EVALUATION OF PROBIOTIC EFFICIENCY OF BACILLUS ISOLATED FROM FERMENTED FOOD PRODUCT

By

SMRITI SHARMA – 071701

SHAGUN WADHWA – 071707

PROJECT SUPERVISOR

Dr.Gargi Dey

MAY-2011

Submitted in Partial fulfillment of the Degree of Bachelor of Technology

In

BIOTECHNOLOGY

DEPARTMENT OF BIOTECHNOLOGY AND BIOINFORMATICS

JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY-WAKNAGHAT

TABLE OF CONTENTS

JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY

CERTIFICATE

This is to certify that the work entitled, "EVALUATION OF PROBIOTIC EFFICIENCY OF TWO BACILLUS SPECIES ISOLATED FROM FERMENTED FOOD PRODUCT " submitted by ***Smriti (071701) and Shagun Wadhwa (071707)*** in partial fulfillment for the award of degree of Bachelor of Technology in Biotechnology of Jaypee University of Information Technology has been carried out under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

PROJECT SUPERVISOR
Dr. Gargi Dey
Assistant Professor
Department of Biotechnology and Bioinformatics
Jaypee University of Information Technology
Waknaghat, Dist. Solan, HP-173215, India

ACKNOWLEDGEMENT

Any assignment puts to litmus test of an individual's knowledge, credibility or experience and thus sole efforts of an individual are not sufficient to accomplish the desired work. Successful completion of a project involves interest and efforts of many people and so this becomes obligatory on our part to record thanks to them.

Therefore, first of all we would like to thank our guide and mentor **Dr. Gargi Dey**, for her guidance, help and constant encouragement throughout this training. Working under her was an enriching experience.

We express our heartfelt thanks to our Head of Department **Dr. R.S Chauhan** for providing us, with the opportunities of doing this final year project.

We are also thankful to Shivani Sood, as without her cooperation, this project and thesis would not have been possible, all my seniors, and to the lab staff, especially Mr. Baleshwar, for their guidance and friendly cooperation.

We would also express our gratitude towards our families and our friends for their support throughout the execution of this study.

Smriti Sharma
(071701)

Shagun Wadhwa
(071707)

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ABBREVIATIONS	*EXPANDED FORM*
AMP	Adenosine Mono Phosphate
BSHs	Bile salt hydrolases
conc.	Concenteration
CFU	Colony Forming Unit
FDA	Food And Drug Administration
GIT	Gastro Intestinal Tract
GALT	Gut Associated Lymphoid Tissue
LAB	Lactic Acid Bacteria
MTCC	Microbial Type Culture Collection
MLN	Mesenteric Lymph Node
MRS	Man Rogosa Sharpe
ml	Milli litres
mg	Milli grams
mM	Milli Molar
min.	Minutes
nM	Nano Molar
nm	Nanometer
rpm	Rotations per minute
sp.	Species
var.	Variety
WHO	World Health Organisation
w/v	Weight/volume
µg	Micro grams
%	Percentage
°C	Degree Celsius

ABSTRACT

Alkaline-fermented food condiments play an important role in the diets of many people in the developing and a few developed countries. The rise in pH during production of these foods is due to the ability of the dominant Microorganisms, *Bacillus* sp., to hydrolyze proteins into amino acids and ammonia. This study was aimed at comparing the phenotypic characteristics of Bacillus species with Lactobacillus in order to investigate important prerequisites of probiotic interest, such as the capability to survive at low pH, to have Bile Salt Hydrolase activity, to show Antibiotic susceptibility and the ability to grow in anaerobic conditions. We have taken two Bacillus strains isolated from fermented bamboo, *Bacillus subtilis* MTCC-2451 and *Bacillus licheniformis* MTCC-2450 and these were compared with well known probiotic strains *Lactobacillus plantarum* MTCC 2941 and *Lactobacillus rhamnosus* MTCC 1408 all obtained from IMTECH Chandigarh. We have also reviewed the various alkaline fermented food products of Oriental and African regions to study various raw material used by Bacillus to ferment and the nutritional and health benefits provided by these food upon ingestion, showing that like Lactobacillus, Bacillus also has a potential to provide a huge repertoire of fermented food products which can have conceivable probiotic effects. We have done in-vitro tests of Gastric Juice tolerance, antibiotic resistance tests, ability to grow in anaerobic conditions, also we have done BSH assay. It was found that *Bacillus subtilis and Bacillus licheniformis* show considerable tolerance to gastric juice at pH 2 in comparison to *Lactobacillus plantarum and Lactobacillus rhamnosus*. Also our test organisms had good amount of BSH activity but it was less than the control organisms. Antibiotic resistance test that was done by disk diffusion method for Ampicillin (10 µg/ml), Streptomycin (10 µg/ml), Streptomycin (25 µg/ml) (for *Bacillus subtilis and Bacillus licheniformis*), Tetracycline (30 µg/ml), Kanamycin (30 µg/ml), Chloramphenicol(30 µg/ml),showed that our test strains were sensitive to all these antibiotics.

The experimental evidence from this study emphasizes that both *Bacillus subtilis and Bacillus licheniformis* show the potential probiotic characteristics.

CHAPTER -1

INTRODUCTION

The WHO food safety unit has given high priority to the research area of fermentation as a technique for preparation/storage of food. Fermentation is defined as the action of selected microorganisms by which a biochemically and organoleptically modified substrate is produced, resulting in an acceptable product for human consumption.

Since prehistoric times the microorganisms are being used for the preparation of food from available plant and animal material by traditional methods. These microorganisms play an essential role in biochemical changes in the substrates during fermentation.

There are various kinds of fermented products for example Lactic Acid Bacteria fermented products (LAB), Acetic acid bacteria fermentation and Alkaline fermented food products.

Alkaline fermentation is the process in which hydrolysis of protein to amino acids and peptides takes place, thus, releasing ammonia, which increases the alkalinity and makes the substrate unsuitable for the growth of spoilage organisms. Bacillus species are the pronounced organisms carrying out the alkaline fermentation. Alkaline fermentations are more common with protein rich foods such as soybeans and other legumes, although there are a few examples utilising plant seeds. For example water melon seeds (*Ogiri* in Nigeria) and sesame seeds (*Ogiri-saro* in Sierra Leone) and others where coconut and leaf proteins are the substrates (Indonesian *semayi* and Sudanese *kawal* respectively).

Alkaline fermented food products are very important as they provide flavouring attributes as well as contribute significantly to the intake of Proteins, essential amino acids, essential fatty acid,

Group B vitamins also A and C and Minerals like Ca, Fe, Zn, Cu ,Mg , P, Se, Na, Mn . They not only serve as nutritious non meat substitutes, but also as condiments and flavouring agents.

Alkaline fermentation plays some very important functions in order to make fermented food products safe to consume for an example:

- The high pH inhibits invasion by pathogenic and spoilage microorganisms.
- During fermentation inedible foods are made edible by the elimination/decrease of anti nutritional factors.
- Alkaline fermentation increases digestibility due to extensive hydrolysis of proteins and lipids respectively to peptides, essential amino acids and fatty acids.
- Degradation of non digestible oligosaccharides such as stachyose and raffinose into simple sugars.
- Decreases toxicity by the elimination/decrease of toxic components of raw materials like African locust beans contains high levels of toxic substances such as oxalic acid, phytic acid and hydrocyanic acid which are reduced during the fermentation.

There are number of Bacillus fermented food products indigenous to Oriental and African continents, which not only serve as a source of nutrition but also have other health benefits like Kinema which is used by the tribal people as prophylactic agent, for curing diarrhea. Many of these products are being studied for the probiotic potential of there strains like *Bacillus subtilis var.natto*, strains from Kinema, Bikalga are also under study.
Huge amount of studies are being carried to test various species of Bacillus for their probiotic potential in order to have wide range of food products that can render health benefits to consumers other than Lactic acid bacteria fermented food products .

A **Probiotic** has been defined by a working group of the International Life Sciences Institute Europe (ILSI Europe) as "a viable microbial food supplement which beneficially influences the health of the host". Probiotics are usually members of the healthy gut micro biota and their addition can assist in returning a disturbed micro biota to its normal beneficial composition. Natural probiotics exist in several foods including yogurt, certain cereals and fermented milk. Specifically, probiotics consist of lactic acid producing bacteria (LAB), non-lactic acid producing bacterial species, and non-pathogenic yeast.

Some of the commonly used microflora in probiotics include: *L. acidophilus, L. fermentum, L. paracasei, L. brevis, L. gasseri, L. plantarum, L. bulgaricus, L. helveticus, L. reuteri, L. casei, L. jensenii, L. rhamnosus, L. crispatus, L. johnsonii, L. salivarius, B. adolescentis, B. breve, B. longum, B. animalis, B. infantis, B. thermophilum, B. bifidum, B. lactis, Streptococcus thermophilus, Enterococcus faecium, Bacillus subtilis, B. coagulans, B. licheniformis, B. cereus Proprionibacterium, S. boulardii, L. rhamnosus, Lactobacillus strains and Bifidobacterium strains.*

Criteria for selecting probiotics that are specific for a desired target have been developed, but a general criterion that must be satisfied includes:

- It should be isolated from the same species as its intended host
- It should have a demonstrable beneficial effect on the host
- It should be non-pathogenic
- It should be able to survive transit through the gastrointestinal tract
- On storage, large number of viable bacteria must be able to survive prolonged periods.

Probiotics – mechanism of action:

Mechanisms for the benefits of probiotics are incompletely understood. However, as a general rule, includes:

- Adherence and colonization of the gut
- Suppression of growth or epithelial binding/invasion by pathogenic bacteria and production of antimicrobial substances
- Improvement of intestinal barrier function
- Controlled transfer of dietary antigens
- Stimulation of mucosal and systemic host immunity.

Probiotics in Health:

Lifestyle and eating habits are partly responsible for each individual's overall health status.The potential benefits that are claimed include improved nutrition and growth and prevention of various gastrointestinal disorders. Probiotic-containing products are available for human nutrition, as animal feed supplements, and also for aquaculture. In some countries Probiotics are taken as prophylactic agents (for example, to prevent childhood diarrhea), while in Southeast Asia they are also used as therapeutic agents.

The basic probiotic Functions are:

- Probiotics obstruct harmful bacteria life and reproduction.
- Good flora feed on the same nutrients as harmful bacteria, thereby starving them.
- Probiotics cause a reduction in harmful bacteria by taking the best adhesion sites on the gut wall.
- Probiotics balance pH in the gut.
- Probiotics enhance overall digestion.
- Healthy bacteria ensure best Micro flora balance for the natural immune system.

Some of the more commonly documented functions of probiotics include: alleviation of eczema, irritable bowel syndrome and food allergies, inflammation management, vitamin production and enhanced mineral absorption. It also prevents diseases like –

- Colon Cancer,
- Cardio Vascular diseases
- lactose intolerance
- Liver diseases
- Inflammatory Bowel Disease

Safety Aspects

Probiotic supplements are easily accessible and available in the market worldwide. They are not regulated by the US FDA because of their classification as a nutritional product rather than as a pharmaceutical product. This has made them available without prescription; however, the lack of regulation necessitates increased awareness from those who use them.

Probiotics are generally considered safe. As evidenced by epidemiologic studies, bacteremia or sepsis from lactobacilli is extremely rare. Numerous probiotics have a long history of safe use and no health concerns have been observed. There are, however, isolated reports of fungemia with Saccharomyces following its use as a probiotic especially in immune compromised or ICU patients. Thus although administration of probiotics generally can be considered safe, each strain of probiotic has specific properties that should be considered before its use in any patient. Novel microbes, including probiotics and genetically modified probiotics need to be assessed for their safety on a strain- by-strain basis. The safety of probiotics in conditions where the mucosal integrity of gastrointestinal tract is compromised requires more studies before sweeping safety recommendations can be formulated.

CHAPTER-2

REVIEW LITERATURE

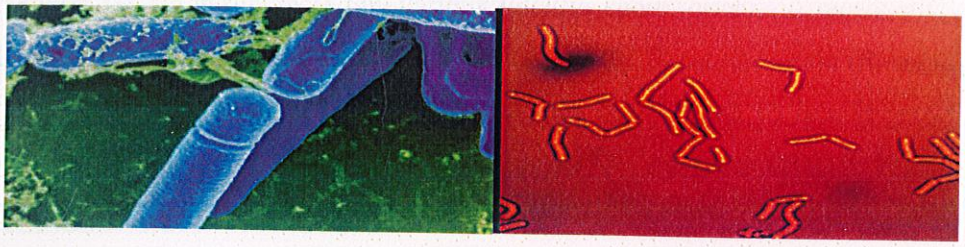
2.1 BACILLUS AS PROBIOTIC:

Lactobacillus is a most widely used micro organism for fermenting many dairy products .Till now, an extensive research has been done on the probiotic efficiency of the various Lactic Acid Bacteria (LABs), and there has been a list of LAB species which have been characterized as well known probiotic, but it's important to keep in mind that not all LABs exhibit probiotic effects.

Recent advances in genome sequencing projects, molecular tools, and genomic-based strategies for functional studies of lactobacilli contribute greatly to the identification of adaptation and probiotic biomarkers (Lebeer.S *et al.* 2008). Other than LAB's there are species which have probiotic potential and needs to be focused in order to diversify the scope of research.

Bacillus a gram +ve endo spore forming organism (fig 2.1) which resides in multiple niches like soil, dust, and water as well as in the air.Their primary reservoir though, has long been considered soil, and indeed, they can be found there in abundance. By associating with plant matter, it would be expected that *Bacillus* spores could enter the gastrointestinal tract (GIT) of animals by ingestion (Nguyen K. M. *et al.* 2006). Example of various species are – *Bacillus subtilis , Bacillus licheniformis , Bacillus pumilus , Bacillus cereus, Bacillus megaterium, Bacillus clausii ,etc.*

Fig 2.1: Bacillus species from different niches



There has been studies carried on the intestinal sustenance of Bacillus sp. and it has been documented that after consuming Bacillus spores they germinate and the primary signals that induce spore germination are nutritional , and in some *Bacillus* species, low pH of the gut has also been shown to help activate the germination process thus showing that spores may not be transient passengers in the gut or that if they are, they may still have an intimate interaction with the host cells or microflora that can enhance their potential probiotic effect(Nguyen K. M. *et al.* 2006)

The following three basic mechanisms have been proposed for how orally ingested nonindigenous bacteria can have a probiotic effect in a host:

- immunomodulation (that is, stimulation of the GALT) (e.g., induction of cytokines)
- competitive exclusion of gastrointestinal pathogens (e.g., competition for adhesion sites), a
- secretion of antimicrobial compounds which suppress the growth of harmful bacteria.(Duc .H *et al.* 2003)

Few studies have demonstrated a direct probiotic effect of *Bacillus* spores, showing that they can interact with the gut-associated lymphoid tissue (GALT) also it have been shown that orally ingested *B. subtilis* spores are immunogenic and can disseminate to the Peyer's patches and mesenteric lymph nodes (MLN)(Duc .H *et al.* 2003).

Preliminary studies with poultry have provided evidence that there is competitive exclusion of *Escherichia coli* 078:K80 by *B. subtilis* and a number of studies have demonstrated that *Vibrio harveyi* in shrimp is suppressed by various *Bacillus* spore formers. A recent study has described the characterization of an antibiotic produced by the *B. subtilis* strain (*B. subtilis* 3) found in the commercial product Biosporin, which has been shown to inhibit growth of *Helicobacter pylori* (Duc .H *et al.* 2003).

There are number of commercial products available having *Bacillus* spores formulations which are known to have probiotic potential they are studied for various probiotic biomarkers like –

- Tolerance of gastric juice
- pH tolerance
- Bile salts tolerance ,
- Adherence to the gut
- Anti microbial Effect
- Antibiotic production
- Immunomodulatory activities
- Prophylactic actions

Examples of some of the commercial formulations are - Bactisubtil (*B. cereus* IP 5832) ,Enterogermina(*B. clausii*), Biosubtyl Nha Trang (referred to here as BiosubtylNT; a strain of *B. pumilus*) , Biosubtyl Da Lat (referred to here as BiosubtylDL; a *B. cereus* strain), and Subtyl (a strain similar to *B. cereus* spp. and designated *B. cereus* var. vietnami) (Duc .H *et al.* 2003).

Other than these bacterial formulations another way of supplementing these probiotic strains is through Bacillus fermented food products. A huge repertoire of alkaline fermented food products is available and many of them are under study for checking the probiotic potential of the strains.

2.2 ALKALINE FERMENTED FOOD PRODUCTS

Today, we need to diversify our scope of research and so need to focus on other fermented food products i.e. other than Lactic Acid fermented food products. There are wide varieties of alkaline fermented food products which are indigenous to Oriental and African origin as mentioned in Table 2.1. We have reviewed these products in order to understand the diversity in the raw materials being fermented by different types of *Bacillus* species, various techniques and conditions that are adopted by tribal people across the globe, different mixed cultures used for the fermentation and the increase in the nutritional content of the product after fermentation (Parkouda.C *et al.* 2009).

For example kinema a soya bean fermented food product of north east region. The process of fermentation is very traditional native to a particular tribe. *Bacillus subtilis* isolated from kinema has been studied for its prophylactic properties and is being used by tribal people to cure diarrhea (Singh *et al.* 2007)

Similarly Bikalga from hibiscus sabdariffa consumed in African countries which is majorly fermented by Bacillus species, has been shown to have some prophylactic properties and the organism has probiotic potential (Parkouda.C *et al.* 2008).

An other a very well known Alkaline fermented food product Natto, fermented by *Bacillus subtilis var. natto*, a popular food of Japan contains numerous nutrients originating from both soyabeans as well as from intact cells and metabolites of *B. subtilis* (natto) and many of these have physiological activity (Hosoi, T., Kiuchi, K; Bacterial spore formers: probiotics and emerging applications;pp200-220)

Natto is health-enhancing and these claims are backed by medical research. For an example Pyrazine is a compound that, in addition to giving natto its distinct smell, reduces the likelihood of blood clotting. It also contains a serine protease type enzyme called nattokinase which may also reduce blood clotting both by direct fibrinolysis of clots, and inhibition of the plasma protein plasminogen activator inhibitor 1.

This may help to avoid thrombosis, as for example in heart attacks, pulmonary embolism, or strokes. An extract from natto containing nattokinase is available as a dietary supplement. Studies have shown that oral administration of nattokinase in enteric capsules leads to a mild enhancement of fibrinolytic activity in rats and dogs. It is, therefore, plausible to hypothesize that nattokinase might reduce blood clots in humans (Sumi .H *et al.* 1987)

In Japan Natto cheese traditionally has not only been consumed for cardiovascular support, but to lower blood pressure as well. Recent studies have confirmed Japan's tradition usage of the Nattokinase enzyme in Natto. Researchers have studied the effects of nattokinase on blood pressure in both human and animal subjects. The researchers confirmed the presence of inhibitors of angiotensin converting enzyme [ACE], which converts angiotensin 1 to its "active" form angiotensin 2 within the test extract. ACE causes blood vessels to narrow and blood pressure to rise - by inhibiting ACE, nattokinase has a lowering effect on blood pressure (Sumi .H *et al.* 1987).

Also it has been suggested that Natto fractions might help to prevent arteriosclerosis, as they appear to reduce lipid per oxidation and improve lipid metabolism.

Not only the product has number of health benefits the strain isolated from Natto i.e *Bacillus subtilis var. natto* has also been tested for its probiotic potential like the adherence of the strains to caco-2 cell lines which is a very important property in order to sustain in the gut, tolerance to various stresses during their gut transit,also the cells induce cytokine responses of human intestinal epithelial-like Caco-2 cells with less cytotoxicity than nonpathogenic *Escherichia coli, pathogenic Salmonella enteritidis*, and *Pseudomonas aeruginosa*. In addition, a serine protease, subtilisin, produced by B. subtilis degrades soyabean allergens and shows fibrinolytic activity. Also the Natto strains are shown to form biofilm which helps them to adhere and germinate in the gut. (Hosoi, T., Kiuchi, K; Bacterial spore formers: probiotics and emerging applications;pp200-220)

Similarly, Thua nao and other naturally fermented soybean foods harbor *B. subtilis* strains, which exhibit high potential for producing enzymes such as amylase and protease, and for

producing health-promoting compounds such as (PGA) gamma-polyglutamic acid and proteases.

Bacillus has an upper hand over Lactobacillus due to its high anti microbial effects towards harmful bacteria and moulds, i.e. Bacillus has ability to inhibit both gram positive and gram negative bacteria .Similarly, it has been found that. Bacillus strains isolated from these fermented products have an ability to produce antifungal, antimicrobial agents like surfactants. Other than these Bacillus adds to the nutrient level of the food products like an increase, in vitamin B2 and B3 levels during kinema formation (Parkouda.C *et al.* 2009)

Table 2.1: REVIEW ON INDEGENOUS ALKALINE FERMENTED FOOD PRODUCTS OF ORIENTAL AND AFRICAN COUNTRIES

SOURCE	PRODUCTS	PLACE	*MICROBIAL STRAINS*	REFERENCE
Legume based (soya)	Kinema	Nepal	*Bacillus subtilis(dominant), Entrococcus faecium, Candida sp.*	J.P Tamang : Ind. Journal of trad. Knowldg : Indigenous fermented soybean foods knowledge of Northeast women on production of ethnic food
Legume based (soya)	Hawaijar	Manipur	*Bacillus subtilis, B.licheniformis, B.cereus, Staphylococcus aureus, S.sciuri, Alkaligenesis sp.*	J.P Tamang : Ind. Journal of trad. Knowldg : Indigenous fermented soybean foods knowledge of Northeast women on production of ethnic food
Legume based (soya)	Tungrymbai	Meghalaya	*Bacillus subtilis and other Bacillus sp.*	J.P Tamang : Ind. Journal of trad.

				Knowldg : Indigenous fermented soybean foods knowledge of Northeast women on production of ethnic food
Legume based (soya)	Aakhone	Nagaland	*Bacillus subtilis and other Bacillus sp.*	J.P Tamang : Ind. Journal of trad. Knowldg : Indigenous fermented soybean foods knowledge of Northeast women on production of ethnic food
Legume based (soya)	Bikang	Mizoram	*Bacillus subtilis and other Bacillus sp.*	J.P Tamang : Ind. Journal of trad. Knowldg : Indigenous fermented soybean foods knowledge of Northeast women on production of ethnic food
Legume based (soya)	Thua-nao	*Northern Thailand*	*Bacillus subtilis strains*	Yasuhiro Inatsu: National Food Res. Inst: Charac. of Bacillus subtilis strains in Thua nao, a trad. fermented soybean food in northern Thailand
Legume based (soya)	Natto	*Japan*	*B. subtilis var natto*	Kiuchi (2004)
Legume based (soya)	Soy-Daddawa	*West Africa*	*Bacillus subtilis, B.licheniformis ,B.pumilus.*	B. O. Omafuvb S. H. Abioseand O. O. Shonukan :Food Microbiiology sci direct :

				Fermentation of soybean (*Glycine max*) for soy-*daddawa* production by starter cultures of*Bacillus*
Legume based (African Locust Bean)	Iru	*Nigeria*	*Bacillus subtilis, B.licheniformis ,B.pumilus.*	Dr. S. A. Odunfa , O. B. Oyewole :Journal of Basic Microbiology : Identification of *Bacillus* species from iru a fermented African locust bean product
Legume based (African Locust Bean)	Soumbala	*Africa*	*B. subtilis, B. thuringiensis, B. licheniformis, B. cereus, B. badius, Paenibacillus alvei, B. firmus, P. larvae, Brevibacillus laterosporus, B. megaterium, B. mycoides and B. sphaericus*	P. K. Sarkar, B. Hasenack and M. J. R. Nout : Int jour of food mirobio : Diversity and functionality of Bacillus and related genera isolated from spontaneously fermented soybeans (Indian Kinema) and locust beans (African Soumbala)
African locust bean (Parkia filicoidea Welw)	Dawadawa	*Africa*	*Bacillus subtilis, Leuconostoc mesenteroides and L. dextranicus*	S. P. Antai M. H. Ibrahim : Journal of applied microbio: Micro-organisms associated with African locust bean (Parkia filicoidea Welw) fermentation for 'dawadawa'

				production
Seed based (castor seeds Ricinus communis)	Ogiri-igbo	*Southern Nigeria*	*B. subtilis, B. licheniformis, B. metaterium, Staphylococcus spp., Pseudomonas aeruginosacoccus sp.*	L. Barber, S. C. Achinewhuand E. A. Ibiama : Sci Direct: The microbio of ogiri production from castor seed (Ricinus communis)
Seed based (Prosopis africana seeds)	Okpehe	*Nigeria*	*Bacillus subtilis, B. licheniformis, B. megaterium, Staphylococcus epidermidis andMicrococcus spp*	F. A. Oguntoyinbo, A. I. Sanni : Sciene Direct :Phenotypic diversity and technological properties of Bacillus subtilis species isolated from okpehe, a traditional fermented condiment
Seed based (seeds of Albizia Saman)	Aisa	*Nigeria*	*Bacillus cereus var. mycoides, B. coagulans, B. licheniformis, B.megaterium, B. pumilus, Staphylococcus cereus and S. saprophyticus*	Adenike A.O. Ogunshe, Abiodun E. Ayodele2 and Iheanyi O. Okonko: Pakistan Jour of nutrition : Microbial Studies on *Aisa*: A Potential Indigenous Laboratory Fermented Food Condiment from *Albizia saman* (Jacq.) F. Mull
Fermented fish(Puntius sophore)	Ngari	*Manipur*	*Bacillus subtilis and Bacillus pumilus, Micrococcus, species of Candida and Saccharomycopsis, Lactococcus lactis*	Namrata Thapa, Joydeb Pal and Jyoti Prakash Tamang : World Journ of microbio : Microbial

			subsp. cremoris, Lactococcus plantarum, Enterococcus faecium, L. fructosus, L. amylophilus, L. coryniformis subsp. Torquens	diversity in ngari, hentak and tungtap, fermented fish products of North-East India
Sun-dried fish (Esomus danricus) powder and petioles of aroid plants (Alocasia macrorhiza)	Hentak	*Manipur*	*Bacillus subtilis and Bacillus pumilus, Micrococcus, species of Candida and Saccharomycopsis, Lactococcus lactis subsp. cremoris, Lactococcus plantarum, Enterococcus faecium, L. fructosus, L. amylophilus, L. coryniformis subsp. torquens and L. plantarum*	Namrata Thapa, Joydeb Pal and Jyoti Prakash Tamang : World Journ of microbio Microbial diversity in ngari, hentak and tungtap, fermented fish products of North-East India

2.3 BIOMARKERS OF PROBIOSIS

As already mentioned there are some criteria that need to be fulfilled by an organism in order to be probiotic. We have focused on some of the important criteria's and the reasons which make them important for the selection of the organism.

2.3.1 Bile Salt Tolerance :

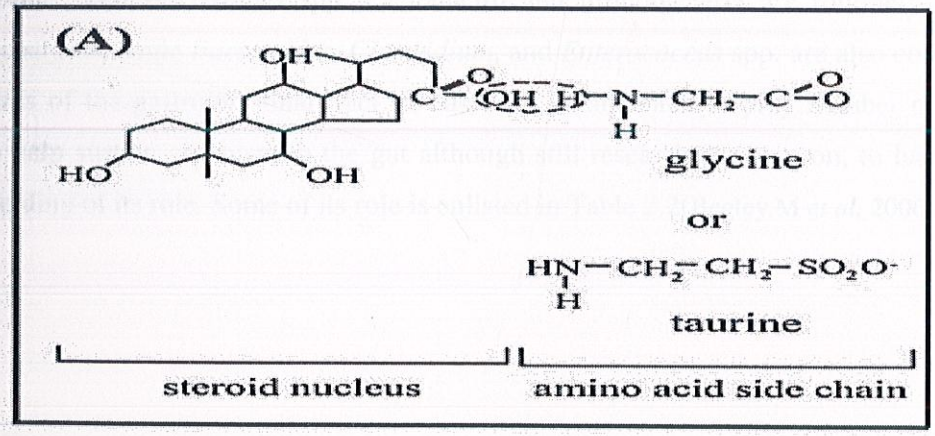
The ability of probiotic strains to hydrolyze bile salts has often been included among the criteria for probiotic strain selection, and a number of bile salt hydrolases (BSHs) have been identified and characterized.

Bile is a yellow-green aqueous solution whose major constituents include bile acids, cholesterol, phospholipids, and the pigment biliverdin. It is synthesized in the pericentral hepatocytes of the liver, stored and concentrated in the gallbladder interdigestively, and released into the duodenum after food intake.

Bile functions as a biological detergent that emulsifies and solubilizes lipids, thereby playing an essential role in fat digestion. This detergent property of bile also confers potent antimicrobial activity, primarily through the dissolution of bacterial membranes.

The primary bile acids, **cholic and chenodeoxycholic acid** , are synthesized de novo in the liver from cholesterol. The solubility of the hydrophobic steroid nucleus is increased by conjugation as an *N*-acyl amidate with either glycine (glycoconjugated) or taurine (tauroconjugated) prior to secretion (Fig. 2.2). The resulting molecules are therefore amphipathic and can solubilize lipids to form mixed micelles.(Begley.M *et al.* 2006)

Fig.2.2: Chemical structure of bile acids.

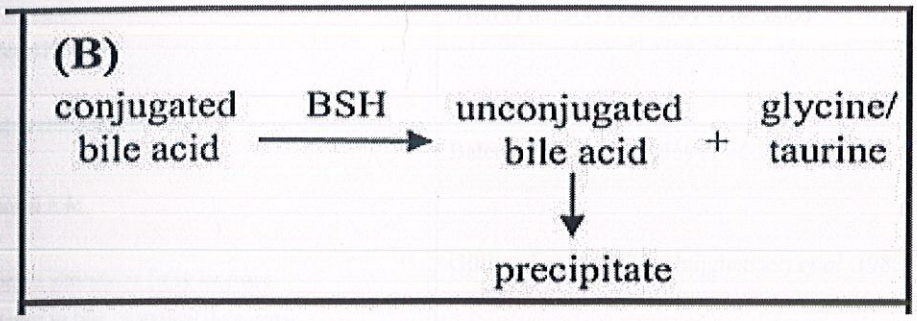


Primary bile acids are synthesized in the liver from cholesterol and are conjugated with Either glycine or taurine prior to secretion. The carboxyl group of the bile acid and the amino group of the amino acid are linked by an amide bond.

One important transformation is deconjugation, a reaction that must occur before further modifications are possible . Deconjugation is catalyzed by bile salt hydrolase (BSH) enzymes

(EC 3.5.1.24), which hydrolyze the amide bond and liberate the glycine/taurine moiety from the steroid core (Fig.2.3).

Fig 2.3: Reaction catalyzed by BSH enzymes.



BSHs cleave the peptide linkage of bile acids, which results in removal of the amino acid group from the steroid core. The resulting unconjugated bile acids precipitate at low pH.

BSH activity has been detected in *Lactobacillus*, *Bifidobacterium, Enterococcus* , *Clostridium* , and *Bacteroides* spp. Lactobacilli and bifidobacteria are routinely used as probiotic strains, while *Bacteroides*, *Clostridium*, and *Enterococcus* spp. are also commensal inhabitants of the gastrointestinal tract. It has been documented to play number of role in order to help sustain organism in the gut although still research is going on, to have better understanding of its role. Some of its role is enlisted in Table 2.2(Begley.M *et al.* 2006).

Table 2.2: Role of BSH

Role or impact of BSH activity	References
Microbial role	
Bile detoxification	Ahn *et al.*.2003 , Begley *et al.*.2005
Gastrointestinal persistence	Bateup e al.1995,Begley *et al.*.2005
Nutritional role	
Membrane alterations (may increase Resistance to bile, intestinal defensins, Lysozyme, etc.)	Gilliland *et al.* 1977, Huijghebaert *et al.* 1982
	Boggs, J. M. *et al.* 1987., Dambekodi *et al.* , 1998
Impact on the host	
Altered digestive functions (lipid Mal absorption, weight loss)	De Smet *et al.* 1994 , Feighner *et al.* 1987
Cholesterol lowering	De Smet *et al.* 1994, De Smet *et al.* 1995
Cancer/activation of carcinogens	fernstein *et al.* 2005 , Huijghebaert *et al.* 1982
Formation of gallstones	Berr, F., *et al.* 1962 , Low-Beer *et al.* 1987

Now it's important to find out whether this particular biomarker is present in different *Bacillus sp*. or not. This can be done by in-vitro assays of BSH enzyme or by genetic analysis of the genome of *Bacillus sp*.

2.3.2 Adherence:

It is generally assumed that a good adherence capacity is a desirable trait for any organism to be a probiotic , as it can promote-

- the gut residence time,
- pathogen exclusion, and

- Interaction with host cells for the protection of epithelial cells or immune modulation.

Mostly, adherence to epithelial cell lines (e.g., Caco-2 or HT-29 human-derived adenocarcinoma cells) or immobilized intestinal mucus or extracellular matrix molecules (e.g., collagen and fibronectin) can be investigated in short-term assays. However, in most natural niches, adherent bacteria can form microcolonies and multi cellular structures, recognized as biofilm-like communities. In the human gut of healthy individuals, isolated bacteria and micro colonies seem to be the predominant colonization form. This more dynamic adherence process can also be simulated *In vitro* for organisms in order to select for potential probiotic.(Lebeer.S *et al.* 2008)

Lot of work has been done on Lactobacillus sp. in order to find the genes responsible for adherence also number of in-vitro assays are done to check the adherence in order to prove the probiotic potential of an organism. A few studies are also done to investigate the adhesion and colonization dynamics of lactobacilli *In vivo* in animal models or humans. Similarly the adhesion abilities of the *Bacillus* vegetative cells and spores were characterized *In vitro* using mucin, Caco-2 cell monolayers and Matrigel as matrices. Globally, vegetative cells adhered less well than did the corresponding spores. Also the corresponding genes responsible in *Bacillus sp.* are being explored with the help of various functional analysis tools (Sanchez.B *et al.* 2009)

2.3.3 Tolerance to gut stresses:

Probiotic organisms encounter various environmental conditions upon ingestion by the host and during transit in the GIT, firstly, they need to survive the harsh conditions of the stomach. Humans secrete approximately 2.5 liters of gastric juice each day, generating a fasting pH of 1.5, increasing to pH 3 to 5 during food intake (Lebeer.S *et al.* 2008).
The effect of Acid stress on the physiology of the organism can be varied like lowering of the intracellular pH reduces the transmembrane pH difference, which determines the proton motive force used as an energy source in numerous transmembrane transport processes. Also Internal acidification reduces the activity of acid-sensitive enzymes and results in damage to

proteins and DNA of the organism thus a probiotic organism in order to perform its function has to sustain high acidic conditions of the gut and for testing the tolerance of an organism in-vitro experiments are performed giving similar stress conditions in order to see their response. Given that the liver excretes approximately 1 liter of bile each day into the small intestine, exposure to bile represents another challenge for bacteria entering the GIT. Bile acids are surface active, amphipatic molecules with potent antimicrobial activity and act as detergents, disrupting biological membranes. Moreover, bile salts also seem to induce an intracellular acidification so that many resistance mechanisms are common for bile and acid stress so it's very important to check for Bile tolerance of an organism, to be a potent probiotic (Lebeer.S *et al.* 2008).

In addition to coping with acid and bile, the contributions of other stress responses like anaerobisis, interactions with other microbes and with cells of the immune system and the various antimicrobial products produced by pathogenic organisms. also impose a serious threat for the probiotic microbes to the survival capacity in the GIT and they should not be overlooked.

Multiple genome-wide studies and functional analyses have now been performed to characterize various stress responses in lactobacilli. Similar functional analysis is required for *Bacillus sp.* in order to widen the scope of probiotic research.

2.3.4 Antibiotic susceptibility :

It has been advocated as an essential selection criterion for potentially probiotic cultures to ensure additionally that technologically used strains are regarded as non-pathogenic and unlikely to participate in undesirable (e.g. antibiotic resistance) gene transfer cascades in-vivo.

There are many types of antibiotics. Chiefly, they have two types of mechanism of action-

- Bacteriostatic : they stop the bacterium from multiplying further by interfering with their DNA. But they do not kill the bacteria.
- Bactericidal: they kill the bacteria.

There are different classes of Antibiotics and there mode of action varies accordingly table 2.3 shows some of the classes of antibiotics and their mode of action:

Table 2.3: Classification of Antibiotics

Chemical Class	Examples	Biological Source	Spectrum (Effective Against)	Mode Of Action
Beta-lactams(penicillins and cephalosporins)	Penicillin G, Cephalothin	*Penicillium notatum And cephalosporium species*	Gram-positive bacteria	Inhibits steps in cell wall (peptidoglycan) synthesis and murein assembly.
Semisynthetic beta-lactams	Ampicillin, Amoxicillin		Gram-positive and Gram- negative bacteria	Inhibits steps in cell wall (peptidoglycan) synthesis and murein assembly
Aminoglycosides	Streptomycin	*Streptomyces griseus*	Gram-positive and Gram- negative bacteria	Inhibits translation (protein synthesis)
Glycopeptides	Vancomycin	*Amycolatopsis orientalis* (formerly designated *Nocardia orientalis*)	Gram-positive bacteria, esp. *Staphylococcus aureus*	Inhibits steps in murein(peptidoglycan) biosynthesis and assembly
Lincomycins	Clindamycin	*Streptomyces lincolnensis*	Gram-positive & Gram-negative bacteria ;anaerobic Bacteroides	Inhibits translation (protein synthesis)
Macrolides	Erythromycin, Azithromycin	*Streptomyces erythreus*	Gram-positive bacteria, Gram-negative	Inhibit translation (protein synthesis)

			bacteria not enterics,Neisseria,Legionella, Mycoplasma	
Rifamycins	Rifampicin	*Streptomyces mediterranei*	Gram-positive and Gram- negative bacteria, Mycobacterium tuberculosis	Inhibits transcription (bacterial RNA polymerase)
Tetracyclines	Tetracycline	*Streptomyces Species*	Gram-positive and Gram-negative bacteria, Rickettsias	Inhibit translation (protein synthesis)
Semisynthetic tetracycline	Doxycycline		Gram-positive and Gram-negative bacteria, Rickettsias Ehrlichia, Borrelia	Inhibit translation (protein synthesis)
Chloramphenicol	Chloramphenicol	*Streptomyces Venezuelae*	Gram-positive and Gram- negative bacteria	Inhibits translation (protein synthesis

Each potent probiotic strain is tested for Antibiotic susceptibility by number of in-vitro tests like Kirby- Bauer method as important selection criteria.

2.3.5 Antimicrobial Activity :

Besides competition for nutrients, probiotics are known to produce a variety of compounds that exert a direct antimicrobial action toward competing bacteria and viruses.

Lactic acid can be considered to be a key antimicrobial compound produced by lactobacilli. Many lactobacilli are also reported to secrete antimicrobial peptides called bacteriocins. These bacteriocins are usually active against closely related bacteria that are likely to reside in the same ecological niche. Other antimicrobial compounds include organic acids, fatty acids, hydrogen peroxide, and diacetyl(Lebeer.S *et al.* 2008).

Similarly there are antimicrobial compounds synthesized by *Bacillus sp.* like there are gene encoded Bacteriocins for eg subtilisin produced by *B.subtilis* which have Bactericidal and immunomodulating activities (Zheng.G *et al.* 1999).

Also Surfactin, a cyclic lipopeptide antibiotic and biosurfactant produced by *Bacillus subtilis*, is well-known for its interactions with artificial and biomembrane systems (e.g., bacterial protoplasts or enveloped viruses) has been assessed for its number of biological activities like (Vollenbroich. D *et al.* 1996)

- It exhibits antifungal properties,
- moderate antibacterial properties, and
- hemolysis; inhibits fibrin clot formation ;
- induces the formation of ion channels in lipid bilayer membranes ;
- inhibits enzymes such as cyclic AMP phosphodiesterase ;
- exhibits antiviral and antitumor activities

More work is being carried on *Bacillus sp.* for exploring such kind of characteristics which makes it stand as a potential probiotic.

CHAPTER-3

MATERIALS & METHODS

3.1 MATERIALS

3.1.1 Bacterial strains

Four strains *Bacillus subtilis* MTCC 2451, *Bacillus licheniformis* MTCC 2450, *Lactobacillus plantarum* MTCC 2941 , *Lactobacillus rhamnosus* MTCC 1408 were obtained from IMTECH, Chandigarh in lyophilized form (preparation date:25th Sep. 2010). Lyophilized forms were stored as glycerol stocks for long term preservation.

For this, 50% glycerol was prepared and autoclaved. For each glycerol stock, 0.85 ml glycerol and 0.15 ml water were put in an eppendorf tube and the lyophilized culture was inoculated and stored at -80 ° C.

The lyophilized culture was revitalized in Nutrient Broth for *Bacillus subtilis* and *Bacillus licheniformis* at 37^{0}C for 48hrs and in MRS Broth for *Lactobacillus plantarum* at 30^{0}C and *Lactobacillus rhamnosus* at 37^{0}C both for 24hrs.. For carrying out daily experiments, cultures were incubated on Nutrient Agar (for *Bacillus subtilis* and *Bacillus licheniformis*) and MRS Agar (*Lactobacillus plantarum* and *Lactobacillus rhamnosus*) plates and slants at 37°C and then stored at 4°C.

3.1.2 Chemicals

3.1.2.1 Nutrient Broth(Himedia)

INGREDIENTS	*GRAMS*
Peptone	10.0
Meat Extract	10.0
Sodium Chloride	05.0
Distilled Water	1000 mL

3.1.2.2 Nutrient Agar(Himedia)

INGREDIENTS	*GRAMS*
Peptone	5.0
Beef Extract	1.5
Yeast Extract	1.5
Sodium Chloride	5.0
Agar	15.0
Distilled Water	1000 mL

3.1.2.3 MRS Broth(Himedia)

INGREDIENTS	*GRAMS*
Peptone	10.0
Yeast Extract	05.0
D – Glucose	20.0
Polysorbate-80	01.0
K_2HPO_4	02.0
Sodium Acetate	05.0
Tri – ammonium citrate	02.0
$MgSO_4.7H2O$	0.1
$MnSO_4.4H2O$	0.05
Distilled Water	1000 mL

3.1.2.4 MRS Agar (Himedia)

INGREDIENTS	*GRAMS*
Peptone	10.0
Beef Extract	8.0
Yeast Extract	5.0
Ammonium citrate	2.0
Sodium Acetate	5.0
$MgSO_4.7H2O$	0.20
$MnSO_4.4H2O$	0.05
K_2HPO_4	2.0
D- Glucose	20.0
Polysorbate 80	1.0
Distilled Water	1000 mL

3.1.2.5 Crystal violet (primary stain)

3.1.2.6 Iodine solution/Gram's Iodine (mordant that fixes crystal violet to cell wall)

3.1.2.7 Decolorizer (e.g. ethanol)

3.1.2.8 Safranin (secondary stain)

3.1.2.9 Distilled Water (preferably in a squirt bottle)

3.1.2.10 Anaerobic Culture Jar

3.1.2.10.1 Petri Plate Carrier: The carrier can accommodate upto 10 Petridishes of 100 mm (4") diameter.

3.1.2.10.2 Plain Lid: It is used when anaerobic conditions are generated through ready made gas packs available in the market. The jar capacity is 3.5 litres, hence gas pack of 3.5 litres capacity to be used.

3.1.2.10.3 Three Finger Clamp with Screw : This is used to tight the lid on the jar on three sides to avoid any kind of leakage from the jar.

3.1.2.10.4 Silicon 'O' Ring: It is made up of pure Silicon rubber. It is to be fitted on the flange of the anaerobic jar.

3.1.2.10.5 Gas Pack: This is disposable oxygen absorbing and carbon dioxide generating agent for use in anaerobic jars. No catalyst or pressure gauge is necessary for this pack since no gas pressure is generated.

3.1.2.10.6 Indicator Tablet: These indicator tablets are pink in colour and remain pink in the jar if the anaerobic conditions are generated. If there is any leakage in the jar it means the anaerobic conditions are not generated then these tablets converted into purple.

3.1.2.11 Saline

Saline was prepared by adding 0.5% w/v of NaCl in distilled water.

3.1.2.12 Pepsin

Pepsin was used at a concentration of 0.3% w/v in saline.

3.1.2.13 Antibiotics

3.1.2.13.1 Ampicillin

Ampicillin was used at a concenteration of 10 µg/ml for all the four strains. Stock was made of 50 mg/ml in distilled water.

3.1.2.13.2 Chloramphenicol

Chloramphenicol was used at a concenteration of 30 µg/ml for all the four strains and the stock was made of 34 mg/ml in 100% ethanol.

3.1.2.13.3 Kanamycin

Kanamycin was used at a concenteration of 30 µg/ml for all the four strains and the stock was made of 10 mg/ml in distilled water.

3.1.2.13.4 Tetracycline

Tetracycline was used at a concenteration of 30 µg/ml for all the four strains and the stock was made of 5 mg/ml in 70% ethanol.

3.1.2.13.5 Streptomycin

Streptomycin was used at a concenteration of 10 µg/ml for *Lactobacillus plantarum* and *Lactobacillus rhamnosus* and on the other hand, 25 µg/ml concenteration was used for *Bacillus subtilis* and *Bacillus licheniformis*. The stock was made of 50 mg/ml in distilled water.

3.1.2.14 Bile Salt

Sodium Glychocholate (1mM concenteration).

3.1.2.15 Glycine

Glycine stock was made at a concenteration of 10 nmol/litre.

3.1.2.16 Diethylthriotol (10 mM)

10 mM working solution was prepared from 100 mM stock of DTT.

3.1.2.17 Sodium Phosphate Buffer (0.1 M sodium-phosphate buffer pH 7.0)

100ml stock solution of Sodium Phosphate Buffer was made by adding 1.4 grams of Sodium dihydrogen phosphate dihydrate ($NaH_2PO_4.2H_2O$) (monobasic) , 1.56 grams of Di Sodium hydrogen phosphate(Na_2HPO_4) (dibasic) in 50ml of distilled water. The pH of the solution

was adjusted to 7 by addition of sodium hydroxide solution (1 N) and volume was made up to 100 ml with distilled water.

3.1.2.18 Sodium Phosphate Buffer (0.1 M sodium-phosphate buffer pH 6.0)

100ml stock solution of Sodium Phosphate Buffer was made by adding 1.4 grams of Sodium dihydrogen phosphate dihydrate ($NaH_2PO_4.2H_2O$) (monobasic) , 1.56 grams of Di Sodium hydrogen phosphate(Na_2HPO_4) (dibasic) in 50ml of distilled water. The pH of the solution was adjusted to 6 by addition of Hydro chloride solution (1 N) and volume was made up to 100 ml with distilled water.

3.1.2.19 Sodium Citrate Buffer (0.5 M sodium-citrate buffer pH 5.5)

200 ml stock solution of Sodium Citrate Buffer was made by adding 21.01 grams of citric acid (monohydrate), 29.41 grams of sodium citrate (dihydrate) in 150 ml of distilled water.The pH of the solution was adjusted to 5.5 by addition of sodium hydroxide solution (1 N) and volume was made up to 200 ml with distilled water.

3.1.2.20 Ninhydrin Reagent

200 ml stock of ninhydrin reagent was prepared by adding 52.63 ml of 1% (wt/vol) ninhydrin in 0.5*M* sodium-citrate buffer,pH 5.5, 31.58 ml of glycerol, and 5.26 ml of 0.5 *M* sodium-citrate buffer pH 5.5.

3.1.2.21 TCA solution

TCA solution was made up of 15% (wt/vol) in distilled water.

3.2 METHODS

3.2.1 Gram Staining

Gram staining is a common technique used to differentiate two large groups of bacteria based on their different cell wall constituents. The Gram stain procedure distinguishes between Gram positive and Gram negative groups by coloring these cells red or violet. Gram staining

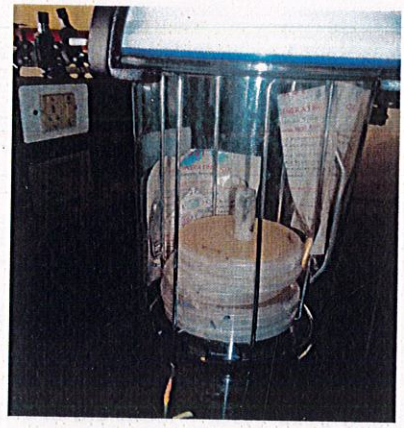
involves three processes: staining with a water-soluble dye called crystal violet, de colorization, and counterstaining, usually with safranin. Due to differences in the thickness of a peptidoglycan layer in the cell membrane between Gram positive and Gram negative bacteria, Gram positive bacteria (with a thicker peptidoglycan layer) retain crystal violet stain during the de colorization process, while Gram negative bacteria lose the crystal violet stain and are instead stained by the safranin in the final staining process. We made slides of each of our strain colony sample. We heated the sample to the slide by carefully passing the slide with a drop or small piece of sample on it through a Bunsen burner three times. Then, added the primary stain (crystal violet) to the sample/slide and incubated for 1 minute. Rinsed the slide with a gentle stream of water for 5 seconds to remove unbound crystal violet. Then, added Gram's iodine for 1 minute- this is a mordant, or an agent that fixes the crystal violet to the bacterial cell wall. Rinsed the sample/slide with alcohol for ~3 seconds and rinsed with a gentle stream of water. The alcohol can decolorize the sample if it is Gram negative, removing the crystal violet. However, if the alcohol remains on the sample for too long, it may also decolorize Gram positive cells. The secondary stain, safranin, was added to the slide and incubated for 1 minute. Then, it was again washed with a gentle stream of water for 5 seconds, and then viewed under a microscope. If the bacteria are Gram positive, it can retain the primary stain (crystal violet) and not take the secondary stain (safranin), causing it to look violet/purple under a microscope. If the bacteria are Gram negative, it will lose the primary stain and take the secondary stain, causing it to appear red when viewed under a microscope.

3.2.2 Anaerobisis

Firstly, for checking the anaerobisis, we streaked our grown cultures of *Bacillus subtilis, Bacillus licheniformis, Lactobacillus plantarum, and Lactobacillus rhamnosus* on petri dishes. Apart from these strains, we also took *Bifidobacterium bifidium* NCDC 255 as a control for checking the Anaerobisis, as it is considered to be an obligate anaerobe. After streaking, the plates were properly sealed with parafilm. Then, the petri dishes were placed in the carrier. The anaerobic indicator tablet sachet was cut open and one tablet pack was removed. This pack was inserted into the smaller clip on the plate carrier immediately. The

plate carrier was put into the base of Polycarbonate jar. After this, the Anaerobic Gas Pack was cut off from the top, as indicated by the cut mark and placed it in the bigger clip of the plates carrier and 50ml of water was poured into it. Then, we placed the lid on the jar making sure that the 'O' ring is correctly in place against the flange of jar as a secure fit. Then, the three finger clamp was applied and it was screwed down the knob until tight as shown in figure 4. Then, the whole Anaerobic Gas Jar was put into the incubator at 37° C for 24 hours.

Fig 3.1: Anaerobic Jar

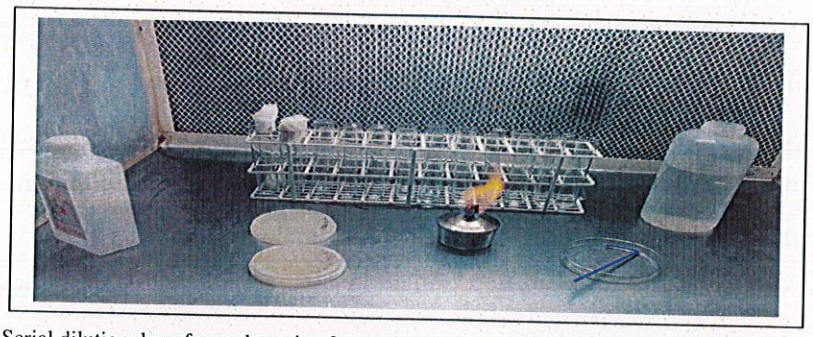


Plates for *L.plantarum,L.rhamnosus,B.subtilis,B.licheniformis and* control plate of *Bifidobacterium*

3.2.3 Acid Tolerance Test

All four cultures were grown at their desired conditions. Then, vials with preweight were taken and each strain was put in respective vials, centrifuged at 10,000 rpm for 10 minutes, supernatant was discarded, so as to get 2% net weight of each culture. Gastric juice was prepared by adding Saline (0.5% w/v NaCl) and Pepsin (0.3% w/v). Following this pH of gastric juice was adjusted to 2.0 and the gastric juice was then filter sterilized by 0.2µ filters. After retaining the pellet, it was washed with Sodium phosphate buffer (pH 7). Gastric juice was added to each vial and then strains were kept at 30, 60, 90, 120 minutes of incubation. (Ashraf.M *et al.* 2009). After completion of the incubation, all the vials were centrifuged at 10, 000 rpm for 10 minutes. The supernatant was discarded and to each vial autoclaved distilled water was added and the solution was made homogenous by vortexing. (Ashraf.M *et al.* 2009).

Fig 3.2: Serial Dilution



Serial dilution done for each strain after each time interval i.e after 30, 60, 90 and 120 min;

Then, we took 1 ml of this homogenous solution and was serially diluted (for *Lactobacillus* strains dilution was done till 10^{-8} and 10^{-6} for *Bacillus* strains) as shown in Figure 4. After this, CFU was done from 10^{-2}, 10^{-4} and 10^{-6} dilution tubes for *Bacillus* strains and 10^{-4}, 10^{-6}, 10^{-8} dilution for *Lactobacillus* strains.

3.2.4 Antibiotic Resistance Test

The disc diffusion (DD) method is the most widely used one for Antibiotic Resistance Test because of its high degree of reliability towards the standardization of the antibiotic concentration and its relative ease of performance. The primary role of the culture medium in the DD technique is to supply an optimal nutritional environment to support growth of the test organism. In addition, it should also provide a suitable gel matrix to allow reproducible and uniform diffusion of the antibiotic agent hereby minimizing possible chemical interactions between undefined medium components and the antibiotic gradient. Also, this method is the most common method for performing Antibiotic resistance test.

In this test, each strain was spreaded uniformly onto the surface of an agar plate, i.e. Nutrient Agar plate for *Bacillus subtilis* and *Bacillus licheniformis*, and MRS Agar plate for *Lactobacillus plantarum* and *Lactobacillus rhamnosus*. Stocks of all the antibiotics to be used were made and were filter sterilized by 0.2µ filter. A filter disk impregnated with a standard amount of an antibiotic was applied to the surface of the plate and each antibiotic was allowed to diffuse into the medium. We checked resistance of five Antibiotics, namely, Ampicillin (10 µg/ml), Streptomycin (10 µg/ml) for *Lactobacillus plantarum and*

Lactobacillus rhamnosus, Streptomycin (25 µg/ml) for *Bacillus subtilis and Bacillus licheniformis* (Hemalatha.S *et al.* 2010), Tetracycline (30 µg/ml), Kanamycin (30 µg/ml), Chloramphenicol(30 µg/ml), for all the four strains (Charteris WP *et al.* 1998). Following this, the plates were incubated for 24 hours at 37°C, and were observed for zone of inhibition. The size of the zone of inhibition is dependent on the diffusion rate of the antibiotic, the degree of sensitivity of the microorganism, and the growth rate of the bacterium. (Temmerman.R *et al.* 2002).

The test was performed under standardized conditions and standard zones of inhibition have been established for each antibiotic. If the zone of inhibition is equal to or greater than the standard, the organism is considered to be sensitive to the antibiotic. If the zone of inhibition is less than the standard, the organism is considered to be resistant (Charteris WP *et al.* 1998).

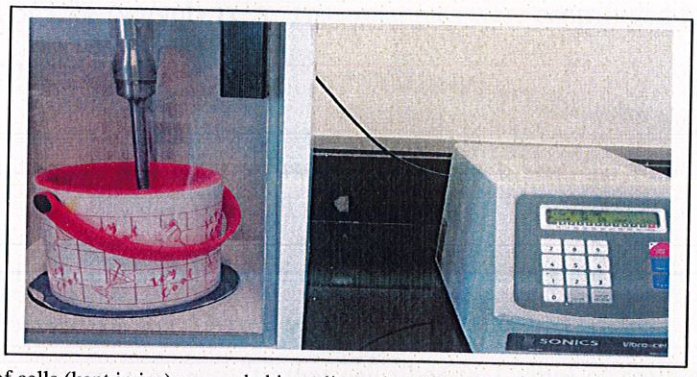
3.2.5 Bile Salt Hydrolase Assay

Bile salt hydrolase (**BSH**) (Cholylglycine hydrolase, E.C.3.5.1.24) is an enzyme produced by the intestinal microflora that catalyses the hydrolysis of glycine or taurine- conjugated bile acids into the amino acid residue and the bile acid. Bile salt hydrolysis is part of the bile salt metabolism in mammals and is dependent on the intestinal microflora.

For the quantitative assay, BSH activity was determined in cell extracts, which were prepared as follows.

Cells of an overnight culture were sedimented for 10 min at 10,000 RPM at 4°C, washed twice in 0.1 *M* sodium-phosphate buffer pH 7.0, re-suspended in the same buffer (20 ml) such that the net weight for each was 5% and vortexed. To reduce oxidation of the enzyme, 10 m*M* DTT (2 ml) was added.

Figure 3.3: Sonication of the Cells



Sonication of cells (kept in ice) suspended in sodium phosphate buffer(pH-7) each for 3min; pulse on for 30 sec ,pulse off for 10 sec;

This cell suspension was sonicated for 3 min (pulse on - 30sec, pulse off – 10 sec) with 50% amplification and constant cooling as shown in Figure 5. The mixture was centrifuged for 10 min at 10,000 RPM at 4°C. The supernatant was stored as cell extract (H. TANAKA *et al.* 1999).

Determination of BSH activity was performed in a two-step process. Firstly by the determination of the amount of the amino acids liberated from the bile salts.

To 900 μl of reaction buffer (0.1 *M* sodium-phosphate, pH 6.0), 50 µl of 1m*M* bile salt, 10 m*M* DTT, and 50 µl of enzyme extract were added in the eppendorf as shown in Figure 6 and the incubation was carried out at 37°C.

Fig 3.4: Addition of Different salts to carry out Enzyme Assay o BSH

Pipeting in 0.1 *M* sodium-phosphate, pH 6.0,1mM bile salt, 10mM DTT to the eppendorf and to this we add Enzyme Extract of each;set for incubation at 37^0C for 10 min

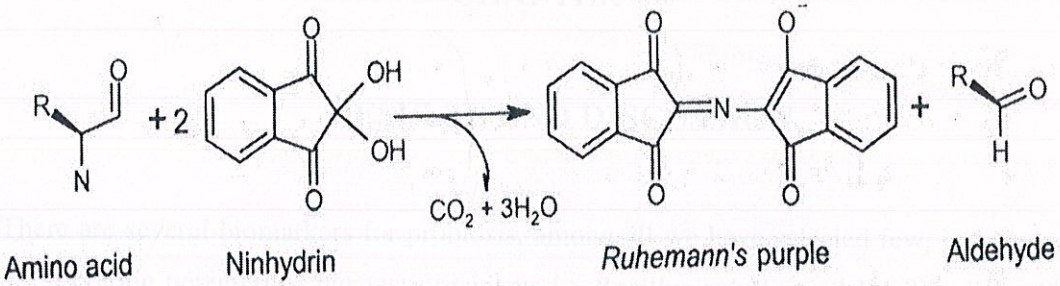
Hundred-microliter samples were taken after 10 min. incubation and were mixed immediately with 100 μl of 15% (wt/vol) TCA. These samples were centrifuged for 10 min at 15,000 RPM at 4°C to remove the precipitate.

For the second reaction, an aliquot of the first reaction was taken. To this , 3.8 ml of ninhydrin reagent
was added, thoroughly mixed, and boiled for 15 min. Then the tubes were cooled down for 3 min in tap water, and the absorbance at 570 nm was measured.
Enzyme unit is defined as the enzyme activity that can liberate 1 μmol of amino acids from the substrate per minute(R. Suresh Kumar *et al.* 2006;H. TANAKA *et al.* 1999).

3.2.5.1 Glycine Standard Curve :

Estimation of Amino Acid (Glycine) was done by ninhydrin method. Ninhydrin, also known as triketohydrindene hydrate reacts with amino acids to give a purple coloured complex (Ruhemann's purple) with an absorption maximum at 570 nm. However, imino acids such as proline and hydroxyl-proline yield a yellow colour with an absorption maximum at 440 nm.
Ninhydrin oxidizes the amino acid (glycine) to aldehyde, releasing carbondioxide and ammonia. During the course of reaction, ninhydrin gets reduced to hydridantin. The hydridantin formed condenses with ninhydrin in the presence of ammonia to yield a purple coloured complex – Ruhemann's complex.(Rao.B, Deshpande.V; Experimental Biochemistry: A Student Companion Textbook; IK Int. Pvt. Ltd;pp100-102)

Fig 3.5: Ninhydrin Reaction with Amino Acids



For preparing 10 µl/ml of stock, 37.53 mg of Glycine was taken and dissolved in 50 ml of distilled water. From this, to make a working standard solution of 500 nmol/ ml , 0.5 ml was taken and 9.5 ml of distilled water was added to it. We worked with various concentrations of glycine starting from 150 nmol/ ml, 200 nmol/ml, 250 nmol/ml, ... upto 500 nmol/ml, and to final 1 ml volume of these standard glycine solutions, 2 ml of ninhydrin reagent was added. Then, we heated the test tubes covered with foil and parafilm, in boiling water for 15 minutes.

The tubes were cooled to room temperature; the purple colour developed was measured against the reagent blank at 570 nm in a spectrophotometer and recorded the absorbance.

Then, we constructed a calibration curve on a graph paper, by plotting the glycine concentration (150-500 nmol/ml) on x-axis and absorbance at 570 nm on y-axis. Computed the concentration of glycine in the sample from the calibration curve.

One unit of BSH activity is defined as the amount of enzyme that liberates 1nano mol of the amino acid from substrate per min.

CHAPTER – 4

RESULTS AND DISCUSSION

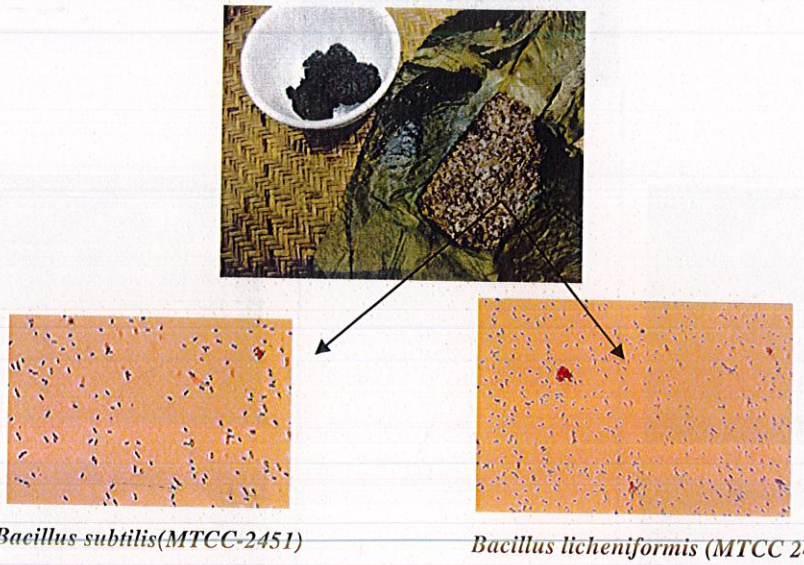
There are several biomarkers for probiosis, among all we have selected few, in order to test the probiotic potential of our test organisms i.e *Bacillus subtilis and Bacillus licheniformis* isolated from fermented food product.

4.1 RESULTS

4.1.1 Gram Staining

It was done for the morphology characterization of the strains which were isolated from fermented bamboo products.

Fig 4.1: Gram stained Bacillus sp. Isolated from Bamboo shoot fermented food product



Bacillus subtilis(MTCC-2451) *Bacillus licheniformis (MTCC 2450)*

B.subtilis(MTCC-2451) and B.licheniformis(MTCC-2450) isolated from fermented Bamboo product are Gram positive rod shaped bacteria seen under 40X Resolution

The strains that were obtained from IMTECH Chandigarh upon Gram Staining showed that both the organisms were Gram positive and were rod shaped as seen under microscope.

4.1.2 Anaerobisis

The environment of the gut is anaerobic so the organism in order to retain in the gut and to grow should have potential to also grow in anaerobic environment.

Bifidobacterium bifidium which is an obligate anaerobe was taken as a control and along with it our test strains i.e *B.subtilis and B.licheniformis* and well known probiotic strains i.e *L.plantarum and L.rhamnosus* were also grown in order to check whether they are growing or not .

Fig 4.2: Plates from anaerobic jar

All the strains showed growth on the plates as comparable to the control organism i.e *Bifidobacterium bifidium* which is an obligate anaerobe thus it was inferred that all have the potential to sustain in anaerobic environment and thus can grow in the gut.

4.1.3 Acid Tolerance

For the organism to sustain in the gut and to be a potential probiotic it is very necessary that it tolerates various stresses one of which is the Gastric juice, which is secreted in stomach having a pH as low as 2, so it's very important that an organism should be checked for gastric tolerance in order to be considered as a candidate for potential probiotic.

Table 4.1: ACID TOLERANCE (pH=2.0) (CFU/ml (done in duplicates))

TIME	*B.licheniformis*	*B.subtilis*	*L.plantarum*	*L.rhamnosus*
T_0	4×10^9	3×10^9	3×10^{11}	5×10^{11}
T_{30}	2×10^9	1×10^9	1×10^{11}	3×10^{11}
T_{60}	2×10^8	4×10^8	2×10^{10}	1×10^{10}
T_{90}	2×10^7	6×10^8	5×10^{10}	5×10^{10}
T_{120}	6×10^7	4×10^8	1×10^{10}	4×10^{10}

Acid tolerance done for four strains, two are the test strains i.e *B. subtilis and B.licheniformis* and two control strains *i.e L.plantarum and L.rhamnosus.* CFU/ml is calculated by plating 100µl of 10^{-8} dilution on MRS Agar Plates (for *l.plantarum and L.rhamnosus*) and 100 µl of 10^{-6} dilution on Nutrient Agar plates(for *B.subtilis and B.licheniformis*);plates incubated at 37^0C for 24hrs;colonies were counted

We have observed strain dependent acid-tolerance in *Lactobacillus* and *Bacillus* at pHs of 2. At pH 2 *L.plantarum and L.rhamnosus* started with a good amount of initial count and there was not much decrease in the viable count of the two after 120 min of exposure to gastric juice as expected as they are well known probiotics. Whereas in *B.licheniformis,* there was a decrease in the viable count after 60 min of incubation and in 120 min the CFU/ml dropped by power 2 on the other hand *B.subtilis* which started with an initial count of 3×10^9, there

was not much drop in the viable count even after 120 min of incubation but as compared to *Lactobacillus*, *Bacillus* showed comparatively less tolerance.

4.1.4 Antibiotic Resistance Determination

Antibiotic resistance is the ability of a microorganism to withstand the effects of an antibiotic. Checking the resistance of organisms towards various antibiotics is an important criterion for selection as the organisms for sustaining in the gut has to have resistance to these daily consumed antibiotics for their survival.

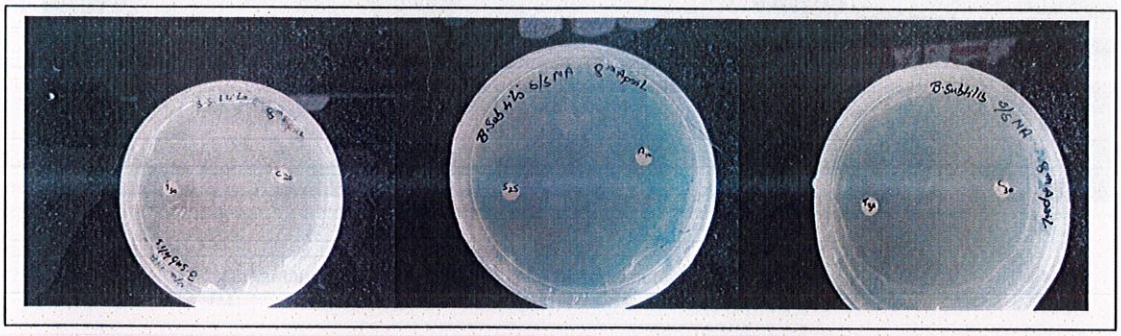
Antibiotic resistance character of all the strains was determined using Ampicillin, Kanamycin, Streptomycin, Tetracycline, Chloramphenicol by Kirby Bauer Method and Diameter of Zone of Inhibition was measured.

Table 4.2: Antibiotic resistance character of the four strains

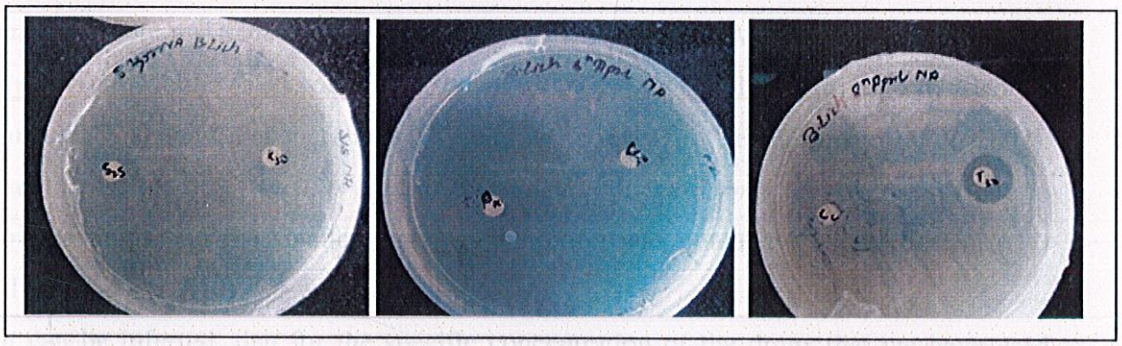
Antibiotic Conc.	*B.licheniformis*	*B.subtilis*	*L.plantarum*	*L.rhamnosus*
Steptomycine(S_{10})	-	-	10	10
Ampicillin (A_{10})	20	22	16	10
Kanamycine (K_{30})	20	20	4	12
Tetracycline (T_{30})	26	18	8	18
Streptomycine(S_{25})	16	20	-	-
Chloramphenicol (C_{30})	10	12	15	14

Ampicillin, Streptomycin, Kanamycin, Tetracycline, Chloramphenicol measured by Kirby Bauer Method and Diameter of Zone of Inhibition is calculated (mm); MRS plates (for *L.plantarum and L.rhamnosus)* incubated at 37^0c for 24hrs and Nutrient plates (for *B.subtilis and B.licheniformis)*

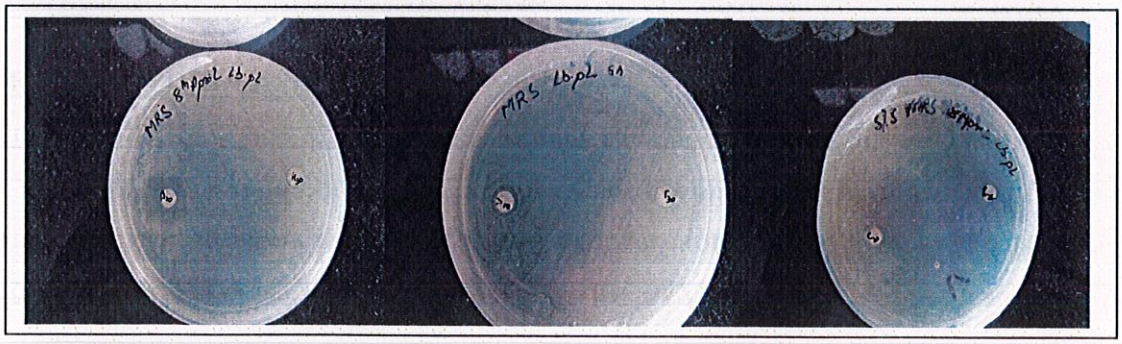
Fig 4.3: Results of disk diffusion of *Bacillus subtilis, Bacillus licheniformis Lactobacillus plantarum, and Lactobacillus rhamnosus*



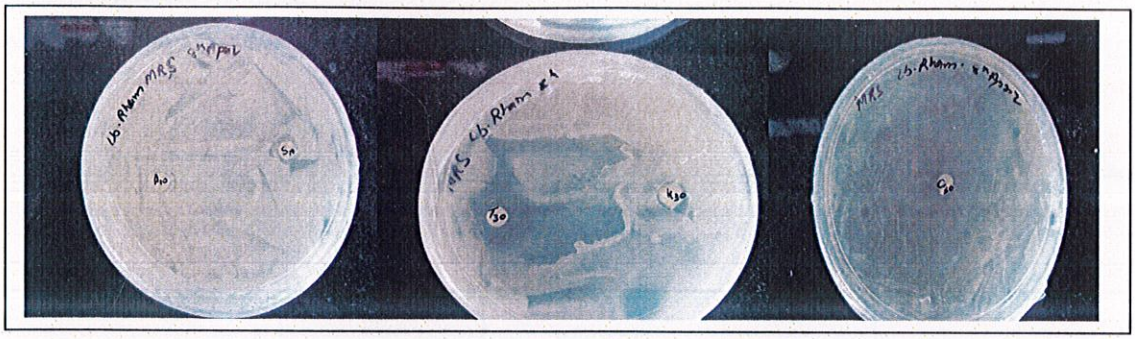
Bacillus subtilis spreaded on Nutrient agar plates;incubated at 37^0C for 24 hrs



Bacillus licheniformis spreaded on Nutrient agar plates;incubated at 37^0C for 24 hrs



L.plantarum spreaded on MRS agar plates;incubated at 37^0C for 24 hrs



L.rhamnosus spreaded on MRS agar plates;incubated at 37^0C for 24 hrs

It was inferred by looking at the diameter of Zone of Inhibition that *L.plantarum and L.rhamnosus* were resistant to kanamycine(K_{30}) and Ampicillin (A_{10}) and *L.plantarum* was resistant to Tetracycline (T_{30}) whereas *L.rhamnosus* was moderately sensitive to it. Also for Steptomycine(S_{10}) and Chloramphenicol (C_{30}) both were resistant (Temmerman.R *et al.* 2002). Whereas in case of both *Bacillus subtilis and Bacillus licheniformis* they were sensitive to most of the drugs and the smallest zone of inhibition was noted in Chloramphenicol (C_{30}) (Hemalatha.S *et al.* 2010).

Thus it can be inferred, that for the specific concentration of the drugs that we have used *Bacillus subtilis and Bacillus licheniformis* are sensitive whereas *L.plantarum and L.rhamnosus* are resistant.

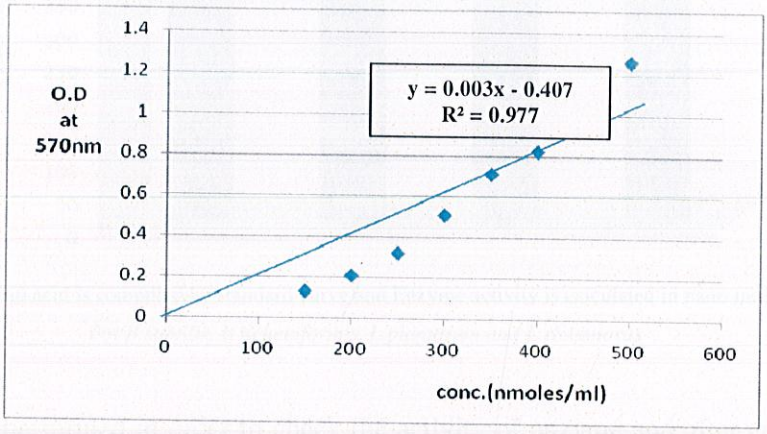
4.5 BILE SALT HYDROLASE ASSAY

BSH is an intracellular enzyme which leads to de conjugation of conjugated Bile salts in the gut thus providing tolerance to organism against bile acids not only this it have number of other benefits as well, therefore it is a important marker for probiosis and it's presence is critical for the survival of organism in the gut.

4.5.1 Glycine Standard Curve

Amino acid standard plot was made by Ninhydrin Test using glycine as standard.

Fig 4.4: Glycine standard curve



Different working conc of Glycine (nanomoles/ml) were taken (100,150….500nanomoles/ml);ninhydrin reaction is carried out and O.D taken at 570nm.

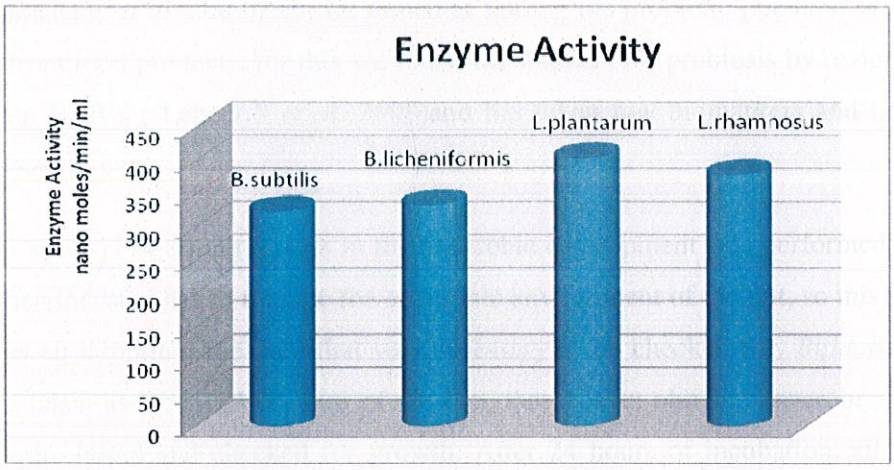
4.5.2 BSH Activity

One unit of BSH activity is defined as the amount of enzyme that liberates 1nano mol of the amino acid from substrate per min per ml and is given as –

$$\text{Enzyme Activity} = \frac{\text{amount of Amino acids liberated (nano moles)}}{\text{Time (min)} \times \text{ml}}$$

(R. Suresh Kumar *et al.* 2006;H. TANAKA *et al.* 1999).

Fig 4.5: Enzyme Activity of different strains



Concentration of amino acid is computed by standard curve and Enzyme activity is calculated in nano mole per min per ml For *B.subtilis, B.licheniformis, L.plantarum and L.rhamnosus*

BSH assay was performed in order to check the activity of enzyme and after calculating the activity from the bar diagram it was inferred that *L.plantarum* had the maximum activity of all and *L.rhamnosus* , *B.subtilis* and *B.licheniformis* had comparable activity showing that *Bacillus* has a capability of deconjugating bile acids to a significant extent.

4.2 DISCUSSIONS

The two strains obtained from IMTECH Chandigarh are well characterized *Bacillus sp.* which were isolated from fermented Bamboo product was morphologically characterized again using Gram staining techniques (Laboratory procedure) and was found to be Gram+ve rod shaped organism. Both of the strains retained crystal violet stain thus showing that cell wall is rich in peptidoglycan. Now these Gram+ve bacillus are known to exist in number of niches like soil, water, dust and also in the GIT of the organisms (Nguyen K. M. *et al.* 2006). There are number of strains of Bacillus which have been tested for their probiotic features but majority of them are taken as spore formulations in the form of drugs (Le H. Duc *et al.* 2003). Like LAB's which are considered to be the well known probiotic and ferment number of food products which have numerous health benefits, there are wide variety of Alkaline

fermented food products which are fermented by Bacillus and also have considerable health benefits(Parkouda.C *et al.* 2009). So, we aimed at finding the probiotic potential of Bacillus sp. isolated from food products, for this we found the markers for probiosis by reviewing the work done on LAB's (Lebeer.S *et al.* 2008)and has taken few biomarkers and tested the potential of our strains.

Study on the survival of all our strains in the anaerobic environment was performed. As any organism when ingested has to tolerate the anaerobic environment of the gut, so this was also considered as an important measure that was necessary to be checked for. *Bifidobacterium bifidum*, was taken as a control because of the fact, that it is an obligate anaerobe, and then our strains were tested and checked for growth. After 24 hours of incubation, all the four strains showed growth suggesting their capability to grow in the anaerobic environment.

Similarly, in order to sustain in the gut it is very important that the potential strains should have the capability to tolerate gastric juices produced by parietal cells in the stomach having pH as low as 2 for about two hours as the transit time of food from stomach is 2-3 hrs. We performed an in-vitro test in which we tried to mimic the gut conditions and exposed our test organisms to pepsin at pH 2 dissolved in saline and compared the results with well known probiotic strains of Lactobacillus (*L.plantarum and L.rhamnosus)* and from the results it was inferred that the tolerance of *B.subtilis and B.licheniformis* to gastric juice was less than the Lactobacillus strains but they had the potential as there was not much fall in the viable count even after 2hrs of exposure(Ashraf.M *et al.* 2009). Then we performed Antibiotic resistance test which is an important biomarker as organisms in order to sustain in the gut have to tolerate various drugs, from our disk diffusion tests for *B.subtilis and B.licheniformis* we have inferred that the organisms were sensitive to all most all the drug concentration taken into account whereas *L.plantarum and L.rhamnosus* were resistant to many of them ((Hemalatha.S *et al.* 2010).Although more indebt characterization of the strains can help to gain more insight into the various characteristics of them.

BSH is an intracellular enzyme and is mainly responsible for deconjugation of Bile salts which is important for gut organisms, as it helps in tolerating the toxic affects of bile also this enzyme helps in lowering the cholesterol level thus proving beneficial for host. Bacillus

strains were tested for the enzyme activity and it was seen that the organisms were showing relatively good activity, comparable to *L.plantarum and L.rhamnosus*. Thus it was inferred that the strains showed the capability to deconjugate the bile salts as they possess good activity and also if these strains would be ingested then they will pose health benefits to the host not only because it possess the enzyme but the activity of the enzyme is also considerable good.

CONCLUSION

The growing and increasing demand of Probiotics and their applications has been a motivation for on – going research activities in the field of fermentation and functional food technology. We reviewed about the various alkaline fermented food products of Oriental and African regions and studied various raw materials used by *Bacillus* to ferment and the nutritional and health benefits provided by these food products upon ingestion, showing that just like *Lactobacilli, Bacillus* also has a potential to provide a huge repertoire of fermented food products which can have conceivable probiotic effects. We tested both *Bacillus sp. and Lactobacillus sp.* for various probiotic markers like Anaerobisis, Bile Salt Hydrolase, Antibiotic Susceptibility Testing, and Acid Tolerance.

Characterization of these two Bacillus strains was done and was found that both belong to the gram positive family of bacteria. Study on the survival of all our strains in the anaerobic environment was performed and all the four strains showed growth suggesting their capability to grow in the anaerobic environment. Upon testing for other probiotic markers like Acid tolerance test, the test strains i.e *Bacillus subtilis and Bacillus licheniformis* showed tolerance to the Gastric juices although the tolerance varied in the two strains but is comparable to well known probiotic strains of Lactobacillus. Studies on the Antibiotic resistance of the strains suggested that both *L.plantarum and L.rhamnosus* were almost resistant to all the formulations as expected but our test strains lied in the sensitive zone. Another observation during the studies on the Bile Salt Hydrolase Assay revealed that enzyme activity that was calculated was better and higher in case of Lactobacillus strains, than observed in the *Bacillus* strains. Across all the four strains, *Lactobacillus plantarum* showed the best enzyme activity and the remaining three (*Bacillus subtilis, Bacillus licheniformis, Lactobacillus rhamnosus*) showed comparable enzyme activity.

In our study, we wanted to test the potential of *Bacillus sp.*, isolated from fermented food product in comparison with well known probiotic Lactobacillus strains and at looking at the test results we hace in turn concluded that with respect to certain biomarkers Bacillus showed comparable activity thus suggesting that it has the potential probiotic features and thus require more indebt characterization in order to be classified among the well known probiotic species.

REFERENCES

Adenike A.O. Ogunshe, Abiodun E. Ayodele and Iheanyi O. Okonko, *Microbial Studies on Aisa: A Potential Indigenous Laboratory Fermented Food Condiment from Albizia saman (Jacq.) F. Mull*, Pakistan Journal of Nutrition, vol. 5 (1), pp. 51-58, 2006

Anamika Singh, Ranjay K Singh, Amish K Sureja, *Cultural significance and diversities of ethnic foods of Northeast India, Indian Journal Of Traditional Knowledge*, vol. 6(1), pp. 79-94, 2007

B. O. Omafuvbe , S. H. Abiose and O. O. Shonukan, *Fermentation of soybean (Glycine max) for soy- daddawa production by starter cultures of Bacillus,* Food Microbiology, vol. 19(6), pp. 561-566, December 2002

B. Sa´ nchez, S. Arias, S. Chaignepain, M. Denayrolles, J. M. Schmitter, P. Bressollier and M. C. Urdaci1 , *Identification of surface proteins involved in the adhesion of a probiotic Bacillus cereus strain to mucin and fibronectin*, Microbiology, vol.155, pp. 1708–1716 , 2005

Charles Parkouda, Dennis S. Nielsen, Paulin Azokpota, Labia Ivette Irène Ouoba, Wisdom Kofi Amoa-Awua, Line Thorsen, Joseph D. Hounhouigan, Jan S. Jensen, K. Tano-Debrah, Bréhima Diawara, and Mogens Jakobsen, *The microbiology of alkaline-fermentation of indigenous seeds used as food condiments in Africa and Asia, Critical Reviews in Microbiology*, vol. 35(2), pp. 139–156, 2009

Charles Parkouda, Bréhima Diawara and Labia Irène Ivette Ouoba, *Technology and physico-chemical characteristics of Bikalga, alkaline fermented seeds of Hibiscus sabdariffa,* African Journal of Biotechnology, vol. 7 (7), pp. 916-922, April, 2008

Charteris WP, Kelly PM, Morelli L, Collins JK, *Antibiotic susceptibility of potentially probiotic Lactobacillus species*, J Food Prot, vol. 61(12), pp. 1636-43, 1998 Dec

David, Oluwole Moses and Aderibigbe, Esther Yemisi, *Microbiology and Proximate Composition of 'Ogiri', A Pastry Produced From Different Melon Seeds,* New York Science Journal, vol. 3(4), 2010

Dirk Vollenbroich,Georg Pauli, Muhsin O¨ Zeland Joachim Vater, *Antimycoplasma Properties and Application in Cell Culture of Surfactin, a Lipopeptide Antibiotic from Bacillus subtilis,* Applied And Environmental Microbiology, Vol. 63, No. 1, pp. 44–49, 1997

Dr. Folasade M. Olajuyigbe, Department of Chemical Sciences, University of Trieste, Via Giorgieri, 1, Trieste , *Some Properties of Extracellular Protease from Bacillus licheniformis Lbbl-11 Isolated from "iru", A Traditionally Fermented African Locust Bean Condiment 1Folasade M. Olajuyigbe and Joshua O. Ajele*, Global Journal of Biotechnology & Biochemistry, vol. 3 (1), pp. 42-46, 2008

Erika Isolauri, Yelda Sütas, Pasi Kankaanpää, Heikki Arvilommi and Seppo Salminen , *Probiotics: effects on immunity,* American Journal of Clinical Nutrition, vol. 73, No. 2, pp. 444S-450s, February 2001

Eszter Pais, Tamas Alexy, Ralph E. Holsworth, Jr., Herbert J. Meiselman, *Effects of nattokinase, a pro-fibrinolytic enzyme, on red blood cell aggregation and whole blood viscosity,* Clinical Hemorheology and Microcirculation, vol. 35, No. 1-2, 2006

Gabriella Casula and Simon M. Cutting, *Bacillus Probiotics: Spore Germination in the Gastrointestinal Tract,* Applied And Environmental Microbiology, vol. 68, No. 5, pp. 2344–2352, May 2002

Guolo Zheng, Liang Z. Yan, John C. Vederas,and Peter Zuber, *Genes of the sbo-alb Locus of Bacillus subtilis Are Required for Production of the Antilisterial Bacteriocin Subtilosin,* Journal Of Bacteriology, vol. 181No. 23, , p p. 7346–7355, Dec. 1999

Hosoi, T., Kiuchi, K; *Bacterial spore formers: probiotics and emerging applications*; pp200-220

H.A. Hong1, J.-M. Huang1, R. Khaneja1, L.V. Hiep2, M.C. Urdaci3, S.M. Cutting1, *The safety of Bacillus subtilis and Bacillus indicus as food probiotics, Journal of Applied Microbiology,* vol. 105, Issue 2, pp. 510–520, August 2008

Hiroshi Tanaka Honoo Hashiba Jan Kok and Igor Mierau, *Bile Salt Hydrolase of Bifidobacterium longum—Biochemical and Genetic Characterization,* Applied And Environmental Microbiology, pp. 2502–2512, vol. 66, No. 6, June 2000

H. tanaka, K. doesburg, T. iwasaki, and I. Mierau, *Screening of Lactic Acid Bacteria for Bile Salt Hydrolase Activity,* Journal of Dairy Science, vol. 82, No. 12, 1999

K.D'Haene, G. Huys, J. Swings' ,*Influence of the culture medium on antibiotic susceptibility testing of food-associated lactic acid bacteria with the agar overlay disc diffusion method,* vol. 34, Issue 6, pp. 402–406, June 2002

K.-L. Chen , W.-L. Kho , S.-H. You , R.-H. Yeh , S.-W. Tang , and C.-W. Hsieh, *Effects of Bacillus subtilis var. natto and Saccharomyces cerevisiae mixed fermented feed on the enhanced growth performance of broilers* , Poultry Science, vol. 88, pp. 309–315, 2009

Le H. Duc, Huynh A. Hong, Teresa M. Barbosa, Adriano O. Henriques, and Simon M. Cutting1, *Characterization of Bacillus Probiotics Available for Human Use*, vol. 70, No., pp. 2161–2171, 4 Apr. 2004

L. Morelli, *In vitro Selection of Probiotic Lactobacilli: A Critical Appraisal,* Curr. Issues Intest. Microbiol., vol. 1(2), pp. 59-67, 2000

L.I.I. Ouoba, B. Diawara, L. Jespersen1 and M. Jakobsen , *Antimicrobial activity of Bacillus subtilis and Bacillus pumilus during the fermentation of African locust bean* (Parkia biglobosa) for Soumbala production, Journal of Applied Microbiology , vol. 102, pp. 963–970, 2007

L.I.I. Ouoba, C. Parkouda, B. Diawara, C. Scotti1 and A.H. Varnam1, *Identification of Bacillus spp. from Bikalga, fermented seeds of Hibiscus sabdariffa: phenotypic and genotypic characterization*, Journal of Applied Microbiology, Journal of Applied Microbiology, vol. 104, Issue 1, pp. 122 – 131, 3 July 2007

M.Ashraf,M.Arshad, *In vitro Screening of Locally isolated Lactobacillus Species For Probiotic Properties*,Pakistan Vet.J., vol. 29(4), pp. 186-190, 2009

M.P. Taranto, F. Sesma, A. Pesce de Ruiz Holgado and G.F. de Valdez, *Bile salts hydrolase plays a key role on cholesterol removal by Lactobacillus reuteri,* Biotechnology Letters, vol. 19, No. 9, pp. 845–847, 1997

Namrata Thapa, Joydeb Pal and Jyoti Prakash Tamang, *Microbial diversity in ngari, hentak and tungtap, fermented fish products of North-East India,* World Journal of Microbiology & Biotechnology, vol. 20, pp. 599–607, 2004

Ma´ire Begley,Colin Hill,and Cormac G. M. Gahan, *Bile Salt Hydrolase Activity in Probiotics,* Applied And Environmental Microbiology, vol. 72, No. 3, pp. 1729–1738, Mar. 2006

Nguyen K. M. Tam, Nguyen Q. Uyen, Huynh A. Hong, Le H. Duc, Tran T. Hoa, Claudia R. Serra, Adriano O. Henriques, and Simon M. Cutting, *The Intestinal Life Cycle of Bacillus subtilis and Close Relatives*, vol. 188, No. 7, pp. 2692–2700, Apr. 2006

Nora Narkie Terlabie, Esther Sakyi-Dawson and Wisdom Kofi Amoa-Awua, *The comparative ability of four isolates of Bacillus subtilis to ferment soybeans into dawadawa,* International Journal of Food Microbiology ,vol. 106, Issue 2, pp. 145-152, February 2006

Orla O'Sullivan, John O'Callaghan, Amaia Sangrador-Vegas, Olivia McAuliffe, Lydia Slattery, Pawel Kaleta, Michael Callanan, Gerald F Fitzgerald, R Paul Ross and Tom Beresford, *Comparative genomics of lactic acid bacteria reveals a niche-specific gene set*, BMC Microbiology, vol. 9 (50), 1471-2180, March 2009

O. K. Achi, *Traditional fermented protein condiments in Nigeria*, African Journal of Biotechnology vol. 4 (13), pp. 1612-1621, December, 2005

Panuwan Chantawannakula, Anchalee Oncharoena, Khanungkan Klanbuta , Ekachai Chukeatiroteb and Saisamorn Lumyonga, *Characterization of proteases of Bacillus subtilis strain 38 isolated from traditionally fermented soybean in Northern Thailand, ScienceAsia,* vol. 28, pp. 241-245, 2002

P. De Boever á R. Wouters á L. Verschaeve, P. Berckmans á G. Schoeters á W. Verstraete, *Protective effect of the bile salt hydrolase-active Lactobacillus reuteri against bile salt cytotoxicity,* Appl Microbiol Biotechnol , vol.53, pp. 709-714, 2000

P. E. Reynolds, *Structure, biochemistry and mechanism of action of glycopeptide antibiotics*, European Journal of Clinical Microbiology & Infectious Diseases, pp. 943-950, 1989

P.Q.Â Wang, P.Â Sun, *Effects of Bacillus subtilis natto on performance and immune function of preweaning calves,* Journal of Dairy Science, vol. 93, Issue 12 , pp. 5851-5855, December 2010

R. Suresh Kumar, James A. Brannigan, Asmita A. Prabhune, Archana V. Pundle, Guy G. Dodson, Eleanor J. Dodson, and C. G. Suresh, *Structural and Functional Analysis of a Conjugated Bile Salt Hydrolase from Bifidobacterium longum Reveals an Evolutionary Relationship with Penicillin V Acylase*, The Journal Of Biological Chemistry, vol. 281, No. 43, pp. 32516–32525, October 27, 2006

R. Temmerman, B. Pot , G. Huys , J. Swings , *Identification and antibiotic susceptibility of bacterial isolates from probiotic products,* International Journal of Food Microbiology, vol. 81 ,pp. 1 – 10, 2002

Rao.B, Deshpande.V; Experimental Biochemistry: A Student Companion Textbook; IK Int. Pvt. Ltd;pp100-102

Sumi H, Hamada H, Tsushima H, Mihara H, Muraki H.,*A novel fibrinolytic enzyme (nattokinase) in the vegetable cheese Natto; a typical and popular soybean food in the Japanese diet*, Experientia,vol.43(10) , Oct 15,1987

Sarah Lebeer, Jos Vanderleyden, and Sigrid C. J. De Keersmaecker, *Genes and Molecules of Lactobacilli Supporting Probiotic Action,* Microbiology And Molecular Biology Reviews, vol. 72, No. 4, pp. 728–764 ,Dec. 2008

Seppo Salminen, Jussi Nurmi and Miguel Gueimonde, *The genomics of probiotic intestinal microorganisms*, BioMed Central Ltd, 2005

S. Hemalatha and S. Shanthi, *In vitro* characterization of bacteriocin producing *Bacillus subtilis* from milk samples; African Journal of Microbiology Research, vol. 4(19), pp. 2004-2010, 4 October, 2010

S. P. Amtai, H. Ibrahim, *Micro-organisms associated with African locust bean (Parkia filicoidea Welw) fermentation for 'dawadawa' production,* Journal of Applied Microbiology, Journal of Applied Microbiology, vol. 61 Issue 2, pp. 145 – 148, 16 May 1986

Wisdom Kofi Amoa-Awua, Nora Narkie Terlabie and Esther Sakyi-Dawson, *Screening of 42 Bacillus isolates for ability to ferment soybeans into dawadawa* , International Journal of Food Microbiology, vol. 106, Issue 3, pp. 343-347 , 15 February 2006

Yanyan Wang, Heping Zhang, Lei Zhang, Wenjun Liu, Yong Zhang, Xinchang Zhang and Tiansong Sun, *In vitro assessment of probiotic properties of Bacillus isolated from naturally fermented congee from Inner Mongolia of China,* World Journal of Microbiology and Biotechnology , vol. 26, No. 8, pp. 1369-1377, 2008

Young Kim, Si Nae Gum, Jean Kyung Paik, Hyo Hee Lim, Kyong-chol Kim, Kazuya Ogasawara, Kenichi Inoue, Sungha Park, Yangsoo Jang and Jong Ho Lee' *Effects of Nattokinase on Blood Pressure: A Randomized, Controlled Trial, Hypertension Research*, vol. 31, pp.1583–1588 , 2008

BRIEF BIODATA OF STUDENTS

Smriti

I am currently pursuing dual degree in biotechnology from Jaypee University of information technology and will be completing my degree in June, 2012. My interests lie in Food Biotechnology and genomics. I want to pursue research as my career in the field of Food technology and its improvements due to my special interests in the same.

Shagun Wadhwa

I am currently pursuing dual degree in biotechnology from Jaypee University of information technology and will be completing my degree in June, 2012. My interests lie in Food Biotechnology .I want to pursue research as my career in the field of Food technology and its improvements.