Screening of potential "functional" properties of Saccharomyces species

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DEPARTMENT OF BIOTECHNOLOGY AND BIOINFORMATICS

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CERTIFICATE

This is to certify that the work titled "***Screening of potential "functional" properties of Saccharomyces species***" submitted by " **Samriti Bedi**" in partial fulfillment for the award of degree of bachelor of technology in biotechnology of Jaypee University of Information Technology, Waknaghat 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.

Signature of Supervisor .....Gargi Dey...

Name of Supervisor: Dr. Gargi Dey

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Date: 21st May, 2011

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On completion of this project, I would like to express my gratitude to all those who had a share in making it a success

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Signature of the student Samriti Bedi

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Date : 21st May, 2010

Summary

The yeast species of *Saccharomyces cerevisiae* has been used in baking, fermented alcoholic beverages, wines, etc. *S.boulardii* has been shown to maintain and restore the natural flora in large and small intestine and is classified as a Probiotic with the potential reducing the symptoms of acute diarrhea, reducing the rate of Clostridium difficile infection, as a prophylactic treatment in antibiotic associated diarrhea etc. Apart from the conventional uses, saccharomyces species have also been used as a reservoir of valuable compounds like Glucans which have the ability to modulate the immune responses.

The present study was performed on *S. cerevisiae* strains which have been isolated from various fermented beverages. These strain were screened for possible "functional" or therapeutic potential. The possible therapeutic potential included identifying the yeast strains with probiotic potential . Another part of the work was also to isolate the cell wall component of the selected yeast strains in order to evaluate the potential bifidogenic activity.

The analysis of the result shows some strains, which survived the gastric juice stress and highly acidic conditions, do have a possible potential Probiotic effect. The cell wall components of the further few selected did not show potential bifidogenic activity

List of figures:

Chapter 1: *INTRODUCTION*

The beneficial properties of strains of some Saccharomyces spp are well documented [58]. In addition to their nutritive value (e.g. provision of vitamins of the B group), probiotic yeasts are generally resistant to gastrointestinal passage and are resistant to most antibiotics. Yeast preparations have also been successfully applied, in combination with antibiotics, to treat Clostridium difficile -related diarrhoea commonly known as antibiotic associated diarrhoea. Probiotic Saccharomyces spp may also help to re-establish a normal gut function after long term antibiotic therapy.

The use of the term *biotherapeutic* agent rather than *probiotic* is because it denotes a microorganism having several therapeutic properties.

The genus Saccharomyces has 16 species, including S. cerevisiae and S. boulardii; of which two , S.cerevisiae and S. boulardii, are described in the literature as containing biotherapeutic agents. These strains have been reported to be efficacious in the prevention or recurrence of different types of diarrhoea and colitis in humans [65]. S.cerevisiae and S.boulardii, have been reported to be effective in the treatment of acute diarrhoea in children [1] and critically ill tube fed patients.

In order to survive and proliferate within the gastrointestinal tract, probiotics must tolerate several environmental hurdles, including the low pH of the stomach, as well as reduced water activity (aw) and bile in the upper small intestine. Furthermore, the ability to persist in the intestine is considered to be a valuable criterion in achieving optimal probiotic efficacy.
For this purpose, the survival of the strains under stressed conditions of the gastrointestinal tract was evaluated by subjecting cells to gastric juice and low pH conditions. Following which the survival of the cells was accounted in terms of cfu(colony forming units).

Apart from this, the strains must also be antagonistic to high salt concentrations which shall prove the feasibility of the strains to be taken to the host as a part of food matrix.

For this, the strain survival was checked with a high salt concentration in media. The therapeutic aspects of yeast is not just limited to it Probiotic efficacy but the activity of the cell wall components is imperative.
The cell walls of S.cerevisiae have been tested in earlier studies to have high concentrations of proteins including β-glucans. Numerous studies have demonstrated that these exhibit antitumor, antimicrobial and even bifidogenic properties. This study was limited to check the content of cell walls for total protein and carbohydrate content and to further evaluate the effect of these cell walls on the growth rate of bifidobacteria, if any.

Chapter 2:

LITERATURE REVIEW

YEAST

Yeast is one of the eukaryotic microorganisms, which has wide applications in various spheres and is being used since very long time. Its application in the breweries and bakeries is well known. The history of application of yeast in industries is very long. This organism is exploited in the production of ethanol, beers, wine and bread. Besides, it is also a source of microbial protein (SCP), and directly used in food, feed and food supplements. Hence, it is also referred as 'nutritional yeast'. Its use as probiotics is not uncommon. Yeast is also a major source of enzymes such as invertase, β-n experiment. galactosidase, alcohol dehydrogenase, glyceraldehydes 3-phosphate, hexokinase, etc. the processed yeast products(yeast extract) are used as food additives or flavours. Some strains of yeast find place in bioremediation. Yeast was the first choice among the eukaryotic microorganisms for the application of recombinant DNA technology. It is the best organism suited for gene manipulation and transformation experiment. Thus, yeast has been used as industrial process organism, as protein source and also as tool in gene manipulation and transformation technologies [39].

Yeast

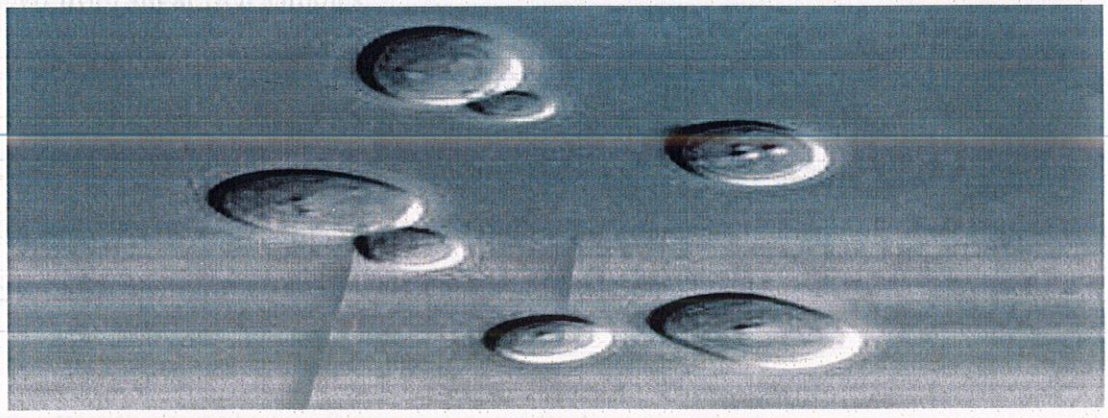

Figure 1: Yeast of the species *Saccharomyces cerevisiae*

Yeasts are a group of eukaryotic microorganisms classified in the kingdom of fungi. Approximately, 1500 species of yeasts have been described, most of which reproduce asexually by budding, although a few cases also reproduce by binary fission[40]. Yeasts are cosmopolitian and widely distributed in almost all natural habitats. The most well known and commercially significant yeasts are the related species and strains of *Saccharomyces cerevisiae.* These organisms have been long utilized to ferment the sugars of rice, wheat,barley and corn to produce alcohol, beverages, and in the baking industry to expand or raise the dough. S. cerevisiae is commonly used as baker's yeast and also for some types of fermentation. Yeast is also often taken as a vitamin supplement because it has 50% protein and also a rich source of B vitamins, niacin and folic acid. During the last two decades, S. cerevisiae has been the model system of the molecular genetic research because of the basic cellular mechanisms of replication , recombination, and cellular metabolism which are generally considered between yeasts and larger eukaryotes, including mammals[15,41,64,].

Growth requirements of yeasts:

Yeasts use organic compounds as the source of energy and donot require light to grow. They are chemoautotrophs. The main source of carbon is obtained from hexose such as glucose or disaccherides such as sucrose and maltose and pentose sugars such as fructose. Some can metabolize alcohols and organic acids. Species of yeast are aerobic,i.e, require oxygen for cellular respiration or are anaerobic, which also have aerobic methods of energy production. Yeasts are ubiquitous and are most frequently isolated from sugar rich samples.

Industrial and biotechnological potential of yeasts:

Yeasts are directly and indirectly very useful microorganisms to mankind. Species and strains of Saccharomyces are widely employed by human beings in

1. Baking industry – S. cerevisiae
2. Distilleries industry- S.cerevisiae and S. diastaticus

3. Wine industry –S. ellipsoideous
4. The production of alcoholic beverages –S. cerevisiae and S. carlsbergengis

The useful physiological properties of yeasts have led to their use in the field of biotechnology. Fermentation of sugars by yeasts is the oldest and the largest application. Many types of yeasts are useful for making many foods such as baker's yeast in the bread making and the brewer's yeast in the process of fermentation to the production of wine and xylitol[41]

As an example, the strains belonging to the yeast species Kluyveromyces marxianus have been isolated from a great variety of habitats, which results in a high metabolic diversity and a substantial degree of intraspecific polymorphism. As a consequence, several different biotechnological applications have been investigated with this yeast: production of enzymes (beta-galactosidase, beta-glucosidase, inulinase, and polygalacturonases, among others), of single-cell protein, of aroma compounds, and of ethanol (including high-temperature and simultaneous saccharification-fermentation processes); reduction of lactose content in food products; production of bioingredients from cheese-whey; bioremediation; as an anticholesterolemic agent; and as a host for heterologous protein production[15].

Yeast as a Nutritional component:

Yeast is used in nutritional supplements popular with vegans and the health conscious, where it is often referred to as "nutritional yeast". It is deactivated yeast, usually *S. cerevisiae*. It is an excellent source of protein and vitamins, especially the B-complex vitamins, whose functions are related to metabolism, as well as other minerals and cofactors required for growth. It is also naturally low in fat and sodium. Some brands of nutritional yeast, though not all, are fortified with vitamin B_{12}. Nutritional yeast, though it has a similar appearance to brewer's yeast, is very different and has a very different taste. Brewer's yeast is a good source of B-complex vitamins but, contrary to some claims, it contains little or no vitamin B_{12}

Baking

Yeast, most commonly *S. cerevisiae*, is used in baking as a leavening agent, where it converts the fermentable sugars present in dough into the gas carbon dioxide. This causes the dough to expand or rise as gas forms pockets or bubbles. When the dough is baked, the yeast dies and the air pockets "set", giving the baked product a soft and spongy texture.[13] The use of potatoes, water from potato boiling, eggs, or sugar in a bread dough accelerates the growth of yeasts. Most yeasts used in baking are of the same species common in alcoholic fermentation. Additionally, *Saccharomyces exiguus* (also known as *S. minor*), a wild yeast found on plants, fruits, and grains, is occasionally used for baking. Sugar and vinegar provide the best conditions for yeast to ferment. In bread making, the yeast initially respires aerobically, producing carbon dioxide and water. When the oxygen is depleted, anerobic respiration, producing ethanol as a waste product; however, this evaporates during baking[48].

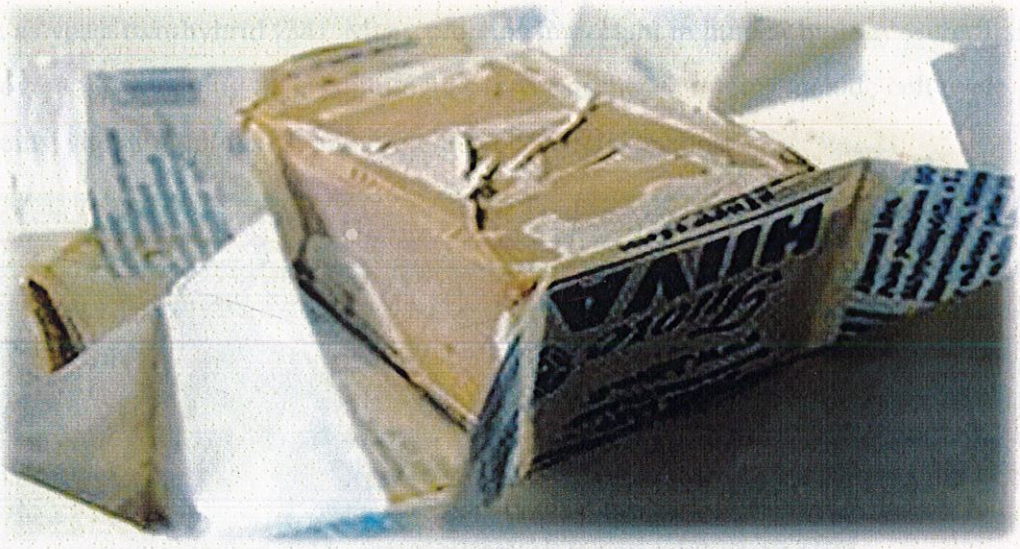

Figure 2: A block of fresh yeast

Bioremediation

Some yeasts can find potential application in the field of bioremediation. One such yeast, *Yarrowia lipolytica*,[51] is known to degrade palm mill[37] effluent TNT(an explosive material) and other hydrocarbons, such as alkalines,[24] fatty acids, fats and

oils. It can also tolerate high concentrations of salt and heavy metals [4], and is being investigated for its potential as a heavy metal biosorbent[5].

Yeast in genetics:

Several yeasts, particularly *S. cerevisiae*, have been widely used in genetics and cell biology. This is largely because *S. cerevisiae* is a simple eukaryotic cell, serving as a model for all eukaryotes, including humans for the study of fundamental cellular processes such as the cell cycle, DNA replication, recombination, cell division and metabolism[72]. Also, yeasts are easily manipulated and cultured in the laboratory, which has allowed for the development of powerful standard techniques, such as yeast two-hybrid [34]. Many proteins important in human biology were first discovered by studying their homologues in yeast; these proteins include cell cycle proteins, signaling proteins, and protein-processing enzymes[73].

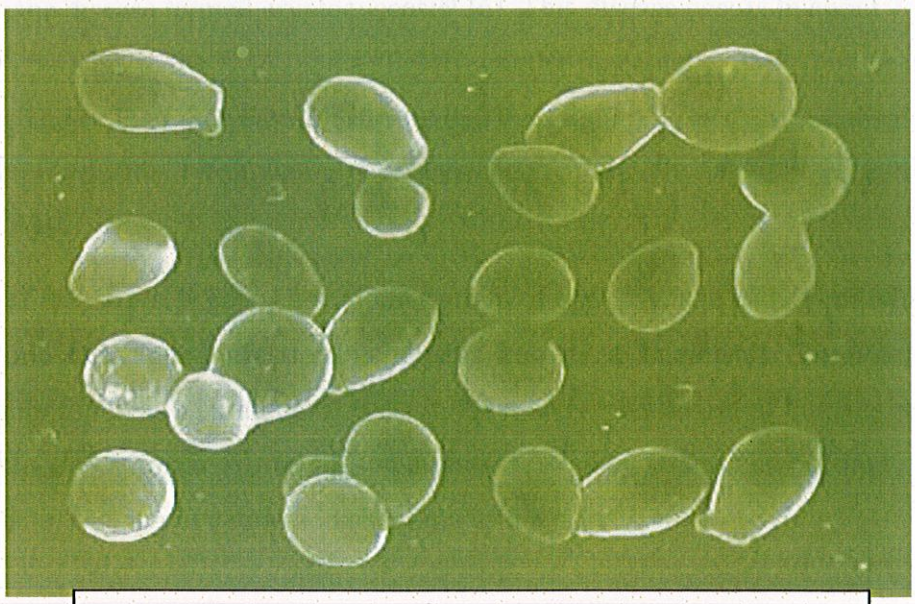

Figure 3: *S. cerevisiae as observed under microscope*

On 24 April 1996 *S. cerevisiae* was announced to be the first eukaryote to have its genome, consisting of 12 million base pairs, fully sequenced as part of the Genome project. At the time, it was the most complex organism to have its full genome sequenced, and took seven years and the involvement of more than 100 laboratories to accomplish. The second yeast species to have its genome sequenced was *Schizosaccharomyces pombe*, which was completed in 2002. It was the sixth eukaryotic genome sequenced and consists of 13.8 million base pairs[57].

YEASTS AS PATHOGENS:

Some species of yeast are opportunistic pathogens where they can cause infection in people with compromised immune systems[67].

Cryptococcus neoformans is a significant pathogen of immunocompromised people causing the disease termed cryptococcosis[36]. This disease occurs in about 7–9% of AIDS patients in the USA, and a slightly smaller percentage (3–6%) in western Europe. The cells of the yeast are surrounded by a rigid polysaccharide capsule, which helps to prevent them from being recognised and engulfed by white blood cells in the human body.

Yeasts of the *Candida* genus are another group of opportunistic pathogens which causes oral and vaginal infections in humans, known as candidiasis. *Candida* is commonly found as a commensal yeast in the mucus membranes of humans and other warm-blooded animals. However, sometimes these same strains can become pathogenic, causing irritation and shedding of the tissues[36].

BIOTHERAPEUTIC POTENTIAL OF YEAST:

- **GENESIS:**

Living microorganisms are widely used for several therapeutic purposes and their beneficial effects as biotherapeutic agents are well known. While certain strains of lactic acid bacteria and bifidobacteria are used as Probiotic in pharmaceutical preparations, feed additives and so-called functional foods yeasts also possess some medicinal efficiency.

The beneficial properties of strains of some Saccharomyces species are well documented [58]. In addition to their nutritive value (e.g. provision of vitamins of the B group), some yeasts are Probiotic which are generally resistant to gastrointestinal passage and are resistant to most antibiotics. Yeast preparations have also been successfully applied, in combination with antibiotics, to treat Clostridium difficile - related diarrhoea commonly known as antibiotic associated diarrhoea. Probiotic Saccharomyces species may also help to re-establish a normal gut function after long term antibiotic therapy [50].

Some Saccharomyces species also have a protective effect, and specific activities, against various enteric pathogens. Saccharomyces species stimulate sIgA production and the phagocytic system of gnotobiotic mice[59]. These probiotic yeasts may also have efficacy in the prevention of Traveler's diarrhoea.

Strains of so-called Saccharomyces boulardii, taxonomic status somewhat unclear since recent work suggests it is a subspecies of Saccharomyces cerevisiae, are regarded as the most prominent representatives of probiotic yeasts within the community of biotherapeutic S. cerevisiae strains. Today, a considerable number of pharmaceutical preparations (capsules, powders, tablets, pellets) containing probiotic yeasts (Saccharomyces spp) cells are commercially available, and are marketed mainly via pharmacies and health stores.

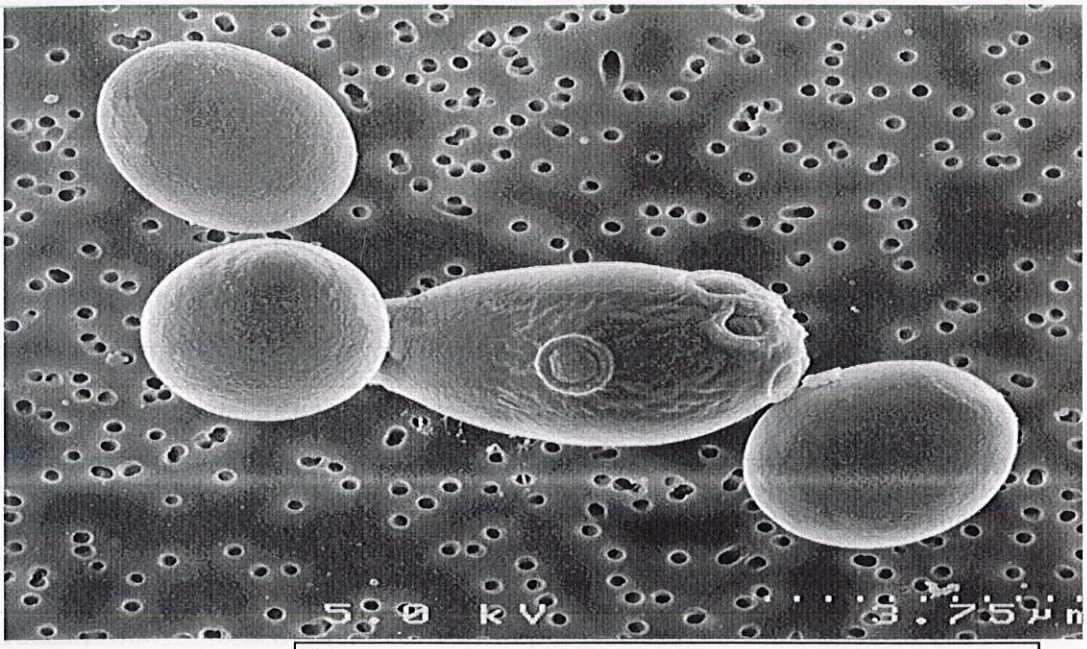

Figure 4: *S.boulardii as seen under microscope*

The use of the term *biotherapeutic* agent rather than *probiotic* is because it denotes a microorganism having several therapeutic properties [46].

Biotherapeutic agents, as with probiotics, must be given in sufficient concentration to exert therapeutic properties, remain stable and viable before use and survive in the intestinal ecosystem of the host to exert their therapeutic properties.

- **PROBIOTICS:**

Probiotics is originally defined as microorganisms promoting the growth of other microorganisms. Recently probiotics have been more precisely defined as " mono or mixed cultures of live microorganisms when applied to animal or man, beneficially affect the host by improving the proportion of indigenous microflora". In relation to food, it can be considered 'viable preparation' in the food or dietary supplements to improve the health of human and animals. Though and impressive number of microbes are considered as probiotics, only those strains that are classifies as Lactic Acid Bacteria and certain Yeasts are considered important with regard to food and nutrition and hence are selected as probiotics, e.g. species of Lactobacillus, Bifidobacterium, Pediococcus, etc.

Besides these some non lactic acid bacteria such as Enterococcus, Bacillus, Clostridium difficle, etc are also considered probiotics[55].

Most probiotics are marketed as foodstuffs or drugs. So safety and functional aspects of probiotics is of the utmost importance while selecting the organisms for use as probiotics. Probiotics help in combating the health related problems including cancer, hypertension, urinogenital infections, enhancement of immune system, lactose intolerance, cholesterol level, ulcer, asthma, etc. Probiotic products are generally milk based. However, efforts are also made for the development or formulation of Probiotic food using other substrates such as cereal and soya. Currently, there is a wide range of Probiotic products commercially viable to consumers. Such products include animal feeds, dairy foods, infant and baby foods, fruit juice based products, and cereal based products and pharmaceuticals [29].

- **HISTORY OF PROBIOTICS:**

The original observation of the positive role played by certain bacteria was first introduced by Russian scientist and Nobel laureate Eli Metchnikoff, who in the beginning of the 20th century suggested that it would be possible to modify the gut flora and to replace harmful microbes with useful microbes. [49] Metchnikoff, at that time a professor at the Pasteur Institute in Paris, produced the notion that the aging process results from the activity of putrefactive (proteolytic) microbes producing toxic substances in the large bowel. [56]Proteolytic bacteria such as clostridia, which are part of the normal gut flora, produce toxic substances
including phenols, indols and ammonia from the digestion of proteins. [30,33]. According to Metchnikoff these compounds were responsible for what he called "intestinal auto-intoxication", which caused the physical changes associated with old age[70].

It was at that time known that milk fermented with lactic-acid bacteria inhibits the growth of proteolytic bacteria because of the low pH produced by the fermentation of lactose. Metchnikoff had also observed that certain rural populations in Europe, for example in Bulgaria and the Russian steppes who lived largely on milk fermented by lactic-acid bacteria were exceptionally long lived. Based on these facts, Metchnikoff proposed that consumption of fermented milk would "seed" the intestine with harmless

lactic-acid bacteria and decrease the intestinal pH and that this would suppress the growth of proteolytic bacteria. Metchnikoff himself introduced in his diet sour milk fermented with the bacteria he called "Bulgarian Bacillus" and found his health benefited. Friends in Paris soon followed his example and physicians began prescribing the sour milk diet for their patients[29].

Bifidobacteria were first isolated from a breast-fed infant by Henry Tissier who also worked at the Pasteur Institute. The isolated bacterium named Bacillus bifidus communis was later renamed to the genus *Bifidobacterium*. Tissier found that bifidobacteria are dominant in the gut flora of breast-fed babies and he observed clinical benefits from treating diarrhea in infants with bifidobacteria. The claimed effect was bifidobacterial displacement of proteolytic bacteria causing the disease.

During an outbreak of shigellosis in 1917, German professor Alfred Nissle isolated a strain of *Escherichia coli* from the feces of a soldier who was not affected by the disease.Methods of treating infectious diseases were needed at that time when antibiotics were not yet available, and Nissle used the *Escherichia coli* Nissle 1917 strain in acute gastrointestinal infectious salmonellosis and shigellosis.

In 1920, Rettger demonstrated that Metchnikoff's "Bulgarian Bacillus", later called *Lactobacillus delbrueckii subsp. bulgaricus*, could not live in the human intestine, and the fermented food phenomena petered out. Metchnikoff's theory was disputable (at this stage), and people doubted his theory of longevity.

After Metchnikoff's death in 1916, the centre of activity moved to the United States. It was reasoned that bacteria originating from the gut were more likely to produce the desired effect in the gut, and in 1935 certain strains of *Lactobacillus acidophilus* were found to be very active when implanted in the human digestive tract. Trials were carried out using this organism, and encouraging results were obtained especially in the relief of chronic constipation.

The term "probiotics" was first introduced in 1953 by Werner Kollath [31]. Contrasting antibiotics, probiotics were defined as microbially derived factors that stimulate the growth of other microorganisms. In 1989 Roy Fuller suggested a definition of probiotics which has been widely used: "*A live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance*".

Fuller's definition emphasizes the requirement of viability for probiotics and introduces the aspect of a beneficial effect on the host.

All effects can only be attributed to the individual strain(s) tested. Testing of a supplement does not indicate benefit from any other strain of the same species, and testing does not indicate benefit from the whole group of LAB (or other probiotics).

- **Saccharomyces species as biotherapeutic agents:**

The genus Saccharomyces has 16 species, including S. cerevisiae and S. boulardii; of which two , S.cerevisiae and S. boulardii, are described in the literature as containing biotherapeutic agents. These strains have been reported to be efficacious in the prevention or recurrence of different types of diarrhoea and colitis in humans [66]. S.cerevisiae and S.boulardii, have been reported to be effective in the treatment of acute diarrhoea in children [1] and critically ill tube fed patients. S.cerevisiae and S.boulardii release polyamines which help in repairing mucous membranes. [6]. These polyamines increase the activity of short chain fatty acids (SCFA) and disaccharide enzymes (lactase, maltase, sucrase). Polyamines stimulate the repair of intestinal cells and the growth of colonic mucosa. Some fermented milks such as kefir and koumiss contain lactic acid bacteria and lactose-fermenting yeasts [65]. Yeasts can also be found in some traditional cheeses. *Debaryomyces hansenii, Kluyveromyces lactis,* and *Yarrowia lipolytica* have frequently been found [38] as predominant species, although they have not been adopted for deliberate use. It is not unusual to find a yeast count of 105 to 107 cells per gram of cheese [21], with beneficial effects such as interaction between starter cultures, production of aroma components, and inhibitory effects against spoilage microorganisms [38]. *Saccharomyces cerevisiae* has been widely applied in industry, and beneficial effects such as promotion of iron absorption [38] and improvement of intestinal conditions [68] have been reported.

Some probiotic supplements use the yeast *Saccharomyces boulardii* to maintain and restore the natural flora in the large and small gastrointestinal tract.*S. boulardii* has been shown to reduce the symptoms of acute diarrhea in children prevent reinfection of *Clostridium difficile* reduce bowel movements in diarrhea predominant IBS patients

and reduce the incidence of antibiotic, traveler's, and HIV/AIDS associated diarrhea. [1,6,20]

S.boulardii is generally administered in lyophilized powder and its application as a food additive has only been reported in a limited number of cases such as in the fermentation of vegetables and incorporation into commercial yogurts [43]. S. cerevisiae and S. boulardii are unique organisms that have the ability to survive in gastric acidity and are not adversely affected or inhibited by antibiotics. They do not appear to alter or adversely affect the normal flora of the GI tract and can be consumed with normal probiotic bacteria [23]. Inclusion of S.boulardii and S.cerevisiae in the standard medical treatment for Clostridium difficile infection has been reported to reduce the risk of recurrence in patients experiencing renewed infection [2].

Children receiving S.boulardii and lactobacilli had a gradual reduction in the number of daily stools, more noticeable after the first day of treatment compared to those in a placebo group. Patients treated with S.boulardii and lactobacilli had a significant faster recovery compared with a placebo control and Lactobacillus species were found to be similarly effective in decreasing the duration of diarrhea [27]. Lactobacilli appear to enhance the beneficial effects of Saccharomyces boulardii on intestinal mucosa . A meta-analysis by Aloysins et al., 2005 suggests that S.boulardii and Lactobacilli can be used to prevent antibiotic associated diarrhea. S.cerevisiae may also have value in the treatment of C.difficile associated diarrhea. S. cerevisiae can also deliver vitamin B and other nutrients like selenium and chromium. S. boulardii.

- **THE GI TRACT:**

The gastrointestinal (GI) microflora ('microbiota') is an extremely complex ecosystem that coexists in equilibrium with the host. When this equilibrium is disrupted, clinical disorders may occur. Microbiota plays a well-established role in infectious GI diseases. Recent research has linked intestinal microbiota disequilibrium to such GI disorders as antibiotic-associated diarrhoea (AAD), ulcers, inflammatory bowel disease (IBD), irritable bowel syndrome (IBS) and colon cancer. Furthermore, the microbiota has been proposed as a major regulator of the immune system outside the gut. Attempts have

been made to improve the health status of affected individuals by modulating the indigenous intestinal flora using living microbial adjuncts called 'probiotics'[32].

Probiotics have been defined as viable micro-organisms that (when ingested) have a beneficial effect in the prevention and treatment of specific pathological conditions. In recent years, the definition of a probiotic has changed, primarily because of the recognition that probiotic bacteria can influence the physiological outcomes, distant from the gut lumen. Moreover, the activation of local mucosal protective mechanisms and the modulation of adaptative immune effector functions can influence protection levels and the degree of inflammation at all mucosal sites. These observations shifted the concept of probiotics from a narrow range of dairy isolates that fermented milk and could 'promote health' to the concept of 'immunobiotics'.[61]
Because viable and biologically active micro-organisms are usually required at the target site in the host, it is essential that the probiotic be able to withstand the host's natural barriers against ingested micro-organisms. Most probiotic micro-organisms are bacteria. Strains of *Lactobacillus acidophilus* and *Lactobacillus rhamnosus* strain GG (formerly *Lactobacillus casei*) probably have the longest history of application as probiotics because of their health benefits. Currently used commercial probiotic products include *Lactobacillus* species, *Bifidobacterium* and even a few non-lactic acid bacteria.

- **Specificity of yeast**

Saccharomyces boulardii, a patented yeast preparation, is the only yeast probiotic that has been proven effective in double-blind studies. [7]. This yeast is used in many countries as both a preventive and therapeutic agent for diarrhoea and other GI disorders caused by the administration of antimicrobial agents. *Saccharomyces boulardii* possesses many properties that make it a potential probiotic agent, i.e. it survives transit through the GI tract, its temperature optimum is 37 °C, both *in vitro* and *in vivo,* it inhibits the growth of a number of microbial pathogens.
However, *S. boulardii* belongs to the group of simple eukaryotic cells (such as fungi and algae) and, it thus differs from bacterial probiotics that are prokaryotes.

- **Yeast in microbial ecology**

Commensal bacteria in the gut constitute a heterogenous microbial system containing approximately 10^{14} bacteria[28]. Yeast are a part of the residual microflora that makes up <0.1% of microbiota. Most yeast isolates from the GI tract are *Candida albicans,* although *Torulopsis glabratra* and *Candida tropicalis* are occasionally recovered.[45] Although yeast account for only a minority of the organisms making up the microbiota, their cell size is 10 times larger than that of bacteria and they could represent a significant stearic hindrance for bacteria.

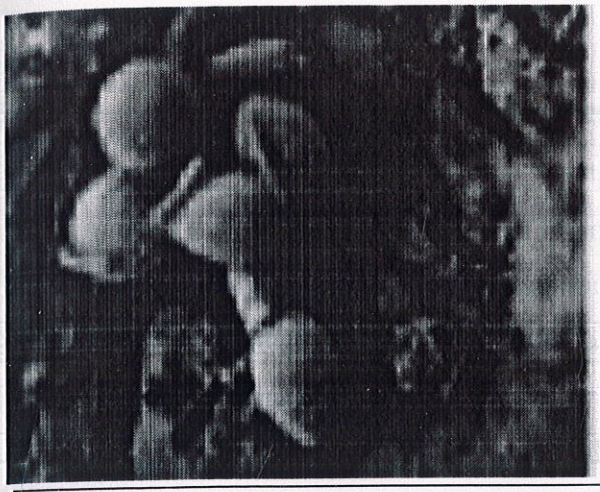

Figure 5: Scanning electron micrographs of T84 cells exposed to *S. boulardii* and *Salmonella typhimurium*.

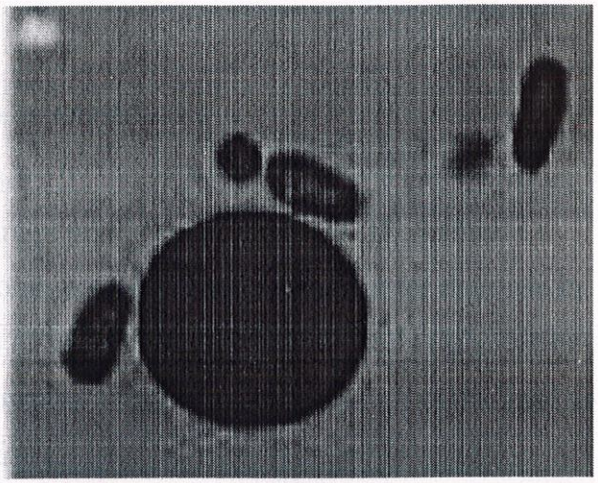

Figure 6: Electron micrographs showing *S. boulardii* and *S. typhimurium*.

Microbial colonization of the human GI tract varies in number and species of bacteria as a function of environmental conditions.[28] The low pH of the stomach, ranging from 2.5 to 3.5, is destructive to most microbes; it grows up towards the distal part of the GI tract. While the pH rises towards the distal part of the GI tract, the presence of aggressive intestinal fluids (e.g. bile and pancreatic juice) and the short transit time in the duodenum, creates a hostile environment, and the duodenum thus contains relatively few microbes. Yeasts are found in the stomach and colon. The presence of yeast in such different conditions can be explained by their resistance to pH variation. In fact, while yeast grows well at pH 7–8, optimal growth is observed between pH 4.5 and 6.5. Most yeast can grow at pH 3.0, and some species can tolerate highly acidic conditions with a pH as low as 1.5. Yeast are thus good candidates as probiotics because probiotics entering the GI tract must be resistant to local stresses such as the presence of GI enzymes, bile salts, organic acids and considerable variations of pH and temperature.

- **Impact of antibiotics on yeast**

The development of antimicrobial resistance by the pathogenic bacteria associated with antibiotic treatment has become an important public health problem. The natural resistance of yeast to antibacterial antibiotics is thus, a major argument for their use in antibiotic-treated patients. Antimicrobial resistance occurs both vertically (inherent or natural resistance of bacterial species or genus) and horizontally because of the transfer of genes between bacteria. The mammalian GI tract provides favourable conditions for the transfer of genetic material between many species of bacteria.[7] Resistance genes might be transferred not only between members of the resident gut flora, but also to and from transient bacterial probiotics. Recently, many investigators have speculated that commensal bacteria, including lactic bacteria, may act as reservoirs of antibiotic resistance genes similar to those found in human pathogens. Genes conferring resistance to tetracycline, erythromycin and vancomycin have been detected and characterized in *Lactobacillus lactis*, Enterococci and, recently, in *Lactobacillus* species isolated from fermented meat and milk products and in strains used as probiotics [11,69]. The main threat associated with these bacteria is that they might transfer resistance genes to pathogenic bacteria. No such transfer of genetic material occurs between bacteria and yeast, making yeast safe for use during antibiotic treatment.

- **Effect on enteric pathogens**

Several studies using animal models or cell models indicated that *S. boulardii* may exert a beneficial effect against various enteric pathogens such as *C. difficile*, *Vibrio cholerae*, *Salmonella, Shigella* and *E. coli*. *Saccharomyces boulardii* appeared to act by two main mechanisms: (i) production of factors that neutralized bacterial toxins and (ii) modulation of the host cell signalling pathway implicated in proinflammatory response during bacterial infection[53].

- **Neutralization of bacterial toxins**

The antitoxin action of *S. boulardii* was demonstrated in cases of *C. difficile* infection. Toxin A, a 308 kDa protein, is a major virulence factor of *C. difficile*. Injection of toxin A into rodent intestines caused fluid secretion, increased mucosal permeability,

mucosal damage and release inflammatory mediators. Oral administration of *S. boulardii* to rats before the addition of toxin A to the intestinal loop reduced toxin A-induced intestinal secretion and permeability.[16] Further investigation demonstrated that the addition of toxin A mixed with *S. boulardii*-filtered supernatant decreased toxin A-induced secretion. Two fractions were identified in *S. boulardii* supernatant: a fraction enriched in a 54 kDa serine protease that acted by proteolysis of both the toxin A and its receptor[17] and, another fraction (<10 kDa) that exerted an anti-inflammatory effect[9]

Saccharomyces boulardii also synthesized a phosphatase that can dephosphorylate endotoxins such as LPS from *E. coli* O55B5 and can partially inactivated its cytotoxic effects[63]. This mechanism may account for the protection afforded in cases of sepsis

- **Antibiotic-associated diarrhoea**

Saccharomyces boulardii is the sole probiotic that has proven a significant efficacy in treating relapsing *C. difficile*-associated diarrhoea. after the administration of *S. boulardii* a 50% reduction of recurrences in patients who had previously experienced a first relapse of *C. difficile* infection was observed.[42]

- **Infectious diarrhoea**

Traveller's diarrhoea

Traveller's diarrhoea is a well-known public health problem, particularly among travellers to developing countries. Enterotoxinogenic*Escherichia coli,* Shigellae and Salmonellae account for about 80% of cases with an identified pathogen in acute diarrhoea in travelers. Kollaritsch *et al.[10]* evaluated the efficacy of *S. boulardii* for the prevention of diarrhoea in 1016 travellers visiting various countries in the world. The incidence of diarrhoea was 40% in patients receiving placebo, 34% in patients receiving *S. boulardii* 250 mg/day ($P = 0.019$) and 29% in patients receiving *S. boulardii* 1 g/day ($P < 0.005$). In a meta-analysis of probiotics for the prevention of traveller's diarrhoea analysing 12 different studies, McFarland[35] concluded that two

probiotics, *S. boulardii* and a mixture of *L. acidophilus* and *Bifidobacterium bifidum,* had significant efficacy

Diarrhoea is a common complication in critically ill patients receiving enteral nutrition. The addition of *S. boulardii* to nutrient supplements administered to patients receiving enteral nutrition decreased the incidence of diarrhoea.

- **Pharmacokinetics**

Pharmacokinetics is the study of the process by which a drug is absorbed, distributed, metabolized, and eliminated by the body. Pharmacokinetics is a branch of pharmacology dedicated to the study of the time course of substances and their relationship with an organism or system. In practice, this discipline is applied mainly to drug substances, though in principle it concerns itself with all manner of compounds residing within an organism or system, such as nutrients, metabolites, endogenous hormones, and toxins.

In this respect, S.cerevisiae and S. boulardii can resist gastric acidity, proteolysis and are able to achieve and maintain high populations in the GI tract. They can permanently colonise the colon and do not easily translocate out of the intestinal tract [11]. They can also be detected alive throughout the digestive system, if they are given daily in freeze dried form .

- **Pharmacodynamics**

Pharmacodynamics is the study of the biochemical and physiological effects of drugs, the mechanisms of drug action and the relationship between drug concentration and effect. Pharmacodynamics is the study of what a drug does to the body, whereas pharmacokinetics is the study of what the body does to a drug .

The pharmacodynamics of S.cerevisiae and S.boulardii involves:

A. Direct antagonism

S.boulardii reduces the growth of Clostridium albicans, Escherichia coli, Salmonella typhi, Shigella dysenteriae, Vibrio cholerae, Salmonella enteritidis, and Clostridium difficile [52]. S.cerevisiae reduces the growth of E. coli , Shigella flexnerii, Clostridium difficile and Vibrio cholerae.

S.cerevisiae and S.boulardii have been shown to protect against various enteric pathogens and members of the family Enterobacteriaceae in animal studies

B. An antisecretory effect by acting specifically on the binding of toxins to intestinal receptors

Pathogenic strains of C. difficile produce two well-characterized toxins, A and B, that cause mucosal damage and inflammation of the colon. S.cerevisiae and S. boulardii significantly reduce the liquid secretion and mannitol permeability caused by C. difficile toxin A in the rat ileum, compared to controls [12].

- **Cell wall components are determinants in the immune response**

Substantial differences in the cell wall composition of bacteria and yeast have an impact on their antigenic responses. All bacteria contain a high-molecular weight sugar associated with protein that forms a rigid structure called peptidoglycan. Gram-negative and Gram-positive bacteria differ in the lipid concentration of their cell wall. Gram-negative bacteria contain up to 20% lipids composed of lipopolysaccharide (LPS) while Gram-positive organisms have much fewer lipids in their cell walls but contain lipoteichoic acids (LTA). The yeast cell wall consists of at least two layers. The outer layer contains a combination of mannose associated with either protein [phosphopetidomannan (PPM), commonly termed mannan] or lipid [phospholipomannan (PLM)]. The inner layer is composed of chitin and 1,3-β- and 1,6-β-glucan[3]. In living species, the first line of defence against microbial aggression is innate immunity.[60] Innate immunity relies on the recognition of pathogen-associated molecular pattern (PAMP) antigens by specific proteins referred to as pattern-recognition receptors (PRRs). Peptidoglycan, LPS and LTA, which are present in bacteria, and PLM, PPM and glycan, which are present in yeast, are all PAMPs and are recognized by different PPRs and thus can account for different responses of these micro-organisms as 'immunobiotics'.[12]

- **Occurrence of Saccharomyces spp in milk and milk products**

Saccharomyces spp e.g. S. burnetii, S. kluveri, S. byanus, S. rosinii, S. cerevisiae and S. boulardii may be isolated from a variety of dairy products including milk, yogurt ,cream, dahi, cheese and kefir. Yeasts rarely grow in milk stored at refrigeration temperature because they are out-grown by psychotropic bacteria. However, in sterilized milk in the absence of competition, Saccharomyces spp are capable of growth to populations of 10 8 -10 9 cfu/ml. Saccharomyces spp. are often present in soft mould ripened cheeses and semi-hard and hard cheeses including Cheddar. Growth of Saccharomyces spp in cheeses is thought to be related to its ability to use lipid and protein products form other species and possibly their ability to utilise lactic acid present in the cheese [35].

- **Resistance to antibiotics**

As *S. boulardii* is naturally resistant to antibiotics, it can be prescribed to patients receiving antibiotic. [72] Research on the administration of *S. boulardii* to patients suffering from recurrent *Clostridium difficile* infections has shown that the faecal yeast count is significantly higher in patients who do not relapse compared to patients that do. The efficiency of *S. boulardii* treatment thus appears correlated with the faecal yeast concentration.

- **Safety and packaging of therapeutic yeast:**

Except for several sporadic reports of fungaemia, in patients with severe general or intestinal disease who had an indwelling catheter,[72]*S. boulardii* is considered to be a safe and well-tolerated treatment. The origin of these cases of fungaemia remains unclear, but is likely related to catheter colonization. Presence of such catheters is thus, a contraindication for the administration of *S. boulardii*.

Saccharomyces boulardii is administered in a lyophilized form, and is prepared, packaged and controlled as such. Therefore, lyophilized*S. boulardii* is clearly distinct from dietary probiotic products which contain diverse strains of micro-organisms and are used either in animals to improve zootechnical yields or in healthy humans (often in form of yogurt) to strengthen host physiology in the absence of any pathological context. *Saccharomyces boulardii* can be considered, an example of a 'probiotic drug'

- **Development of yeast based fermented milk products**

The frequent occurrence of yeasts in dairy and related products indicates their ability to metabolize milk constituents. Saccharomyces species cannot ferment lactose so they develop in milk as a secondary flora, after bacterial growth. Lactic acid bacteria (LAB) ferment milk lactose through hydrolysis to glucose and galactose. The glucose moiety is fermented to lactic acid. Lactic acid creates a high acid environment, however, the ability of some yeasts to utilize lactic acid as a carbon source can create a selective environment for yeast growth and for the growth of less acid tolerant lactic acid bacteria.

Fermented milk products that are manufactured using starter cultures containing yeasts include acidophilus-yeast milk [73]Kefir, Koumiss and Leban. S.boulardii is capable of utilizing the yogurt constituents as growth substrates and maintaining cell counts exceeding 10 ^6 cfu/ml. Various yeast based fermented milk products and their characteristics are shown in table 1.

Table 1. Characteristics of yeast-based fermented milk products

Fermented milk products	**Microorganisms responsible for the fermentation process**	**Description of the products**
Kefir	Lactic acid bacteria, Acetic acid bacteria and yeasts (Lactose fermenting and non lactose fermenting)	A mixed lactic acid and alcoholic fermentation.
Koumiss	*L.bulgaricus* and *S.cerevisiae*	The mare or camel fermented milk of a mare or camel milk. It may be mildly alcoholic

Leban	*L.bulgaricus, S.thermophilus* and yeasts	A concentrated yogurt like product.
Acidophilus yeast milk	*L.acidophilus* and *S.cerevisiae*	Acidic and alcoholic product with probiotic properties
Taette	*S. lactis* var. *hollandicus* and*Saccharomyces taette*	Moderately ropy and sour milk product of slightly flowing consistency that contains not more than 0.3%-0. 5% of alcohol

- **Yeast Probiotic versus Bacteria Probiotic**

Saccharomyces is not part of the naturally occurring gut flora like some bacterial probiotics. Saccharomyces is resistant to stomach acids, bile and pancreatic juices, as it is can tolerate varying pH levels, so its survival through the gut is greater than that of bacteria probiotics. There are also no concerns with the impact of antibiotics on saccharomyces as there are with probiotic bacteria. Yeasts are naturally resistant to antibiotics and are completely safe when taken during antibiotic treatment. Additionally, unlike some bacteria probiotics, saccharomyces does not colonise the intestine; it is a transient. After 3 days of supplementation, it will reach a maximum steady state. As soon as supplementation stops, the body excretes it via the feces.

	Bacteria	Yeast	Probiotic implication
1. LPS, lipopolysaccharide; LTA, lipoteichoic acid; PPM, phosphopetidomannan; LPM, phospholipomannan; TLR, Toll-like receptor; GI, gastrointestinal.			
Presence in human flora	99%	<1%	
Cell size	1 μm	10 μm	Stearic hindrance
Cell wall	Peptidoglycan, LPS (Gram-negative), LTA (Gram-positive)	Chitin, mannose (PPM, PLM), glucan	Immune response via TLRs, lectin receptors
Optimal growth conditions			
pH	6.5–7.5	4.5–6.5	Different sites of action in the GI tract
Temperature (°C)	10–80	20–30	
Resistance to antibiotics	No	Yes	Safety in combination with antibiotherapy

Table 2. Major differences between yeast and bacteria and their probiotic implications

Conclusions

Saccharomyces has been studied in numerous conditions. It is beneficial for treating antibiotic-associated diarrhoea, infectious diarrhoea, such as traveller's diarrhoea and acute diarrhoea in children, AIDS, inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS). It may also be effective for food allergies, candida infection, and parasitic infections.

Saccharomyces is useful in treating diarrhoea because it can adversely affect the pathogenic organisms, *Clostridium difficile*, *Escherichia coli, Shigellae,* and *Salmonella*. In fact, Saccharomyces is the only probiotic to have proven efficacy in Clostridium difficile-associated diarrhoea relapses.[73]

Saccharomyces species are emerging as potential probiotic organisms. Already there is a marked increase in the sale of various yeast based probiotic products. However there is a need to isolate more potential probiotic strains of Saccharomyces and to develop new probiotic yeast based dairy products. Using probiotic Saccharomyces species alone or in combination with lactic acid bacteria can enhance the nutritive value of fermented dairy products.

Chapter 3: MATERIAL AND METHODS:

The yeast strains for the purpose of screening were procured from-Palampur university

The chemicals used in the protocol were mostly the usual laboratory chemicals procured from - Sigma

Pepsin which was obtained from- Labale chemie

a) **Media formulations:**

a. **YPD media-**

Peptone	2% of the media volume
Dextrose	2% of the media volume
Yeast Extract	1% of the media volume

b. **MRS media-**

55.35 gms of media powder in 1000 ml of distilled water.

c. **Gastric juice-**

Pepsin	0.3% (wt/vol)
NaCl	0.5%(wt/vol)

The pH of the same was adjusted to 2 using HCL.

b) **Chemicals**

a. **Lowry's reagent-**

i. 50ml of 2% sodium carbonate mixed with 50ml of 0.1N NaOH solution(0.4 m in 100 ml distilled water.

ii. 10ml of 1.56% copper sulphate soltution mixed with 10ml of 2.37% sodium potassium tartarate solution.

2ml of solution (ii) was mixed with 100 ml of solution (i) to obtain the reagent.

b. **Anthrone reagent-**

i. 0.5g of anthrone was put into a beaker containing absolute ethanol.

ii. Some more ethanol was used to rinse the walls of the beaker removing anthrone particles.

iii. Slowly this was put into a flask containing 75% sulphuric acid 250ml.

iv. The solution was stirred well for use.

c. Sodium phosphate buffer-

i. 0.2 M Monobasic Stock

1. Combine

13.9 g sodium phosphate monobasic

500 mL dH20

ii. 0.2 M Dibasic Stock

1. Combine

53.65 g sodium phosphate dibasic heptahydrate (or 28.4 g of the anhydrous form)

1 L dH20

iii. To prepare the buffer,pH 7.2 84ml of the monobasic solution and 216 ml of the dibasic solution mere mixed and raised further to 1000ml with distilled water.

c) Methods followed::

In order to survive and proliferate within the gastrointestinal tract, probiotics must tolerate several environmental hurdles, including the low pH of the stomach, as well as reduced water activity (aw) and bile in the upper small intestine. Furthermore, the ability to persist in the intestine is considered to be a valuable criterion in achieving optimal probiotic efficacy.

a. Gastric juice tolerance and acid stress:

i. The strains to be tested were grown as fresh cultures of 5ml each.

ii. The fresh cultures were centrifuged to obtain a cell pellet containing cells.

iii. These pellets were suspended in 5ml distilled water, separately

iv. 1ml of the suspentions were inoculated in 5ml of gastric juice which was prepared fresh.

v. The strains were incubated at different time intervals, under gastric juice and acid stress so formed, at 37% without shaking.

vi. After the time intervals, the strains were plated on YPD agar plates to check the cfu and a spectrophotometric result was taken to account the viability rate.

b. **Salt stress**:

To screen out strains which can be put into a food matrix, this salt stress was carried out. The strains which show resistance to high concentration of salt can be considered good subjects for further analysis of their Probiotic or broadly, therapeutic aspects.

i. To subject different strains of yeast to salt stress, YPD media was prepared containing different salt concentrations. 5% salt was taken as the maximum.

ii. The strains were grown under both congenial as well as stressed conditions of salt in the growth media

iii. Following this, the spectrophotometric readings were taken to account the viability under the stressed conditions.

c. **Isolation of yeast cell wall components:**

The cell walls of S.cerevisiae have been tested to have high concentrations of proteins which includes β-glucans. Numerous studies have demonstrated that these exhibit antitumor, antimicrobial and even prebiotic properties. This study was limited to check the total amount of protein and carbohydrate present in the cell walls and to study the effect of the same on the growth rate of bifidogenic activity i.e. a prebiotic efficacy, if any.

i. Yeast cell cultivation

1. Was done fresh in a large media volume of YPD for 48hrs.
2. The culture so obtained was centrifuged at 4◦C, 5000rpm for 10 min.
3. This was done thrice to give proper washing to the yeast cells.

ii. Cell fractionation

1. A suspension of the yeast cells was made in chilled 0.1M sodium phosphate buffer, pH7.2.
2. This suspension was subjected to sonication in an ice bath at 60% amplitude, 30:55sec pulse on: pulse off.
3. Further, the suspension was centrifuged at 7000rpm to separate the cell walls and was diluted by sterile distilled water.
4. Incubation at 100◦C was carried out immediately to deactivate the degrading enzymes released by cell disruption.
5. The cell was obtained were stored at -80◦C for further analysis.

d) Protein and carbohydrate estimation in cell walls

a. To quantify the total protein content in the cell walls of yeast strains, Lowry's method was used.

i. BSA was to plot the standard curve.
ii. 3ml of Lowry's reagent and 300µl of Folin- Ciocalteau reagent (1N) was put into the sample and incubated at 37◦C.
iii. The spectrophotmetric readings were taken at 720nm.
iv. Standard plot was used to estimate the protein content.

b. To quantify the total carbohydrate content in the cell walls of yeast strains, Anthrone method was followed.

i. Dextrose was used to plot the standard curve.
ii. 2ml of Anthrone's reagent was put to the sample and incubated at 60◦C.
iii. The spectrophotometric readings were taken at 620 nm.
iv. Standard plot was used to estimate the carbohydrate content.

e) Analysis of the effect of cell walls on the growth rate of bifidobacteria

a. The cell wall extracts were freshly prepared and lyophilized.
b. The lyophilized amount was suspended in 2ml of distilled water.
c. Bifidobacteria was inoculated in culture tubes containing MRS media
d. A control was maintained along with the culture tubes with a dose of the cell wall extract.
e. After culture growth, the cfu was checked by plating on MRS plates.

RESULTS AND DISCUSSIONS:

a) Salt stress:

The use of probiotic cultures as an additive to food is not a new idea, however, literature on the possibility of their tolerance to various concentrations of salt (NaCl) used in manufacture of these foods like cheese is virtually not existent. Thus the effect of different concentrations of NaCl on the viability of some microbial strains was assessed.[17]

The strains were exposed to 5% NaCl in YPD broth for 18 and 24 hours.

The tolerance to salt stress is indicated by the growth of the cells in this media. Higher the tolerance, more will be the growth and hence OD.

Results obtained in Table show the relationship between the viable counts of all microorganisms and salt concentration along the storage period. This experiment was conducted twice, validating the results.

I. Control-

STRAIN	OD at18hrs	OD at 24hrs
9	1.903	1.860
10	1.825	2.017
11	1.391	1.678
12	1.716	1.794
13	1.996	2.108
14	1.801	1.794
15	1.875	1.825
16	1.928	1.870
17	1.895	1.825

TABLE:

OD of the strains at 600 nm under NO stress

5% salt stress-

STRAIN	OD at 18hrs	OD at 24hrs	% Decrease in growth(18hrs)	%Decrease in growth(24hrs)
9	1.046	1.046	45%	40%
10	0.662	0.865	63.7%	60%
11	1.108	1.046	34%	37%
12	0.860	1.126	49%	39.8%
13	0.881	0.937	54%	55.5%
14	1.242	1.342	31%	25.19%
15	0.605	0.851	67.73%	53.3%
16	0.529	0.715	72%	61%
17	1.396	1.491	26.33%	18.30%

TABLE:

OD at 600nm when cells were subjected to 5% salt stress

II. Control-

STRAIN	18hrs	24hrs
9	1.183	1.124
10	1.961	2.010
11	1.898	1.934
12	1.782	1.754
13	1.823	1.956
14	1.785	1.766
15	1.845	1.879

16	1.889	1.856
17	1.824	1.863

TABLE:

OD of the strains at 600 nm under NO stress

5% salt stress-

STRAIN	OD at 18hrs	OD at 24hrs	% Decrease in growth(18hrs)	%Decrease in growth(24hrs)
9	0.676	0.589	42.85%	47.72%
10	0.943	0.862	51.91%	57.11%
11	1.167	1.332	38.51%	30%
12	0.899	1.133	49.55%	39%
13	0.934	1.167	46.62%	39.65%
14	1.067	1.245	40.22%	29.50%
15	0.568	0.753	68.5%	59.39%
16	0.557	0.785	70.51%	57.70%
17	1.379	1.476	24.39%	20.77%

TABLE:

OD at 600nm when cells were subjected to 5% salt stress

The percentage decrease in the survival rate of the strains indicates their resistance to high salt concentrations. The strains 9, 10, 13 and 15 had a reduction of 50% or more than the control which indicates that these strains are less tolerant to high salt conditions.

The strains 12, 14, 17 had a decrease below 50% of the growth in control. This indicates that these strains are more tolerant to such high salt conditions and hence must be evaluated for tolerance against other stress conditions to screen out as a Probiotic.

So, the strains that offered more resistance towards this stress than the other strains were taken forward towards the Gastric juice and acid stress.

b) Gastric juice tolerance and acid stress:

Before reaching the intestinal tract, probiotcis bacteria must first survive transit through the stomach (Henriksson et al., 1999). There, the secretion of the gastric juice with a pH between 2.0 and 3.4 contitutes a primary defence mechanism against most of the ingested microorganisms. So the survival of Probiotic bacteria in the human gastric juice is more accurate indication of the ability of the strains to survive passage through the stomach (Dunne et al., 2001).

Simulated gastric juice was prepared by suspending pepsin(0.3%w/v) in saline(0.5% w/v). the pH of the solution was adjusted to 2 using HCL.

This developed the conditions under which the strains were screened for their tolerance against the proteolytic activity of pepsin and also the survival at such low pH.

The strains were exposed at intervals of 30 min and 60 min and were compared with control. A cfu was obtained to check the survival of each of the strains under these conditions the results of which are as follows:

a. Strain 12:

Cfu

0 min	30 min	60 min
45×10^6	22×10^6	31×10^6
37×10^6	25×10^6	26×10^6

b. Strain 14:

Cfu:

0 min	30 min	60 min
13×10^6	11×10^6	14×10^6
15×10^6	12×10^6	12×10^6

c. Strain 16:

Cfu

0 min	30 min	60 min
17.7×10^6	16.4×10^6	11.3×10^6
16.2×10^6	18.1×10^6	12×10^6

d. Strain 17:

Cfu:

0 min	30 min	60 min
32×10^6	12.5×10^6	8.9×10^6
42.5×10^6	28.9×10^6	14.3×10^6

e. Strain 19:

Cfu :

0 min	30 min	60 min
22.4×10^6	28.9×10^6	17.5×10^6
24.9×10^6	25.6×10^6	18.9×10^6

The cfu after 24 hours of the strains indicates the survival under stressed conditions. The cfu obtained at 0 min is the control which was compared with the cfu obtained after 30 and 60 min. The strains with the highest cfu are more likely to survive the tough conditions of the GI tract.

Out of the strains screened, the ones which survived best were taken forward to the isolation of cell walls and further analysis.

c) Isolation of cell walls and further analysis:

The cell walls of S.cerevisiae have been tested to have high concentrations of proteins which include β-glucans. Numerous studies have demonstrated that these exhibit antitumor, antimicrobial and even prebiotic properties. This study was limited to check the total amount of protein and carbohydrate present in the cell walls and to study the effect of the same on the growth rate of bifidogenic activity i.e. a prebiotic efficacy, if any.

a. Total protein content-

To check the total protein content, Lowry's method was used.

Principle: The phenolic group of tyrosine and trytophan residues (amino acid) in a protein produce a blue purple color complex ,with Folin-Ciocalteau reagent which consists of sodium tungstate molybdate and phosphate. Thus the intensity of color depends on the amount of these aromatic amino acids present and will thus vary for different proteins. Most proteins estimation techniques use Bovin Serum Albumin (BSA) universally as a standard protein, because of its low cost, high purity and ready availability and so the same was used as standard for the purpose of this experiment. The method is sensitive down to about 10 µg/ml and is probably the most widely used protein assay despite its being only a relative method, subject to interference from Tris buffer, EDTA, nonionic and cationic detergents, carbohydrate, lipids and some salts. The incubation time is very critical for a reproducible assay. The reaction is also dependent on pH and a working range of pH 9 to 10.5 is essential.

The standard curve, made by varying concentrations of BSA, was as shown in the figure below:

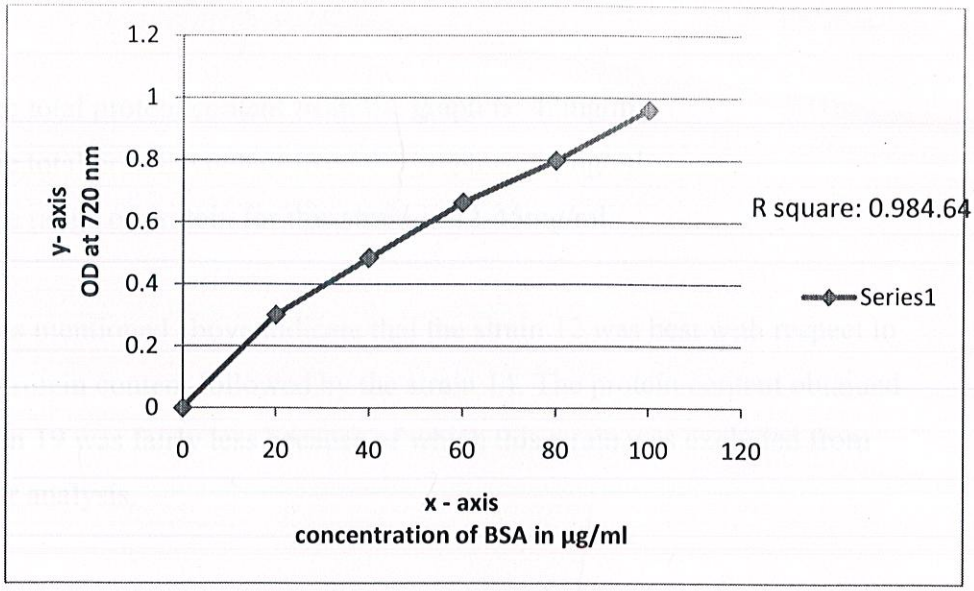

Figure 7: standard curve for Lowry's test

Sample data:

To obtain the sample data the cell walls were extracted and checked for the total protein content by Lowry's method. The OD of the samples at 720 nm was obtained. The standard curve of BSA was used to quantify the total amount of protein in each sample.

Each sample was evaluated twice to authenticate the results.

For each strain the total protein content obtained is as follows:

i. **Strain 14:**

I. The total protein content from the graph is: 38mg/ml.

II. the total protein content from the graph is: 28mg/ml

Hence, the protein content in strain 14 is in the range 28- 38mg/ml.

ii. **Strain 19:**

I. The total protein content from the graph is: 0.2mg/ml

iii. Strain 12:

I. the total protein content from the graph is: 42mg/ml

II. the total protein content from the graph is: 45mg/ml.

Hence, the range of protein for this strain is 42-45mg/ml.

The results mentioned above indicate that the strain 12 was best with respect to the total protein content followed by the strain 14. The protein content obtained from strain 19 was fairly less because of which this strain was excluded from the further analysis.

b. **Total carbohydrate content**:

To evaluate the total carbohydrates in the cell walls, Anthrone's method was used.

The Anthrone method is an example of a colorimetric method of determining the concentration of the total sugars in a sample. Sugars react with the anthrone reagent under acidic conditions to yield a blue-green color. The sample is mixed with sulfuric acid and the anthrone reagent and then boiled until the reaction is completed. The solution is then allowed to cool and its absorbance is measured at 620 nm. There is a linear relationship between the absorbance and the amount of sugar that was present in the original sample. This method determines both reducing and non-reducing sugars because of the presence of the strongly oxidizing sulfuric acid.

The standard curve was made using dextrose, which is as shown in the figure below:

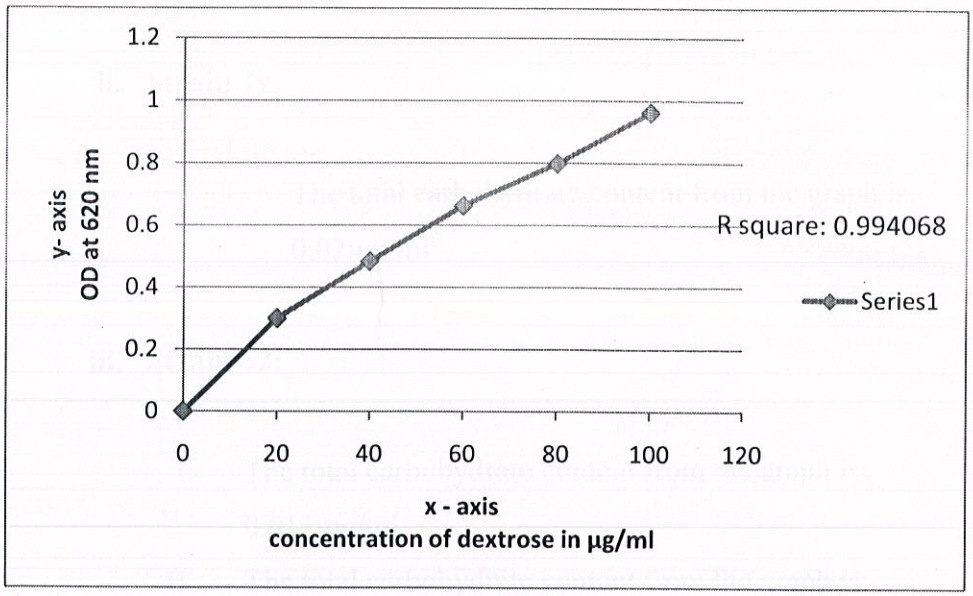

Figure 8: standard curve for Anthrone's test

To obtain the sample data the cell walls were extracted and checked for the total carbohydrate content by Anthrone's method. The OD of the samples at 620 nm was obtained. The standard curve of Dextrose was used to quantify the total amount of carbohydrates in each sample.

Each sample was evaluated twice to authenticate the results.

For each strain the total carbohydrate content obtained is as follows:

i. **Strain 14**:

I. The total carbohydrate content from the graph is: 1600mg/ml.

II. The total carbohydrate content from the graph is: 2200mg/ml.

Hence, the total carbohydrate content is in the range 1600-2200 mg/ml.

ii. Strain 19:

I. The total carbohydrate content from the graph is: 0.02mg/ml

iii. Strain 12:

I. The total carbohydrate content from the graph is: 0.011mg/ml.

II. The total carbohydrate content from the graph is: 0.021mg/ml.

Hence, the total carbohydrate content for this strain is with the range: 0.011-0.021mg/ml.

The results mentioned above indicate that the strain 14 was best with respect to the total carbohydrate content followed by the strain 12. The carbohydrate content obtained from strain 19 was fairly less, because of which, this strain was excluded from the further analysis.

d) Study of bifidogenic activity of the cell walls obtained:

Bifidobacteria, naturally present in the dominant colonic microbiota, represent up to 25% of the cultivable faecal bacteria in adults and 80% in infants. As probiotic agents, bifidobacteria have been studied for their efficacy in the prevention and treatment of a broad spectrum of animal and/or human gastrointestinal disorders, such as colonic transit disorders, intestinal infections, and colonic adenomas and cancer

A bifidogenic factor increases bifidobacteria either in the intestine, or in other conditions (fermented dairy products for example). When bifidobacteria in the intestine are stimulated, a bifidogenic factor may be considered prebiotic, but only if this stimulation has a beneficial effect on the host. In other conditions, bifidogenic factors are not considered prebiotics.

It is assumed that a prebiotic should increase the number and/or activity of bifidobacteria and lactic acid bacteria, as these groups of bacteria are claimed to have several beneficial effects on the host. A product that stimulates (or claims to stimulate) bifidobacteria is considered a bifidogenic factor.

For this purpose the cell wall extract was prepared fresh and a dose of this extract was put in the media with bifidobacteria.

The cfu count of the bifidobacteria grown with this extract was used to analyse the effect of this extract on this bacterial growth.

The results of this are as shown below:

10.8mg/ml of the cell extract from strain 12 was put into the MRS broth after filter sterilization.

Bifidobacteria was allowed to grow in this media for 24hours.

A cfu count was obtained for this which shows the effect of the extract on the growth of this bifidobacteria. The exteriment was done in triplets to validate the result.

Cfu after 48hrs:

S.No	Control cfu	cfu with extract from strain 12
1	22×10^6	26×10^6
2	36×10^6	33.5×10^6
3	28×10^6	24×10^6

The cfu results show that there has been no visible change in the growth rate of bifidobacteria.

Hence, the extract of the strain 12 has no potential bifidogenic aciviity.

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Bio-data

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