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Isolation of *Probiotic Lactobacillus Bulgaricus* and their Antagonestic Activity against Foodborne Pathogens

Susmitha V
Integrated Rural Technology Centre (IRTC), Palakkad, Kerala
E-mail: nimyamanikandan28@gmail.com

ABSTRACT: The aim of this study was to *isolate* and identify *probiotic Lactobacillus bulgaricus* from rice poridge and further synthesised bacteriocin to study the antimicrobial activity against food borne pathogens. The isolated Lactobacillus *bulgaricus* was separately cultured in MRS Agar and identified through various biochemical test. The antimicrobial activity against food borne pathogens like *Klebsiella sp, E.Coli*, Streptococcus sp, Staphylococcus aureus and Salmonella typhi was carried out in vitro by Agar Well Diffusion Method. Effect of p^H and tolerance to bile salt and common salt on the growth rate of *Lactobacillus bulgaricus* was also studied. It was found that *Lactobacillus bulgaricus* remain active in the intestine even after consumption and can tolerate the intestinal p^H and bile concentration. It was concluded that bacteriocins inhibit food borne pathogens like *Klebsiella sp, E.Coli, Streptococcus sp, Staphylococcus aureus and Salmonella typhi*. A Maximum zone of 21mm inhibition was observed with *Streptococcus typhi* and a minimum zone of 17mm inhibition was observed with *Salmonella typhi.*

Keywords: *Probiotic; lactobacillus bulgaricus*; rice porridge; bacteriocins; pathogen; inhibition

INTRODUCTION

The origin of "probiotics" concept rests with Metchnikoff, director of Pasteur Institute, Paris, in the early years of the 20th century. Metchnikoff proposed that humans would benefit by encouraging the correct balance of microbial types in the large bowel, especially from the reduction of putrefactive activity (Metchnikoff, 1908). Milk fermented by lactic acid producing bacteria had been noted to inhibit the multiplication of proteolytic (putrefactive) bacteria because of low pH produced by the fermentation of lactose. Probiotics are defined as "living microorganism, which upon ingestion in certain amount exerts health benefits beyond inherent basic nutrition" (Guarner and Schaafsma, 1998; Tannock, 2002). The concept of orally taking mixtures of microorganisms for improved health is not new. Yoghurt has been thought to have health benefits. The therapeutic use of microorganism antagonistic to pathogen would have the potential to decrease antibiotic use. Biotherapeutic agents hav been used to describe a microbe having specific therapeutic activity against a specific disease. An example of effective use of biotherapeutic agent is the oral administration of *Saccharomyces baulardii* to treat recurrent *C. difficle* associated disease (Elmer, 1999).

Most probiotic microorganisms belong to Lactic Acid Bacteria (LAB), such as *Lactobacillus, Bifidobacterium* and *Enterococcus.* The Lactic Acid Bacteria (LAB) comprise a group of Gram Positive rod shaped bacilli or cocci, low-GC, acid tolerant, generally non-sporulating, no respiring rod or cocci that are associated by their common metabolic and physiological characteristics. Probiotics are defined as the "living microbial food/feed supplements which beneficially affect host human animal by improving its intestinal microbial balance" (Fuller, 1989). Probiotics feed supplements containing these LAB strains are one of the important class of functional foods. They perform both nutritional and pharmaceutical functions and hence the term neutraceuticals. They can be defined as "any substance that may be considered a food or part of a food and provides medical and health benefits including the prevention and treatment of disease" (Hasler**,** 1997). Probiotic bacteria must be resistant to the acidity of the stomach lysozyme, bile, pancreatic enzymes and antibiotics used as growth enhancers in animal nutrition and in therapy. These characteristics may be observed in vitro and can be used for selection of strains (Salminen *et al*., 1998). High acidity in the stomach and the high concentration of bile component in the proximal intestine are the first host factors, which affect strain selection.

Lactic Acid Bacteria (LAB) is heterogeneous group of bacteria found widely in nature. They colonize the gastrointestinal and urogenital tracts human and animals. Present in food such as dairy products, fermented meats, fruits & vegetables. LAB also added to several probiotics products because of their potential health benefits. Many LAB species are generally recognized as safe (GRAS) & several LAB species have received Qualified Presumption of safety (QPS) status given by European Food Safety Authority (EFSA). Fermented foods are those which have been subjected to the action of microorganism or enzymes that the desirable biochemical changes cause significant modi-

fication to food. Many fermented milk products which are eaten as they are, contains living microorganisms. The fermented product is basically a culture of *Lactobacillus bulgaricus* growing in association with *Streptococcus thermopiles*. The two organisms are mutually beneficial in the association with both organisms converting nearly the sugar to lactic acid producing only traces are not pressed. Lactic acid bacteria are regarded as major group of probiotic bacteria. They are non-pathogenic technologically suitable for industrial process. Acid-fast, bile tolerant, adhere to cut epithelial tissue, and produce antimicrobial substance including organic acids, hydrogen peroxide & bacteriocins (Biologically active proteins) (Dunne *et al.*, 1999).

Present study deals with isolation and identification of *Lactobacillus bulgaricus* from fermented rice for potentials to produce bacteriocin and their antibacterial activity against common pathogens.

MATERIALS AND METHODS

Probiotics are defined as "living microorganism, which upon ingestion in certain amount exerts health benefits beyond inherent basic nutrition" (Guarner and Schaafsma, 1998; Tannock, 2002). The concept of orally taking mixtures of microorganisms for improved health is not new.

Sample Preparation: The red and white rice (75g) were cooked with water (rice:water 1:3) for 30min to obtain soft consistency. Fermentation was carried out by soaking cooked rice in clean drinking water over night in clay pots at 27°C.

Isolation of Probiotics

Serial Dilution: 9 ml of sterile distilled water was taken in sterilized 9 test tubes and that was labeled from 10^{-1} to 10^{-9}. 1ml of sample was added into first dilution 10^{-1} and gently mixed. 1 ml was transferred into next dilution 10^{-2} test tube and this was repeated till 10^{-9}. The final 1 ml was discarded.

Spread Plate Technique: Prepare MRS agar and pour into petriplates. After solidification of media, 0.1 ml sample was taken from the dilutions of 10^{-4}. Incubate the plates at 37° C for 48 hrs under anaerobic condition created by anaerobic gas packs.

Identification of Microorganism

Identification of the isolates were performed according to their morphological, cultural, and biochemical characteristics by following Bergey's Manual of Systemic Bacteriology. All the isolates were subjected to Gram staining and specific biochemical tests.

Growth on Man Ragosa Sharpe Agar: The collected sample is carefully spread on the surface of sterile Man Ragosa Sharpe Agar plates and incubated 37° C for 48 hours. The organisms in Man Ragosa Sharpe Agar plates were observed and the results were recorded.

Microscopy

Gram Staining: The colony from MRS agar plates was smeared on a clean, grease free slide and heat fixed it. Then the smear was flooded with crystal violent solution and allowed to react for one minutes. Grams iodine was add to wash off crystal violet solution and the smear was allowed to react for one minutes. The slide was rinsed with running tape water. Absolute alcohol was added drop wise at the upper end of the inclined slide till the washing contain violet color. The smear was rinsed with running tape water flooded with 0.24% saffranin solution for 30 to 60 seconds. Then the slide was washed with water, air dried and examined under oil immersion (100x)

Capsule Staining: One drop of Indian ink was placed over a clean free glass slide. A loop full of bacterial culture was placed on the stain and spreaded gently as a uniform smear over the slide and allowed to air dry at room temperature. The smear was washed with distilled water,air dried and observed under oil immersion(100x).

Motility Test: Spread Vaseline or petroleum jelly on the four corner of a clean cover slip by using tooth pick. Place a small drop of bacterial suspension in the center of the cover slip. And then it observed under 40x objectives.

Biochemical Test

Indole Test: Tryptophan broth and test reagents were prepared. The tubes of tryptophan broth was inoculated with the organisms and incubated for 24 hours at 37ºC. After incubation 0.2% of kovac′s reagent was added to the tubes and allowed to stand for few minutes. Then the results observed and recorded.

Methyl Red and Voges Proskauer Test: MRVP broth was prepared. The organism were inoculated in to MRVP broth and incubated at 37ºC for 24 hours. The broth was divided into equal half and to one 0.5ml of MR reagent was added. To the other half 0.2ml of VP reagent A and 0.2ml of VP reagent B was added. The tubes were mixed gently and allowed tom stand for 15 minutes. Then the results were observed and recorded**.**

Citrate Utilization Test: Citrate agar tubes were prepared. Autoclave at 121^0C for15minutes and allowed to solidify in a slanting position. A drop of 4-6 hours old culture was inoculated into the medium and incubated for 18-24 hours and results were read and recorded. Incubate at 37^0C for 24 hours. The culture was obtained

Catalase Test: Place a drop of 3% hydrogen peroxide (H2O2) on a clean glass slide and ad a drop of bacte-

rial culture. Then the slide was observed for effervescence.

Oxidase Test: Place a sterile strip of filter paper on a clean petridish and add 2 to 3 drops of oxidase reagent or readymade disc's are used. A drop of culture is placed on disc and observed color change.

Sugar Fermentation Test: Sugar fermentation broth was prepared , with different sugar such as glucose , lactose , sucrose , mannitol and durhum's tube were inserted into it and autoclaved at 121lbs pressure for 50 min. After sterilization, a loop full of culture is inoculated into the media and incubated for 18 to 24 hrs. Results were recorded.

Extraction of Bacteriocins: Extraction inoculated with a strain of *Lactobacillus bulgaricus w*ere incubated a 37 ^{0}C for 48 hrs. After incubation a cell free solution were obtained by centrifugation 6000 rpm for 15 min at 4^{0}C to separate bacterial cells and supernatents. The supernatents were adjusted to pH 6.5 by adding 1N NaOH and then filtered through 0.2 µm cellulose acetate filter. The neutralised and filtered supernatants were assayed for antibacterial of bacteriocins is carried out from *L. bulgaricus.* 10 ml MRS broth is activity.

Effect of pH Tolerance

Acidification was measured by change in pH. The inoculums was inoculated to MRS broth adjusted with various pH ranges with conc. NaOH and HCl. Incubate at 37˚c for 24 h and check the OD at 600 nm using spectrophotometer. And the results were recorded.

NaCl Tolerance

The isolates were grown in MRS broth having different NaCl concentration (1-9%) and incubated at 37˚c for 24 hrs. The culture tubes were observed for the presence or absence of growth. After 24 h incubation, growth was determined using a spectrophotometer reading the optical density at 600nm. And the results were recorded.

Bile Salt Tolerance

Bile salt tolerance of the isolates was investigated by determining their growth in MRS broth containing different levels (1, 2, 3, 4, and 5%) bile salts (Oxgall). And optical densities were measured using a spectrophotometer at 600nm after 24 h of incubation.

Antibacterial Activity

Antimicrobial activity of free supernatant was checked against *Staphylococcus aureus, Escherichia coli, Klebsiella pneumonia, Pseudomonas aeruginosa.* These cultures were inoculated in MH broth and incubate at 37˚c for 24 hrs. Muller Hinton Agar plates prepared and lawn cultures of indicator microorganisms were made by swab inoculation. The well with holding volume of 50 µL was made in the center of the plate using well cutter. 24 hrs old cultures of isolates were centrifuged at 10,000 x g for 10 min, and 50 µL of the supernatant was loaded in the well and the plates were incubated at 37˚c for 24 hrs. The antibacterial activity was determined and zone of inhibition was measured in millimeter (mm).

RESULTS AND DISCUSSION

Isolation: In the present study lactobacillus species was isolated from the rice porridge. The rice samples collected from in and around Palakkad for the isolation of microorganisms the samples were inoculated in to the culture medium such as nutrient agar and Man Ragosa Sharpe Agar. Large,white spherical, convex colonies seen in MRS agar

Microscopy: The colonies appeared on nutrient agar and MRS agar plates were taken for microscopic examination using different staining methods, such as simple staining, gram staining, motility test and capsule staining. After the microscopic studies the isolated bacteria were Gram positive, rod shaped, convex, smooth, shiny, irregular, circular, non-spore forming and nonmotile which indicate them to be the member of *Lactobacillus sp*.

Fig1: *Lactobacillus bulgaricus* on MRS agar

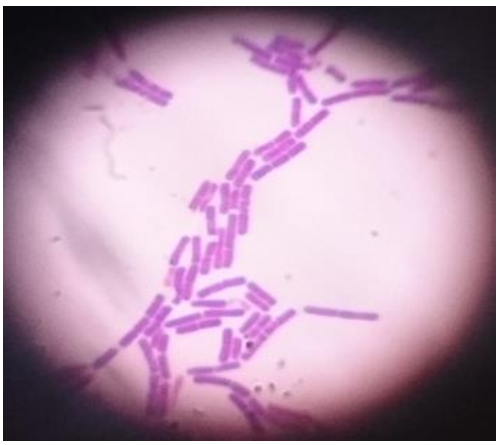

Fig2: Microscopic view of *Lactobacillus bulgaricus*

Biochemical Characterization: Different biochemical test are used for the biochemical characterization. Growth at 35˚c-40˚C,the isolates showed good growth at 37˚c.Whereas the isolates showed could not grow at low and elevated temperature. The isolates showed that indole negative, MR positive, VP negative, citrate negative and oxidase negatve. Most of the microorganism obtain their energy through a serious of orderly and integrated enzymatic reactions leading to the bio-oxidation of a substrate, frequently a carbohydrate.

Fig 3. IMVIC test result of Lactobacillus bulgaricus

Carbohydrate Fermentation Test: *Lactobacilli,* the production of lactic acid fermentation organism and can be safety used for medical & veterinary application. These organism are strictly fermentative, aero tolerant or anaerobic and have a complex nutritional requirements. Using glucose as carbon source, *Lactobacilli* may be either homo fermentative. The organism shows lactose positive resultand others are negative.

Antibacterial Activity: The antibacterial activity was performed by agar gel diffusion method against the indicator microorganisms *Klebsiella, Pneumonia, Escherichia coli, Staphylococcus aureus, Streptococcus and Salmonella typhi.* The zone of inhibition measured in mm. The inhibitory effect of *Klebsiella pneumonia*(20mm),*Escherichiacoli*(18mm),*Staphylococcusaureus*(19mm),*Streptococcus*(21mm) *and Salmonella typhi* (17mm).

Effects of pH: The acidic tolerance of the isolated microorganism was determined by growing the isolates from different pH range (1-9).Maximum growth of *Lactobacilli* sp. at the Ph 4- 7 (OD-1.24). Probiotic bacteria must be resistant to the acidity of the stomach, lysozyme, bile, pancreatic enzymes and antibiotics used as the enhancers in animal nutrition therapy. These characteristics may be observed in vitro and can be used for selection of strains (Salminen et al., 1998).High acidity in the stomach and the high concentration of bile component in the proximal intestine are the first host factors, which affect strain selection. The objectives of the study were to investigate the acid and bile tolerance of some lactic acid bacteria.

NaCl Tolerance: Previous study reported that NaCl tolerance is important physiological parameter for the growth of cells as the physiological saline could prevents the cell from osmotic shock. Hence the tolerance ability of Lactobacillus sp., was studied by subjecting them to grow from 1-9% concentration NaCl in the growth media. The highest growth is occur in 7% (OD-.11).

Bile Tolerance: The bacteria to be used as probiotics should be able to resist inhibitory factors in the gastrointestinal tract such as bile salts (Pyar H,et al.,).Bile tolerance is an important factor for the survival and growth of *Lactobacilli* in the intestinal tract. The resistance ability of the isolates to bile salts was revealed after 24 hrs incubation at 37ºc.It is found that all the Lactobacillus sp, maintained maximum growth in 5% bile.

CONCLUSIONS

The probiotic organism *Lactobacillus bulgaricus* is a bacterial subspecies traditionally isolated from yogurts.It is a Gram positive rod shaped bacteria. The consumption adequate amounts of *Lactobacillus bulgaricus* confer benefit on the hos, normally associated with positive effects on the digestive and immune system.

The identified *Lactobacillus bulgaricus* were subjected in to various tests such as Bacteriocin production, Antibacterial activity, NaCl tolerance, Bile tolerance, Effect of pH. From the above test results, it was clear that they were able to tolerate intestinal pH and Bile acids which indicate the organisam can tolerate the intestinal pH and Bile acids which indicate that the organisams remain active in the intestine even after consumption. The inhibitory action of *Lactobacillus bulgaricus* can due to the accumulation of main primary metabolites such as lactic acid and baceriocins. From the finding it is concluded that even though they can tolerate the intestinal Ph and bile concentration, and several benefits to the hosts after consumption. The bacteriocins inhibit food borne pathogens *Klebsiella, E coli, staphylococcus aureous, Streptococcus, Salmonella.*

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