ORIGINAL RESEARCH PAPER

Microbiology

KEY WORDS: Isolation, Characterization, Lactic acid bacteria, 16s rRNA and Bacillus amyloliqufaciens

ISOLATION AND CHARACTERIZATION OF PROBIOTIC LACTIC ACID BACTERIAISOLATION AND CHARACTERIZATION OF PROBIOTIC LACTIC ACID BACTERIA

G.Buvaneswari Dr.UmayalRamanathan college for women, karaikudi, sivaganga district,Tamilnadu,India,Pin-630001

Dr.S. Annapoorani* Dr.UmayalRamanathan college for women, karaikudi, sivaganga district,Tamilnadu,India,Pin-630001*Corresponding Author

ABSTRACT

Lactic acid bacteria usually used as starter cultures in food technology are known to manufacture antimicrobial products having immense potential. Our study aims to isolate potential strain of lactic acid bacteria with probiotic characters. Five Lactic acid bacterial strains were isolated from the milk product samples. The culture supernatant was screened for antimicrobial activity against E.Coli. Among the five BJJ12 sample shows the maximum activity (12mm). So,the BJJ12 strain was assigned to the genera Bacillus, on the basis of their morphological, biochemical, physiological characteristics. The 16s rRNA gene sequencing revealed that the BJJ12 strain belongs to Bacillus amyloliqufaciens.

INTTRODUCTION

The term 'probiotic' originates from Greek language 'pro bios' which resources 'for life' different to 'antibiotics' which means 'against life'. The history of probiotics initiated with the history of man by intense fermented nutriments that are well known Greek and Romans consume very much (Gismondo, et al,. 1999, Guarner, et al,. 2005). In 1908 a Russian scientist Ellie Metchnikoff, who has a nobel prize, firstly proposed the valuable effects of probiotic microorganisms on human health. Metchnikoff theorized that Bulgarians are strong and long lived people because of the intake of fermented milk products which contains of rod shaped bacteria (Lactobacillus spp.). Therefore, these bacteria distract the gut microflora positively and reduction the microbial toxic activity (Gismondo, et al., 1999, Chuayana, et al., 2003).

The term 'probiotic' primarily used in 1965 by Lilly and Stillwell to define materials which encourage the growth of supplementary microorganisms. After this year the word 'probiotic' was used in different meaning according to its mechanism and the affected on human health. The significance was improved to the nearby one we use nowadays by Parker in 1974. Parker defined 'probiotic' as 'materials and organisms which donate to gut microbial balance'. In 1989, the meaning of use today was enriched by Fuller. Thus, probiotic is a living microbial enhancement which affects the host's health positively by improving its stomach microbial balance. Then this description was developed by Havenaar and Huis isn't Veld in 1992 containing mono or mixed culture of live microorganisms which useful for animal and man (Guarner, et al.,2005, Sanders 2003).

More than 400 bacterial organisms exit in human intestinal tract. It is and hugely complex biota that contains both facultative anaerobic and anaerobic microorganisms (Naidu, et al., 1999). The numbers of kinds is closely stable, because they each have their own growing niches (Fooks, et al., 1999). The alignment of the gut micro flora is continual but can be affected by some factors such as; age, diet, environment, pressure and medicine. To have a strong intestine the balance of the bacteria must be conserved but this is problematic as the lifestyles alteration. Lots of issues may alteration the stability away from possibly suitable or health.

Stimulating bacteria like lactobacilli and bifid bacteria to potentially destructive or pathogenic microorganisms like clostridia, sulphate reducers and Bacteroides species. It makes the host more susceptible to the illnesses. In this situation the prevalence of the valuable bacteria must be sustained. Using of probiotics help to protect the host from various intestinal diseases and disorders while increasing the number of beneficial bacteria and make the balance steady again (Fooks, et al., 1999). Probiotics are suggested as food to provide for the balance of intestinal flora (Holzapfel, et al., 1998).

Probiotics are used for long intervals in food components for human and also to feed the animals lacking any side effects. Also probiotics are acceptable because of being certainly in intestinal tract of healthy human and in nutriments.

Lactose Intolerance

Most of human normally non-Caucasians develop lactose intolerant after preventing. These lactose intolerant people cannot digest lactose due to the lack of vital enzyme -galactosidase. When they drink milk or lactose-containing products, indications including abdominal pain, bloating, flatulence, cramping and diarrhoea ensue. If lactose permits through from the small intestine, it is transformed to gas and acid in the large intestine by the colonic microflora. Also the presence of inhalation hydrogen is a signal for lactose maldigestion. The studies deliver that the addition of certain starter cultures to milk products, permits the lactose intolerant people to drink those products without the normal rise of breath hydrogen or associated symptoms (Fooks, et al., 1999, Scheinbach 1998).

The valuable properties of probiotics on lactose intolerance are described by two ways. One of them is minor lactose concentration in the fermented foods due to the high lactase activity of bacterial preparations used in the manufacture. The other one is; increased lactase active lactase enzyme enters the small intestine with the fermented invention or with the viable probiotic bacteria (Salminen, et al., 2004).

When the yogurt is compared with milk, cause the lactose is changed to lactic acid and the yogurt consist of bacterial -galactosidase enzyme; it is appropriate end valuable to consume by lactose intolerant. Furthermore, the LAB which is used to harvest yogurt, Lactobacillus bulgaricus and Streptococcus thermophilus, are not unaffected to gastric acidity. Hence, the products with probiotic bacteria are more resourceful for lactose intolerant human.

It is thought that the main factor recovers the digestibility by hydrolyses of lactose is the bacterial enzyme -galactosidase. Another factor is the slower gastric discharging of semi-solid milk products such as yogurt. So the -galactosidase activity of probiotic strains and other lactic acid bacteria used in dairy products is really essential. -galactosidase activity within probiotics differs in a vast variety. It has to be considered both the enzyme activity of probiotic strain and the activity left in the final product for their use in lactose intolerant focuses (Salminen, et al., 2004).

Immune System and Probiotics

The effects of immune system are hopeful. However, the mechanism is not well agreed. Human studies have exposed that probiotic bacteria can have positive properties on the immune system of their hosts (Mombelli and Gismondo 2000). Several

researchers have studied on the effects of probiotics on immune system stimulation. Some in vitro and in vivo searches have been carried out in mice and some with human. Data designate that oral bacteriotherapy and living bacteria feeding in fermented milks supported the immune system against some pathogens (Scheinbach 1998, Dugas, et al., 1999). Probiotics affect the immune system in different ways such as; producing cytokines, stimulating macrophages, increasing secretory IgA concentrations (Scheinbach 1998, Dugas, et al., 1999).

Dugas, et al (1999) examined whether eating fermented milk containing Lactobacillus acidophilus La1 and bifid bacteria could modulate the immune response in human. They give volunteers the test fermented milk over a period of three weeks during which attenuated Salmonella typhi Ty21a was administered to mimic an enteropathogenic infection. After three weeks, the specific serum IgA titre rise to S. typhi Ty21a in the test group was >4-fold and significantly higher (p=0.04) than in the control group which did not ate fermented foods but received S. typhi Ty21a. The total serum IgA increased. These results showed that LAB which Cn survive in the gastrointestinal tract can act as adjuvants to the humoral immune response.

Hirayama et al., (2000) feed the mice with lactobacilli or yogurt and it stimulated macrophages and increased secretory IgA concentrations (Scheinbach 1998). Also in a human trial Halpern et al., (1991) feed human with 450 g of yogurt per day for 4 months and at the end a significant increase is detected in the production of -interferon (Fooks, et al., 1999). Brady et al., (2000) showed that Lactobacillus rhamnosus GG and Bifidobacterium lactis Bb-12 derived extracts suppress lymphocyte proliferation in vitro. Further evidence for immune modulation by these two strains a children trial with severe atopic eczema resulting from food allergy. Children fed with Lactobacillus rhamnosus GG and Bifidobacterium lactis Bb-12 showed development in clinical symptoms related to the placebo group.

MATERIALS AND METHOD

ISOLATION OF BACTERIA

The sample were collected in sterile carriers and stored on ice until delivery to the laboratory. It was taken to the procedure for isolation. Sample were serially diluted up to 10-9 dilutions using sterile saline and diluted samples were plate on the sterile nutrient agar (pH-6.8) plates using spread plate method. The plates were incubated at 37˚C for 24 hours after incubation; individual colonies were selected and transferred into sterile broth mediums. The following step is purifying the selected colonies with streak plate onto nutrient agar.

SCREENING FOR LACTIC ACID BACDERIA

The isolated pure strains were streaked on MRS (deMan,Rogosa and Sharpe) agar (pH6.2andpH5.5) for the presence of lactic acid production. The positive cultures were selected based on the colony morphology showing white creamy colour colonies on MRS agar plates. The positive isolates were maintained in MRS agar slants and used for further studies.The composition of MRS agar is Pepton-10.0grm, Lab-Lemco meat extract - 10grm, Yeast extract-5grm,D(-)Glucose- 20grm, Tween 80-1ml, K2HPO4 -2, Sodium acetate- 5grm,Triammonium citrate- 2grm, MgSO4.7H2O- 0.2, MnSO4.4H2O- 0.05, Agar -15.0 and add 1000ml Distilled water. All ingredients were dissolved in distilled water and pH was adjusted to 6.3.Medium was sterilized by autoclaving at 121°C for 15 min.

PHYSIOLOGICAL AND BIOCHEMICAL CHARACTERIZATION GRAM STAINING

The gram reaction of the isolates was determined by light microscopy after gram staining. LABS are known to be gram positive. It means that they give blue-purple colour by gram staining. Cultures were grown in appropriate mediums at 37 °C for 24 h under anaerobic conditions. Cells from fresh cultures were used for gram staining. After incubation cultures were transferred aseptically into 1.5 ml eppendorff tubes and centrifuged for 5min at 6000 rpm. Then, supernatant was removed and cells were re-suspended in sterile water. Gram staining procedure was applied. Then, under light microscopy gram positives and purified isolates were determined.

MOTILITY TEST

The motility reaction of the isolates was determined by microscope .LAB are known to be non motility. Cultures were grown in appropriate mediums at 37˚Cfor24h under anaerobic conditions. Cells from fresh cultures were used one drop of cover slip on cavity slide .then, under microscopy and isolates were determined.

CATALASE TEST

Catalase is an enzyme produced by many microorganisms that breaks down the hydrogen peroxide into water and oxygen and causes gas bubbles. The formation of gas bubbles indicates the presence of catalase enzyme. 2H2O2 2 H2O + O2Catalase test was performed to isolates in order to see their catalase reactions. For this purpose, two methods can be applied. Overnight cultures of isolates were grown on MRS agar at suitable conditions. After 24 h 3% hydrogen peroxide solution was dropped onto randomly chosen colony. Also fresh liquid cultures were used for catalase test by dropping 3% hydrogen peroxide solution onto 1 ml of overnight cultures. The isolates, which did not give gas bubbles, were chooses. Since, LAB is known as catalase negative.

ENDOSPORE TEST

Bacterial smear was made on microscopic slide under aseptic conditions and head fixed. Then slide was placed over the steaming water both and malachite green (primary stain) was applied for 5min slide was removed from the water both and rinsed with water until water run clear. Then the slide was flooded with the counter stain safranin for 20 S and rinsed with water. After these slides were blot dried. They were observed under the light microscope.

OXIDASE TEST

Oxidase tests an experiment to distinguish among the groups of bacteria on the basis of cytochrome oxidase activity. a solution of 1% para phenylean diamine dihydro chloride is prepared in distilled water (5mg in 5ml). Soak the paper with few drops of the test organism and controls rub over the filter paper use for test organism. Observe the colour change.

INDOLE TEST

The organisms able to produce indole by degrading the amino acid by tryptophane. Preparation of (1%) Tryptone broth dissolve of peptone in distilled water sterilize in the autoclave at(121˚C) for 15 minutes. Added sample culture. Incubate inoculated culture at 37oC for 48 hours. After add 1ml of Kovac's reagend to each tubes and control. Shake the tubes gently after intervals for 10 min. Allow the tubes as to colour in the reagent layer.

METHYL –RED TEST

The methyl red (MR) test are used to differentiate two major types of anaerobic bacteria that produce large amounts of acid and those that produce the neutral product acetone as end product .preparation of MRVP broth (pH6.9) tubes. Pour the 5ml broth in each tubes and sterilize by autoclaving at 15lb pressure for 15 min. Inoculate MRVP tubes with sample culture and one tube control. Incubate all tubes at 35˚C for 48 hours. After all tubes add 5dropof methyl red indicator and observe the change in colour (red).

SUGAR FERMENTATION TEST

Approximate 100 ml of the nutrient broth solution was prepared in conical flask and 1ml phenol red was added to it. This medium was autoclave at (121˚C) for 15 min and cooled at room temperature. A syringe filter sterilized solution of 1% glucose was prepared under aseptic conditions. In all sterilized test tube, 5ml of the broth and 100 µl of the glucose solution and inoculated with freshly grown bacterial culture and incubated at 37˚C) for 48 hours. in case of homo fermentation, there will be production of acid along with the change in colour of the medium from red to yellow .and in hetero fermentation there will be gas production in Durham tubes alongside the change in the colour.

GROWTH AT DIFFERENT TEMPERATURE

Temperature test media, MRS containing bromecresol purple indicator, was prepared and transferred into tubes as 5 ml. Then fifty µl of overnight cultures inoculated to tubes and incubated for 7 days at 10 °C, 15 °C, and 45 °C. During these incubation time cells growth at any temperatures was observed by the change of the cultures, from purple to yellow

GROWTH AT DIFFERENT NaCl CONCENTRATION

Isolates were tested for their tolerance against different NaCl concentrations. For this purpose 4% and 6.5% NaCl concentrations were selected. Test mediums containing bromecresol purple indicator were prepared according to the appropriate concentration sand transferred into tubes in 5 ml. these tubes were inoculatedwith1% overnight cultures and then incubated at 37 °C for 7 days. The change of the colour from purple to yellow was proofed the cell growth.

Table1. BioChemical test

S.NO	CHARACTERIZATION	OBSERVATION	INTERFERENCE				
			BJJ11	BJJ12	BJJ13	BJJ14	BJJ15
1	Gram staining	Gram stain add glass slides culture under the microscope result for positive(purple)	+	+	-	-	+
2	Cell shaped	Microscope observed	Rod	Rod	Comma	Cocci	cocci
3	Motility test	One drop culture add cavity slide and under microscope	–	–	–	–	–
4	Oxidase test	Glass slide under oxidase paper on one drop of culture added after colour change	–	–	–	–	–
5	Catalase test	5 ml of culture add H2O2 added 3 drop after see air bubbles	+	–	+	–	+
6	Endospore test	Culture smears by glass slide and added malachite green under microscope	–	–	–	–	–
7	Indole test	Tryptone broth culture added Kovac's reagent after colour change yellow to cherry red ring	+	+	+	–	–
8	Methyl red	MRVP broth culture add 3 drop methyl red reagent after change colour yellow to red	+	+	–	–	+
9	Glucos fermentation	Observe the tube red to yellow colour change acid produced bacteria	+	+	+	+	–

ANTIMICROBIAL ACTIVITY

The antimicrobial activity of isolated LAB S .aureus was performed by well-diffusion methods. E.coli was incubated in nutrient broth 37˚C at 24 hrs. 15ml of Muller Hinton agar was prepared and 150µl of E.coli culture was inoculated into it. Once solidified the plate ,well of 6mm diameter were made and 40µl of each concentration of LAB isolates were filled into well. Then the plates were incubated at 37˚C for 24 hrs. And antimicrobial activity was determined by measuring the clear zone around the well.

RESULT AND DISCUSSION

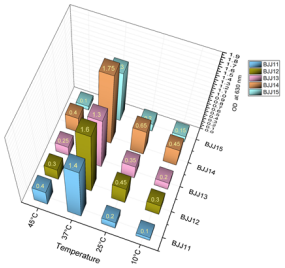
Isolate different type of bacteria from curd sample. With the help of phenotypic characteristic screening then the bacteria were isolated five different groups. Microscopic examination of the bacterial cell showed that varying morphology (yellow, white and pale color). In this study it was observed that the majority of the cultivable bacterial candidates belong to the Lactobacillus sp., E.coli sp., these bacteria have a different morphological and biochemical characterization. The gram staining results indicated that the isolated bacteria could be identified as Bacillus sps. All the pure bacterial candidates were sequentially subcultures on to MRS agar medium. Bismita Nayak, 2013, illustrate the growth of probiotic bacteria, it is similar to our report.

(+) positive, (-) negative.
Biochemical tests subjected on the following bacterial cultures (BJJ11, BJJ12, BJJ13, BJJ14 and BJJ15) were tabulated (table 1).

Among the 5 bacterial species BJJ11, BJJ12, BJJ15 species gives gram positive and BJJ13, BJJ14 species gives negative result after Gram staining reaction. To observe the bacterial morphology Cell shaped is analyzed BJJ11 BJJ12 shows Rod shape, BJJ13 views comma shape and BJJ14, BJJ15 Views cocci shaped. All bacterial isolates gives negative result for Motility test & Oxidase test. Among the 5 species 3 strains (BJJ11, BJJ13 & BJJ15) gives positive result for Catalase test. The isolated 5 strains were does not produced endospore. 3 isolates produce positive result for indole production (BJJ11, BJJ12, BJJ13). With the help of MRVP broth the isolates screened for methyl red test 3 (BJJ11, BJJ12, BJJ15) strains gives positive result. Glucose fermentation was analyzed 4 (BJJ11, BJJ12, BJJ13 & BJJ14) strains gives positive result.

50 µl of overnight culture were transferred into the tubes which contain 5ml of MRS broth at different temperatures and incubate 7days at 10˚C, 25˚C, 37˚C, 45˚C. The colour is change from purple to yellow as taken the evidence for cell growth (BettacheGuessas et al., 2004). It was observed at colorimeter 630nm. The OD value is measured at 630nm (Fig.1). The optimum temperature 37˚C was suitable to growth all the bacteria. BJJ14 is able to grow at 25˚C and BJJ12 is growing above 37˚C.

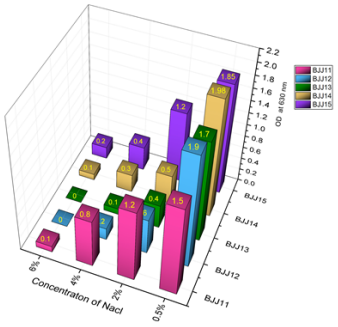
Figure1. Effect of Temperature on growth patterns of isolates



50µl of overnight cultures were transferred into the tubes which contain 5ml Nacl tested to growth 0.5%, 2%, 4%, 6%, Nacl concentrations. The results showed that the isolated bacteria. Further Experiments was selected as the standard microorganism, as it showed the best resistance in both solid and liquid medium. The effect of different Nacl on the bacterial growth was measured at calorimeter. The OD value is measured at 630nm. The optimum concentration of Nacl 0.5% was found in all isolates (Fig.2). They

were incubated for 7 days at 37˚C (Ashwainkumar et al., 2014)

Figure2. Effect of sodium chloride on growth patterns of isolates



The selected strains were examined according to their antimicrobial activity for this purpose, strain were detected against the indicator microorganisms Escherichia Coli and their diameter of inhibition zones. All the five isolates were inoculated against E.coli gut pathogenic bacteria. (Shruthy vv, 2011) Among the five isolates 3 bacterial isolates were have the probiotic activity against E.coli bacteria. The result indicates that maximum zone of inhibition was produced by BJJ12 isolate (1.2mm). The isolates BJJ11 & BJJ13 gives the moderate zone of inhibition such as 0.7mm & 0.3mm respectively. So, the present study indicates that 3 of the isolate have the probiotic activity.

Table2. Antimicrobial activity Against Escherichia Coli

S.NO	ORGANISM	ZONE OF INHIBITION (mm)				
		BJJ11	BJJ12	BJJ13	BJJ14	BJJ15
1	E. coli	0.7mm	1.2 mm	1.3 mm	NIL	NIL

CONCLUSION

Lactobacillus sps., Bacillus sps.,were isolated from isolates from the different curd samples can be exploited in the probiotic activity against pathogenic gut bacteria (E.coli). The term of probiotic is efficiency. The Lactobacillus sps., probiotic organism was, temperature and Nacl environment. Therefore novel microorganism should be intensively screened for probiotic activity.

ACKNOWLEDGEMENT

The authors are grateful to the Management and the Principal of, Dr.UmayalRamanathan college for women, karaikudi, sivaganga district,Tamilnadu, India for providing all the necessary facilities and encouragement. We would like to acknowledge the helpful and extensive recommendations of anonymous reviewers made to this paper.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

REFERENCES

1. Ashwainkumar, Dineshkumar, 2014. isolation and characterization of bacteria from dairy samples of solan in Himachal Pradesh for identification of lactobacillus spp. International journal of pharmaceutical science review and research.,25(2) pp;110-114
2. BettacheGuessas and Mebrouk KIHAL,2004 characterization of Lactic acid bacteria isolated from raw goat's milk. African journal of biotechnology vol 3 (6) pp-399-342
3. Bismita Nayak,2013. Screening and characterization of potential probiotic lactic acid bacteria isolated from vegetable waste and fish intestine .international journal current microbiology and applied science.(2013) 2(8 pp;234-244.
4. Brady, L.J., Gallaher, D.D., Busta, F.F. 2000. The role of probiotic cultures in the prevention of colon cancer. The Journal of Nutrition.130:410S-414S.
5. Chuayana, Jr. E.L., V. Ponce, C., Rivera, M.R.B., Cabrera, E.C. 2003. Antimicrobial activity of probiotics from milk products. Phil J. Microbiol. Infect. Dis. 32(2):71-74.
6. Dugas, B., Mercenier, A., Lenoir-Wijnkoop, I., Arnaud, C., Dugas, N., and Postaire, E.1999.Immunity and probiotics.Trends Immunology Today 20(9):387-390
7. Fooks, L.J., Fuller, R., Gibson, G.R. 1999. Prebiotics, probiotics and human gut microbiology. International Dairy Journal 9:53-61.
8. Gismondo, M.R., Drago, L., Lombardi, A. 1999. review of probiotics available to
9. modify gastrointestinal flora. International Journal of Antimicrobial Agents.12:287-292.
10. Guarner, F., Perdigon, G., Corthier, G., Salminen, S., Koletzko, B. and Morelli, L. 2005. Should yoghurt cultures be considered probiotic.British Journal of Nutrition.93:783-786.
11. Hirayama, K., and Rafter, J. 2000. The role of probiotic bacteria in cancer prevention. Microbes and Infection 2:681-686.
12. Holzapfel, W.H., Haberer, P., Snell, J., Schillinger, U., Huisin't Veld, J. 1998. Overview of gut flora and probiotics.International Journal of Food Microbiology 41:85-101.
13. Mombelli, B., Gismondo, M.R. 2000.The use of probiotics in medical practice. International Journal of Antimicrobial Agents 16:531-536.
14. Naidu, A. S., Bidlack, W. R. and Clemens, R. A. 1999. Probiotic spectra of lactic acid bacteria (LAB). Critical Reviews in Food Science and Nutrition 39(1):13-126.
15. Quwehand, A.C. and Vesterlund, S..eds. 2004. Antimicrobial components from lactic acid bacteria. Lactic Acid Bacteria Microbiological and Functional Aspects. New York: Marcel Dekker Inc.
16. Salminen, S. 1999. Probiotics: scientific support for use. Food Technology 53(11):66.
17. Salminen, S., Gorbach, S., Lee, Y.K., Benno, Y. 2004. Human studies on probiotics:What is scientifically proven today?. Lactic Acid Bacteria Microbiological and Functional Aspects, New York: Marcel Dekker Inc.
18. Sanders, M.E. 2003. Probiotics: Considerations for human health. Nutr.Rev. 61:91-99.
19. Scheinbach, S. 1998. Probiotics: Functionality and commercial status. Biotechnology Advances 16(3):581-608.
20. Schrezenmeir, J. and Vrese, M, 2001. Probiotics, prebiotics, and synbiotics – approaching a definition. Am. J. Clin. Nutr.73:361S-364S.
21. Shruthy vv, 2011 Probiotic potentials among lactic acid bacteria isolated from curd. International journal of research in Ayurveda and pharmacy,2(2),2011 602-609.