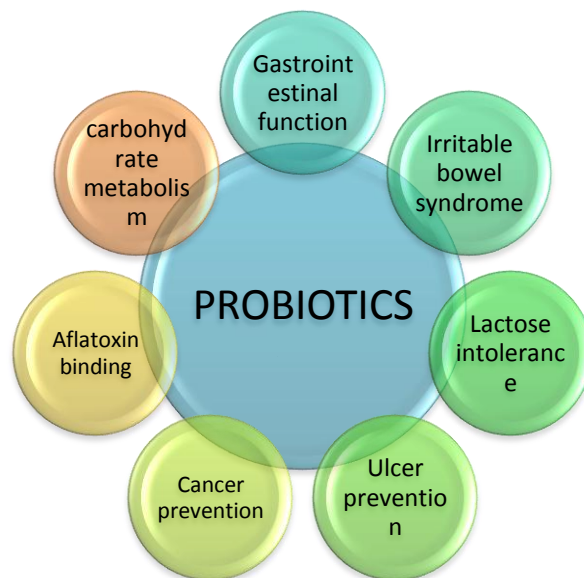


Fig.4. Diagrammatic representation of proven clinical benefits of probiotics

1. Gastrointestinal function: Probiotics have proven to be effective in two types of diarrhea, antibiotic diarrhea and traveler's diarrhea. *Clostridium difficile* is known to be causative agent of diarrhea after antibiotic treatment. This is due to colonization of *C. difficile* on the intestinal walls and production of toxin that causes the diarrhea. Oral administration of probiotics following antibiotics have proven to be effective in checking antibiotic induced diarrhea (Brown et al., 2000)

2. Chronic inflammatory gut condition: A group of inflammatory disorder of gut, collectively called as "Irritable Bowel Syndrome" is accompanied by abdominal pain, microscopic inflammation, visceral hypersensitivity, bloating and altered bowel habits. Probiotics help in overcoming various aspects of IBS in many ways, such as preventing the growth of pathogenic bacteria by producing bacteriocins, reducing lactose intolerance, modulation of intestinal microenvironment, suppression of inflammation by reducing the TNF α (Tumor Necrosis Factor α) and stimulation of growth of normal microflora (Bixquert, 2013)

3. Reduction of lactose intolerance: Hypolactasia or otherwise called as Lactose Intolerance is a medical condition found in almost 70% population around the globe, though most of it goes unnoticed, yet it is very prevalent in American and European ethnic groups. Lactose is a disaccharide made up of a glucose molecule and a galactose molecule. The enzyme β -galactosidase/lactase breaks lactose to give the two monosaccharides. . Lactose intolerance is characterized by inability of an individual to use lactose due to hyopsecretion of β -galactosidase/lactase enzyme. As a result people with this syndrome cannot consume milk and other dairy products. For lactose intolerance to be asymptomatic, correct functioning of only 50% of lactase is enough. Fig.5 represents the action of lactase enzyme on lactose to produce glucose and galactose.

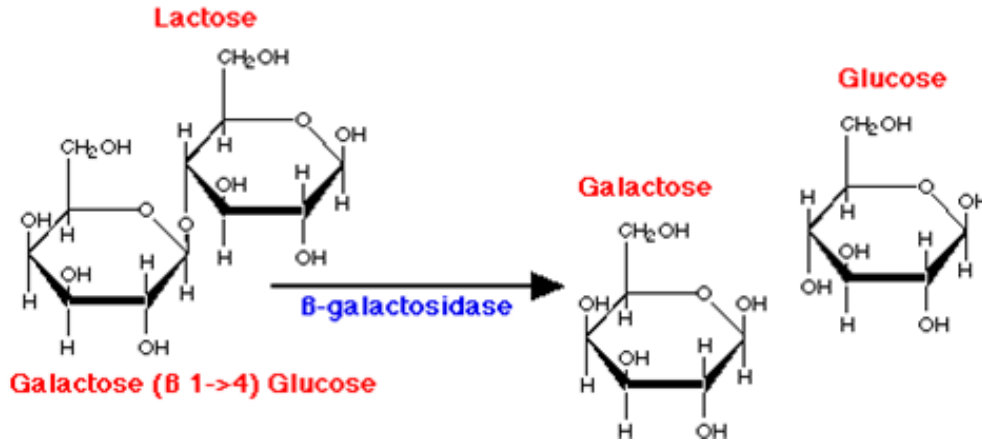


Fig.5: Action of lactase on enzyme on lactose to produce a molecule each of glucose and galactose.

Probiotics help to overcome lactose intolerance by fermenting lactose to give lactate, hydrogen, methane, carbon dioxide and short chain fatty acids. The lactase enzyme contained in probiotics breaks down lactose by hydrolysis to produce glucose and galactose, which later can either be absorbed or fermented (Lomer et al., 2007)

4. Prevention of ulcers: Ulcers that are induced by *Helicobacter pylori* are prevented by consumption of probiotics. This action is due to the colonization of probiotics on intestinal mucosa, thus unavailability of space for *H. pylori* to adhere.

5. Prevention of cancer: Though the mechanism is not clear, but it has observed that probiotics are successful in preventing colon and rectum cancer. But it has been estimated that this action might be due to inactivation of carcinogenic compounds, contest with pathogenic microbes, boosting host's immune system, regulation of apoptosis, fermentation of undigested food and inhibition of tyrosin kinase signaling pathway (Uccello et al., 2012)

6. Aflatoxin binding: Fermentation is a process of preservation of food items. Probiotics, especially Lactic Acid Bacteria have been reported to inhibit growth of molds and aflatoxin biosynthesis due to production of lactic acid and lactic acid metabolites. Probiotics have the ability to bind aflatoxin to their surfaces and then degrade them (Gratz, 2007)

7. Carbohydrate metabolism: Our diet contains a number of complex and simple compounds. The simple compounds are easily broken down by the organism while the complex food components are difficult to assimilate. Like in case of carbohydrate uptake, mammals lack the necessary machinery to degrade complex polysaccharides such as starch, glycans, xylans etc. these complex polysaccharides move to the large intestine that is colonized by normal microflora. Synergistic action of normal microflora and probiotics break these complex molecules into simpler ones that are then readily absorbed (Resta, 2009)

2.2.3 Encapsulation of bacteria

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 mouth 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 (Krasaekoopt et al., 2003)

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 (Champagne et al., 1994)

2.2.3 *Lactobacillus sporogenes*

The probiotic organism used in this project was *Lactobacillus sporogenes*, available by the commercial brand name of “SPORLAC”

2.2.3.1. Systemic classification:-

Kingdom: Bacteria

Phylum: Firmicutes

Class: Bacilli

Order: Bacillicales

Genus: Bacillus

Species: *coagulans*

Bacillus coagulans is usually marketed by the name of *L. Sporogenes* because of its spore forming ability. It is Gram Positive, spore forming and lactic acid producing bacillus which was first discovered in 1933. *L. sporogenes* has got the GRAS (Generally Recognized As Safe) status and is used as a probiotic. The brand name for *L. sporogenes* is “sporlac”, which is available in powder and tablet form. This probiotic is orally administered which passes into the stomach in spore form. Upon reaching the duodenum it becomes active and starts to multiply. *L. sporogenes* is not a permanent resident of the gut. It is removed slowly through fecal matter, after 7 days of dosage (Majeed and Prakash., 1998)

3. AIMS AND OBJECTIVES

The aim of this experiment was to make a probiotic chocolate with the goodness of bael pulp and probiotics. The objectives were:

1. To prepare water extract of bael fruit
2. To isolate and encapsulate *Lactobacillus sporogenes*
3. To prepare probiotic chocolate from bael water extract

4. MATERIALS AND METHODS

4.1 COLLECTION OF BAEI

Aegle marmelos was collected from National Institute of Technology, Rourkela campus in the month of March 2014. The fruits were handpicked from the tree when they were ripe.

4.2 PREPARATION OF BAEI WATER EXTRACT

For the preparation of water extract of bael, semi ripe/ripe fruit was used. The shell was broken and the pulp was mixed in water at the concentration of 0.1 mg/ml. To smoothen the solution the pulp was crushed and seeds were removed and it was strained through a mesh with pore size of 1 mm. The obtained solution was dried at 40°C for 48 hours. The product formed after drying was ground into a fine powder and kept in an air tight container for further use.

4.3 ISOLATION, SCREENING AND ENCAPSULATION OF PROBIOTIC BACTERIA

4.3.1 Collection of probiotic sample

The probiotic sample, Sporlac tablets, containing probiotic organism *Lactobacillus sporogenes* (Uni-Sankyo Ltd) was obtained from Apollo Pharmacy, National Institute of Technology, Rourkela in the month of January 2014

4.3.2 Isolation of probiotic bacteria

The probiotic sample was dispensed in MRS broth and incubated at 37°C for 16 hours. A loop full of this culture was plated on MRSA (de Man Rogosa Sharpe Agar) by spread plate method at concentration of 10⁻³. Incubation of plates was done at 37°C for 48 hours.

4.3.3 Screening of probiotic bacteria

For the screening of the probiotic, it was subjected to test whether they can tolerate the various stress factors in the gut. pH and bile tolerance tests were conducted on the five samples and their results were obtained.

4.3.3.1 pH tolerance test: The cultures were inoculated in MRS broth and incubated at 37°C for overnight. From this fresh culture, serial dilution was made in PBS up to 10⁻³. One ml of this sample was inoculated in MRS broth at pH 6.8, pH 2 and pH 3. At the end of 1 hour, 2 hours and 3 hours 100 µl of this culture was inoculated on plates containing MRSA (HIMEDIA™) (1.0 % peptone, 0.8 % egg extract, 0.4 % yeast extract, 2.0 % glucose, 0.5 % sodium acetate trihydrate, 0.1 % polysorbate 80 (also known as Tween 80), 0.2 % dipotassium hydrogen phosphate, 0.2 % triammonium citrate, 0.02 % magnesium sulfate heptahydrate, 0.005 % manganese sulfate tetrahydrate, 1.0 % agar, pH adjusted to 6.2 at 25°C.) by spread plate method. The plates were incubated at 37°C for 48 hours. The growth obtained in plates were compared to the control (pH 6.8) to deduce the results (Sahadeva et al., 2011).

4.3.3.2 Bile tolerance test: MRSA was prepared with 0.3 % bile salt (HiMedia). A loop full of this sample inoculated at the centre of the plate and the plates were incubated at 37°C for 24 hours. Formation of halo zone at the end of incubation confirmed bile tolerance of the organism (Islam et al., 2012)

4.3.4 Encapsulation of probiotic bacteria

4.3.4.1 Growth, isolation and preparation of probiotic sample: The probiotic sample was inoculated in 250 ml of MRS broth and incubated for 48 hours at 37°C. At the end of 48 hours, the bacterial cells were isolated by centrifugation at 7000 rpm for 15 minutes. The supernatant was discarded and pellet was taken. Sample was prepared by dilution of the bacterial cell pellet in 1 ml autoclaved water.

4.3.4.2 Preparation of capsules/ beads: Three percent sodium alginate and 0.05 M calcium chloride solution was prepared using deionized water and both the solutions were autoclaved at 121°C (15 psi) for fifteen minutes. To the autoclaved sodium alginate solution, bacterial sample was added and the solution was homogenized using vortex. This solution was added drop wise using a syringe with needle diameter of 1 mm to the calcium chloride solution. The capsules/beads formed were allowed to harden for 10 minutes, washed in deionized water twice and spread on a petriplate to dry (Manasouripour et al., 2013)

4.4 PREPARATION OF PROBIOTIC CHOCOLATE

Probiotic chocolate was prepared using dried bael extract, probiotic capsules/beads, sugar and chocolate. A thickening agent (corn starch) is used to thicken the sample. The prepared chocolate can either have a water base or a milk base.

4.4.1 Preparation of thickened extract: Bael extract was added to deionized water or milk at the concentration of 0.05 g/ml and heated at 100°C. Upon heating sugar at the concentration of 0.1 g/ml and corn starch at the concentration of 0.03 g/ml was added to the solution. The mixture

was allowed to thicken and allowed to cool down. Probiotic beads were added and homogenized by mixing.

4.4.2 Preparation of mold and making of chocolate: Milk chocolate (Cadbury) was melted at 50°C in water bath and 1 g of melted chocolate was used to line the walls of the mold of suitable shape. It was cooled at 4°C for 15 minutes to harden the chocolate. Upon hardening, 2 g of thickened bael extract was added to each mold and it was covered with melted chocolate on the top. The mold was allowed to freeze at -20°C for 2 hours to set the mixture. As the mixture sets, it was carefully removed from the mold to obtain the product.

5. RESULTS AND DISCUSSION

5.1 PREPARATION OF WATER EXTRACT OF BAEI

On mixing the pulp of bael with water, most of the seeds were removed in order to reduce the bitterness of the extract. The mixture was homogenized by crushing the pulp and then drying it on a wide, flat and clean surface at 40°C. Figure 6 shows pulp in water solution of bael and dried bael water extract

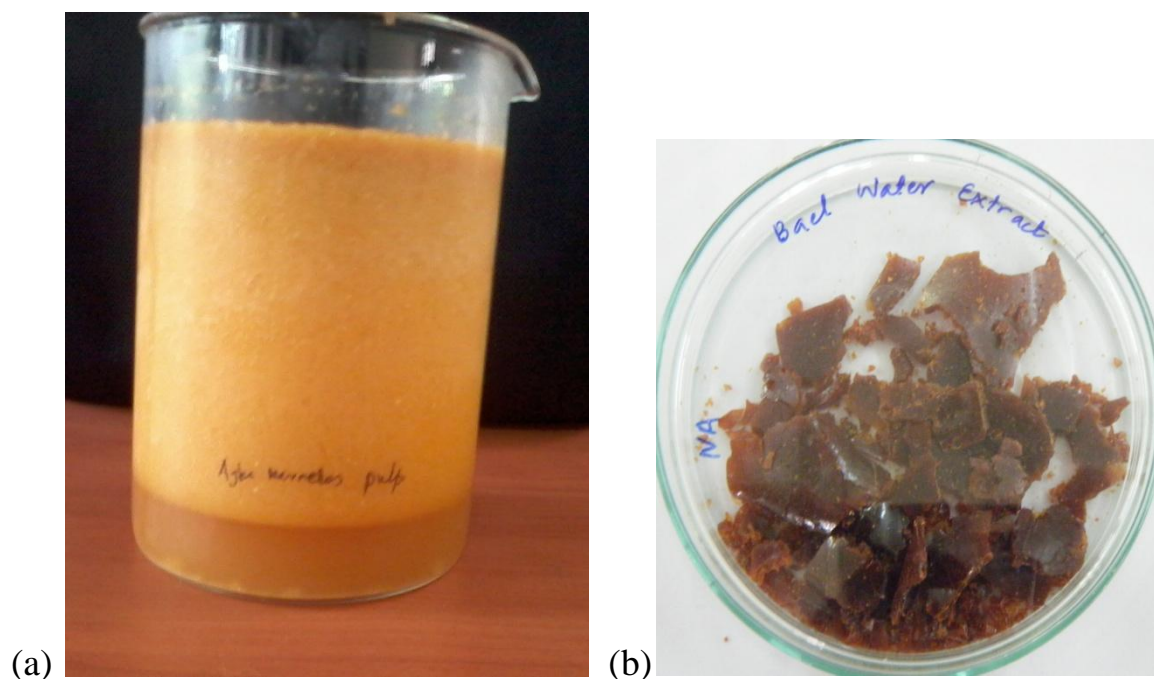


Figure 6: (a) Solution of bael pulp in water (b) dried bael water extract

5.2 SCREENING AND ENCAPSULATION OF PROBIOTIC BACTERIA

Lactobacillus sporogenes showed resistance to stress conditions of pH and bile as found in the gut. Hence it was used in the development of the product

5.2.1 pH tolerance of probiotic bacteria: Table 3 shows the pH tolerance test results of Sporlac (*Lactobacillus sporogenes*)

Table 3: Survival rate of *Lactobacillus sporogenes* at different pH and time interval (no. of colonies)

pH	Number of colonies		
	Hour 1	Hour 2	Hour 3
6.8	15	15	15
3.0	5	4	4
2.0	1	3	0

Figure 7 shows the pH tolerance test at pH 6.8



Figure 7: *Lactobacillus sporogenes* at control pH of 6.8

Figure 8 shows the growth of *Lactobacillus sporogenes* at pH 3 and pH 2

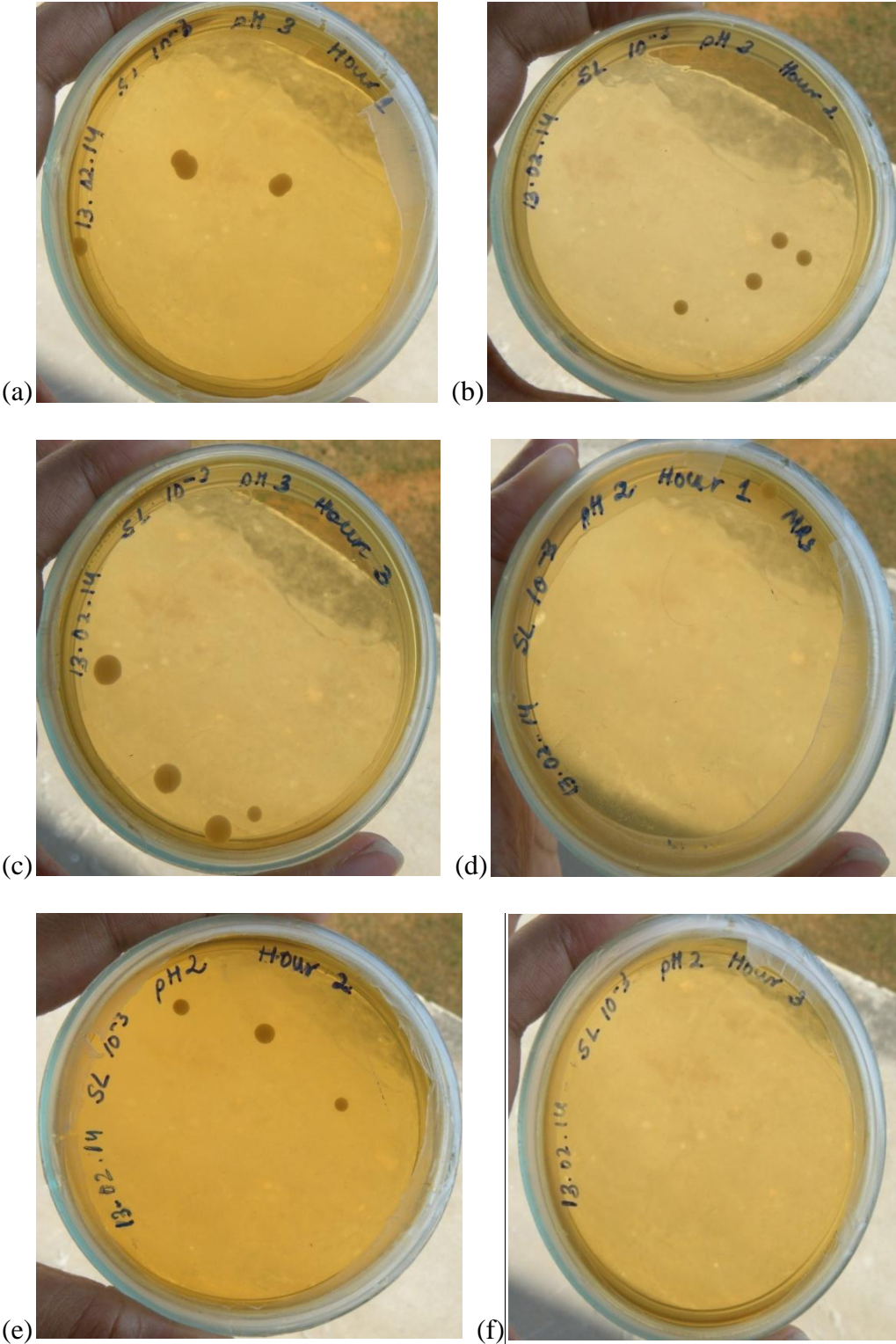


Figure 8: pH tolerance of *Lactobacillus sporogenes* at (a) pH 3 hour1 (b) pH 3 hour 2 (c) pH 3 hour 3 (d) pH 2 hour 1 (e) pH 2 hour 2 (f) pH 2 hour 3

5.2.2 **Bile tolerance test:** Figure 9 shows the bile tolerance test for *Lactobacillus sporogenes* the halo zone formed confirms bile tolerance of the microbe



Figure 9: Halo zone formation for the bile tolerance test by *Lactobacillus sporogenes*

5.2.3 **Preparation of probiotic capsules/ beads:** Figure 10 shows the preparation of beads and dried beads

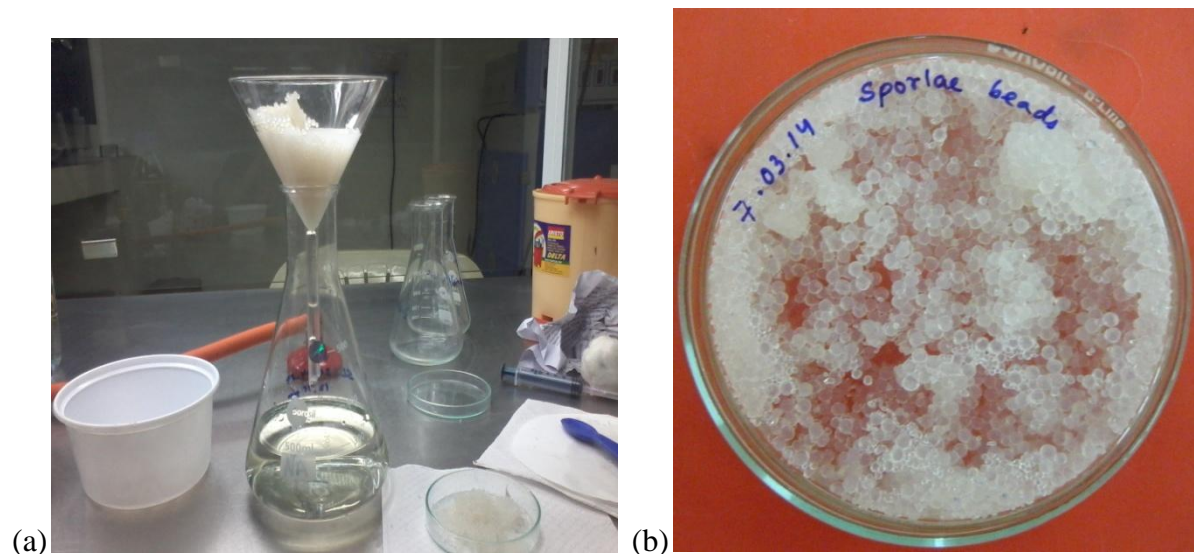


Figure 10:(a) Apparatus for preparation of probiotic beads (b) Prepared *Lactobacillus sporogenes* beads

5.3 PREPARATION OF PROBIOTIC CHOCOLATE

Figure 11 shows the preparation of probiotic chocolate from bael water extract

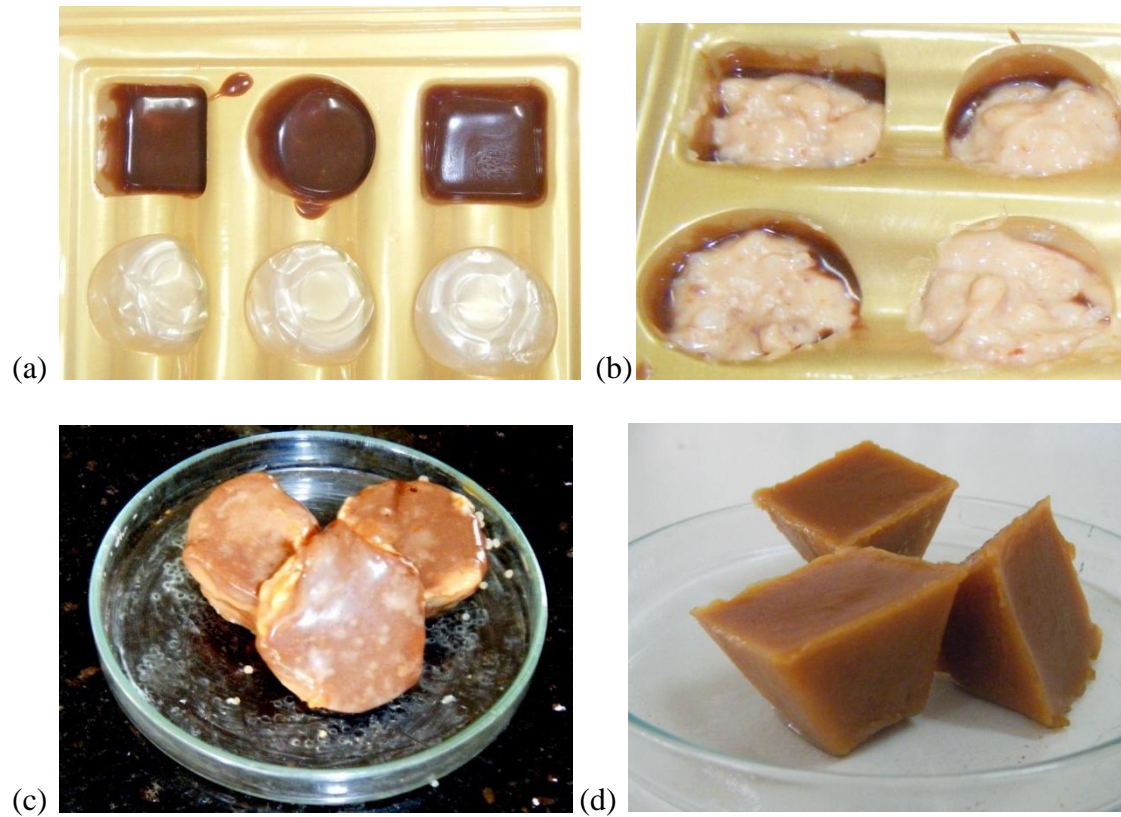


Figure 11: Preparation of probiotic chocolate (a) preparation of mold by lining with liquid chocolate. (b) Filling of mold with thickened bael extract (milk based) (c) Milk based probiotic chocolate. (d) Thickened bael extract (water based)

The chocolate prepared were of two types i.e. milk based and water based. The difference between the two lies in taste and flavor. While the water based chocolate has a stronger aroma and a sharp taste of bael, the milk based chocolate has a milder flavor.

6. CONCLUSION

The probiotic chocolate that was formulated using bael extract, probiotic beads and chocolate is a functional food since it has the beneficial effects of bael, probiotic and chocolate. The idea of combining all these ingredients and making a product with the goodness of each of the ingredients gives the synergistic effect in the functional food. Coating the product with chocolate aims at protecting the inner contents from spoilage creating microbes and it also increases the flavor of the product.

7. FUTURE WORK

For the commercialization of the product, certain works are necessary. The future prospective for this product includes the analysis of the shelf life of the product, its packaging and addition of preservatives to increase the shelf life. Along with this, the storage survivability of probiotics in alginate beads needs to be determined.

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