Isolation, Identification And Characterization of Lactic Acid Bacteria From Rice Wash Sample And Analyse Its Probiotic Nature

Saloni S Samdadiya[1], Shital D Gaikwad[2], Anjali S Hire[3], Ragini P Pansare[4]
Department of Microbiology
[1,2,3,4]New Arts Commerce and Science College, Ahmednagar, Maharashtra, India.

Abstract- *The aim of this study was to isolate lactic acid bacteria (LAB) from rice wash. Rice wash sample was cultured on De Man Rogosa – Sharpe agar (MRS) media with proper dilution for isolation of LAB and pure culture were isolated after continuous subculturing .Identification of LAB was carried out by morphological characteristics, and biochemical characteristics. Different isolates are found from rice wash but single isolate was studied to analyse its properties and then this isolate was checked for the Bile tolerance, NaCl tolerance, pH tolerance, Phenol tolerance, and Antibiotic susceptibility test against different antibiotics such as amikacin, lomefloxacin, cefadroxil, sparfloxacin, netillin, ceffazidime, seffriaxone, ciprofloxacin, cefotaxine, gentamycin, ampicillin and cefoperazone therefore the present study may represent a unique source of isolating LAB.*

Keywords- Lactobacillus, probiotic, rice wash.

I. INTRODUCTION

In nature, there are various organisms present naturally among them some are beneficial and some are harmful to our health. As there is increase in disease day by day its very important to increase the production of beneficial microorganisms to defend with harmful microorganisms and to be heathy. LAB are Gram positive facultative anaerobic or microaerophilic bacteria. In human they are symbiotic and found in gut flora. LAB areusually found in decomposing plants and milk products which produce lactic acid as a major metabolic end product of carbohydrate fermentation. LAB are generally recognised as safe (GRAS) bacteria that have been used in processing of fermented food from centuries. LAB is used for the production of probiotics such as in yakult, nesslac, gain, nestal, yoghurt , milk drinks, cheese, icecream, beer, wine, cider, chocolate and other fermented foods. Samples from which Lactic acid bacteria (LAB) can be isolate are Rice wash, milk, curd, soil, spinach, fruits, idli batter. etc. The term probiotic literally means “for life” was first addressed by lilly and Stillwell (1965) and was used to describe substance produced by different lactobacillus species. probiotic are lives microbes that can be formulated into many different types of products including food, drugs and dietary suppliments. Lactic acid bacteria (LAB) are essential to keep our intestine healthy for long life. Intake of LAB increases the beneficial organisms and reduces the amount of dangerous organisms which causes harmful effect on our body. Mankind exploited these bacteria for thousands of year for the production of traditionally fermented products because they have various abilities to produce desirable changes in taste, flavour, texture and extending the shelf life of food product. The main objective of our study is to isolate LAB and test the probiotic property of those isolates and check growth inhibition activity.

MATERIAL AND METHODS Collection of Sample:

Rice was washed out to remove dirt then water is added to it and kept for 24 hours for soaking purpose. After 24 hours the rice were filter and rice wash water sample was collected. Sample was collected in sterile bottle and stored in an ice box until delivery to the laboratory for analysis.

Characterisation & Identification of lactic acid bacteria:

The isolate was identified based on standard morphological, cultural and biochemical characteristics such as Gram character, Motility, ability to ferment sugars like sucrose, glucose, sorbitol, mannitol, lactose, maltose, catalase test, oxidase test etc.The observed characters were compared to Bergey’s Manual of Determinative Bacteriology.

Morphological characteristics:

Morphological examination was carried out by examining colony characteristics, Gram staining, endospore staining and motility.

i. Gram staining:

Gram staining was performed by using 24 hours old culture of the isolated organism. A heat fixed smear of the culture was prepared to find out cell shape, size, and arrangement and differentiate Gram positive and Gram negative bacteria. A smear was flooded with crystal violet for

1 minute, and then Grams iodine solution was added. Thereafter decolourization was done. Counter staining with saffranin for 5-7 minute was done and dye washed off by rinsing the slide with tap water. The slide was then air dried and examined microscopically under oil immersion objective lens.

ii. Endospore staining:

The endospore staining was done to detect weather the bacteria produced endospore or not. By using aseptic condition, the bacterial smear was prepared and heat fixed. The slide was placed over steaming water bath and malachite green (primary stain) was applied for 5 min. After 5 min the Slide was removed and rinsed with water. Then the slide was flooded with the counter stain saffranin for 2 min. and rinsed with water. After these slides were blot dried, they were observed under the light microscope under oil immersion objective lens.

iii. Motility:

The motility of isolate was detected by using hanging drop method. A thin rig of petroleum jelly was placed around the depression of clean slide. A loopful 24 hours old MRS broth culture was placed in centre of coverslip using nichrome wire loop. A depression slide was inverted over the cover slip, with well over the drop. It then quickly inverted so that cover slip was a top. Motility was observed under microscope near the edge of a culture drop at 40X objective lens.

II. BIOCHEMICAL CHARACTERIZATION

Biochemical characterisation was done by performing catalase test, oxidase test, sugar fermentation test.

i. Sugar fermentation test:

The ability of isolate to ferment sugar was determined by using peptone water based medium with 1% glucose, lactose, mannitol, sucrose, maltose, sorbitol. Isolate was inoculated in different sugar fermentation broths and then tubes were incubated at 370C at 24 hours. A positive reaction i.e. acid production was indicted by colour change of medium from red to yellow and gas production was indicated by bubble formation in inverted Durham's tube.

ii. Oxidase test:

Oxidase test was performed by wet filter paper method. A strip of filter paper soaked with a freshly made 1% solution of N', N',N,N-Tetramethyl-p-phenylene diamine dihydrochloride. Then with the help of sterile loop, a well isolated colony of isolate was rubbed on filter paper strip. A positive reaction was indicated by an intense deep purple colour appeared within 5-10 seconds. A negative reaction was indicated by absence of coloration or by colouration later than 60 second.

iii. Catalase test:

Catalase is an enzyme produced by many organisms that breaks down the hydrogen peroxide into water and oxygen cause gas bubble. Observe the formation of gas bubbles indicates the presence of catalase enzyme. Catalase activity was tested by tested by test tube method. A loopful of culture of each isolate was immerged in tube containing 3% hydrogen peroxide solution separately. Bubble formation shows the positive result.

III. ANALYSIS OF PROBIOTIC PROPERTIES OF THE ISOLATE

Tolerance to Acidic pH:

It was reported that acid such as hydrochloric acid found in the human stomach, interrupt the biomolecules of cells, such as protein, vitamins, fatty acid and DNA(5) and low pH environments can inhibit the metabolism and reduce the growth and viability of lactobacillus. Thus to assess the viability of isolate, it was allow to grow at pH range 9 to 2. MRS broth was adjusted with different pH (2, 3, 4, 5, 6, 7, 8, 9). Then the 1% over night culture was inoculated in each tube and incubated at 370C for 24 hours. After incubation loopful culture was transfer on sterile MRS agar plates which were incubated at 370 C for 24 hrs and growth was checked. Tolerance to low pH found due to the presence of special enzyme H+ translocating ATPase in the cytoplasmic membrane of Lactobacillus spp. Which maintains cytoplasmic pH more alkaline then the outside medium (2).

Assay for NaCl Tolerance:

Probiotic features of Lactobacillus isolate was evaluated by checking its NaCl tolerance, test tubes containing MRS broth were adjusted with different concentrations (2%, 4%, 6%, 8%, and 10%) (w/v) of NaCl. After sterilization 1% over night culture was suspended in each tube and incubated at 370C for 24 hours. After incubation loopful culture was transfer on sterile MRS agar plates which were incubated at 370 C for 24 hours and growth was checked.

Assay for Bile Salt Tolerance:

Bile salt tolerance of isolated lactobacillus was determined by adding different concentrations of bile salt in each tube containing MRS broth. The concentrations used are 0.1%, 0.2%, 0.3% and 0.4%(w/v) of bile salt. After sterilization 1% over night culture was suspended in each tube and incubated at 370C for 24 hours. After incubation loopful culture was transfer on sterile MRS agar plates which were incubated at 370 C for 24 hours and growth was Checked.

Assay for phenol Tolerance:

Phenol tolerance of isolated lactobacillus was determined by adding different concentrations of phenol in each tube containing MRS broth. The concentrations used are 0.1%, 0.2%, 0.3%, 0.4% and 0.5% (w/v) of phenol. After sterilization 1% over night culture was suspended in each tube and incubated at 370C for 24 hours. After incubation loopful culture was transfer on sterile MRS agar plates which were incubated at 370 C for 24 hours and growth was checked.

Antibiotic susceptibility Testing:

The antibiotic susceptibility of isolated Lactobacillus species was assessed using different antibiotic discs on MRS agar plates seeded with the test probiotic organism. The antibiotic discs were placed on the surface of agar and the plates were incubated at 37°C for 24 hours. Resistance was assessed against the different antibiotic namely amikacin, lomefloxacin, cefadroxil, sparfloxacin, netillin, ceffazidime, seffriaxone, ciprofloxacin, cefotaxine, gentamycin, ampicillin, cefoperazone. The antibiotic susceptibility was determined by measuring the inhibition zones around the each disc and expressed in millimetre (mm).

IV. RESULT AND DISCUSSION ISOLATION OF LACTIC ACID BACTERIA

After 24 hours of incubation on MRS agar plate at 370C different types of colonies are observed, out of them creamish colour colony (Fig. no. 1) was selected for further study.

Characterisation & Identification of lactic acid bacteria:

The isolate was identified based on standard morphological, cultural and biochemical characteristics such as Gram character, Motility, ability to ferment sugars like sucrose, glucose, sorbitol, mannitol, lactose, maltose, catalase test, oxidase test etc.The observed characters were compared to Bergey's Manual of Determinative Bacteriology.

Morphological characterisation:

Isolate was Gram positive, rod shaped (Fig. no.2), non motile and non sporulating organism. Colony characters mention in table no. 1

Figure no.1.Growth of isolate after 24 hr. Incubation

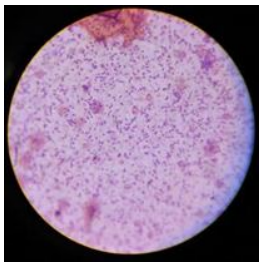

Figure no. 2 Microscopic Observation

Table No. - 1: Morphological & Cultural characteristics of the isolates

Morphological characters	Isolate
Size	1mm
Shape	Circular
Colour	Creamish white
Margin	Entire
Elevation	Convex
Consistency	Sticky
Opacity	Translucent
Motility	Non Motile
Gram character	Gram positive rod

Biochemical Characterisation:

The biochemical Characterization was studied by analysing sugar fermentation, catalase and oxidase test. It was observed that isolate was ferment Lactose, maltose, Sucrose, Sorbitol, with acid and gas production, while Glucose was

ferment with only acid production and manitol was not fermented. The oxidase and catalase test of the isolate was negative (Table no. 2). According to Bergey's manual of determinative bacteriology

from morphological and biochemical characterisation the isolate was tentatively identified as *Lactobacillus spp.*

Table No. 2- Biochemical Characteristics of the isolate :-

	Biochemical characters		**Acid**	**Gas**
1.	Sugars ferment ation	Lactose	+	+
		Maltose	+	+
		Sucrose	+	+
		Sorbitol	+	+
		Glucose	+	-
		Manitol	-	-
2.	Oxidase test		-	
3.	Catalase test		-	

Analysis of Probiotic Properties of the Isolate:

Tolerance to low pH:

The resistant to low pH is one of the major selection criteria for probiotic strains. Therefore to reach the small intestine they have to pass through from the stressful conditions of stomach. Therefore in the present study growth was checked at pH 2 to 9.(7). It is reported that acids such as the hydrochloric acid found also in the human stomach, interrupt the biomolecules of cells, such as proteins, vitamins, fatty acids and DNA. *Lactobacillus* isolated show growth at pH at 3, 4, 5 and 6. The different isolates of Shivaram and Vishwanath (2012), were tolerate pH 2 and 3 (9). While Park et al. (2006) observed that their four isolates were tolerate pH 2,3,4,5 and 7(6).Tolerance to low pH of lactic acid bacteria was determined and following data was obtained.

Table No. 3: Growth of isolate at different pH

pH	**Growth (after 24 hours)**
2	+
3	+
4	+
5	+
6	+
7	+
8	-
9	-

Tolerance to High NaCl concentration:

Lactic acid bacteria generally tolerate high salt concentrations. It allows the bacteria to begin metabolism, which produces acid that further inhibits the growth of non-desirable organisms. Isolated *lactobacillus spp.* Showed growth up to 8% NaCl concentration (Table No.3). Jeronymo-Ceneviva and et al. found that all isolated were able to grow at 1 to 6 % NaCl concentration but failed at 7 to 10 % NaCl concentration(4). Poonam B Chauhan and Divya Daru found tolerance at 4% NaCl tolerance by using milk curd and fecal sample (8).

Table No. 3: Growth of isolate at different NaCl concentration

NaCl%	Growth(After 24 hrs.)
2%	+
4%	+
6%	+
8%	+
10%	-

Tolerance to bile Salt:

Tolerance to bile salts is considered to be a main prerequisite for growth, colonization and metabolic activity of bacteria in the host's gut(3). Therefore, it is generally included in the selection criteria of potential probiotic. In the present study the isolate was screened for their ability to tolerate the bile salt. The isolate showed the 0.4% bile salt tolerance. Dunne, C.et. al. found 14 lactobacillus that showed growth at 0.3 % bile salt concentration. 6 isolate showed growth at 0.5% bile salt concentration (1). While Poonam B Chauhan and Divya Darufound 14 isolate were grown at 0.3% bile salt concentration (8).

Table No.4: Growth of isolate at differant bile salt concentration

Bile salt percentage	Growth (after 24 hours)
0.1%	+
0.2%	+
0.3%	+
0.4%	+

Phenol Tolerance:

Tolerance to phenol is characteristics property because phenol can be formed in the intestinal by bacteria that determine same aromatic amino acid delivered by diet or produced by endogenous protein. The isolate showed 0.5% phenol tolerance concentration. Poonam B Chauhan and DivyaDaru found 14 lactobacillus isolated among that 8 isolates showed growth at 0.2% phenol concentration and 7 isolate showed growth at 0.3% phenol concentration. In there study, Probiotic isolate were tolerated 0.4% phenol concentration which is toxic metabolite produced by deamination of some aminoacids during disintegration by intestinal bacteria (8). Soliman and et al found 3 strains of lactobacilli have ability to tolerate phenol at 0.4 % concentration.(10).

Phenol concentration

Table No. 5: Growth of isolate at different

Phenol Percentage	Growth (After 24 hrs)
0.1%	+
0.2%	+
0.3%	+
0.4%	+
0.5%	+

4.4. Antibiotic Susceptibility Testing:

The isolate was resistant against antibiotic Amikacin, Lomefloxacin, Cefadroxil, Sparfloxacin, Netillin, Ceffazidime, Seffriaxone, Ciprofloxacin, Cefotaxine, Ampicillin, Cefoperazone and sensitive only against gentamycin (2mm). Poonam and Divya Daru found Antibiotic susceptibility of 5 lactobacilli isolates that were resistant to Vancomycin, Tetracycline and sensitive to Erythromycin, Penicillin, Ampicillin(8). According to Soliman A. H. and etal they studied Lactobacillus species and found Lactobacillus acidophilus was resistant to Amikacin, Ciprofloxacin, Cefotaxime, Gentamycine and Kanamycine while sensitive to Ampicillin, Chloramphenicol and Streptomycin. *Lactobacillus casei* was resistant to Kanamycine, Vancomycin and Amikacin while sensitive to Ciprofloxacin, Rifampicin and Erythromycin. Lactobaccilus planetarium was resistant to Kanamycine, Clindamycine and Ciprofloxacin while sensitive to Amoxicillin and Rifampicin.(10).

IV. CONCLUSION

In the present study, we had isolated, characterized and identified lactic acid bacteria from rice wash. The isolated *Lactobacillus spp* .had the ability to survive in acidic condition (low pH),tolerate high bile salt concentration, high salt concentration, phenol concentration and resistant to most of antibiotics which indicates it obey probiotics properties. Based on these tests, there is a high possibility that the isolates would be able to reach the intestinal tract so as to work as probiotics. However the isolated lactobacilli need to be further investigated to established their health benefits as probiotics.

The sample used for the isolation of lactobacillus is rice wash that is cheap, eco-friendly, rapidly available, non-toxic waste product left after washing of rice before cooking. As it follows many characters and properties which are required for an ideal probiotic, if it is further processed it would be the best probiotic drink which may boost humans immune system and keep human healthy.

ACKNOWLEDGEMENT

The authors acknowledge Depatrment of Micrcobiology, College of New Arts, Commerce and Science College, Ahmednagar, Ethiopia for giving permission to use microbiology laboratory.

REFERENCES

[1] Dunne, C ., O'Mahony, L., Murphy, L., Thornton, G., Morrissey, D., O'Hallorn, S., Feeney, M., Flynn, S., Fitzgerald, G., Daly, C., Kily, B., C O' Sullivan, G., Shanahan, F., and Collins J.K., In vitro selection criteria for probiotics bacteria of human origin: Correlation with in vivo findings. America Journal of Clinical Nutrition. (2001). 73:386S-392S.

[2] Fuller R. Probiotics in Man and animal Journal of Applied Bacteriology. (1989)66: 365-378.

[3] J Nowroozi, M Mizaii, M Norouzi (2004) study of lactobacillus as probiotic bacteria. Iranian.JPubl Health, 33(2);1-7.

[4] Jeronymo-Ceneviva AB, et al. (2014) Probiotic properties of lactic acid bacteria isolated from waterbuffalo mozzarella cheese. Probiotic antimicrobial proteins 6:141-156.

[5] M. H Forhad, S. M. Khaledur Rahman, Md. Shahedur Rahman, Forhad Karim Saikot and Krishno Chandra Biswas. (2015) Probiotic Properties Analysis of isolated lactic acid bacteria from buffalo milk. Archeives of Clinical Microbiology. ISSN (1989-8436) iMedPub Journals.

[6] Park S.C., M. H. Hwang, Y. H. Kim, J. C. Kim, J. C. Song, K. W. Lee, K. S. Jeong, M. H. Rhee, K. S. Kim and T. W. Kim,(2006).Comparison of pH and bile resistance of lactobacillus acidophilus strains isolated from rat, pig, chicken and human sources.World Journal of Microbiology and Biotechnology,22:35-37.

[7] Patil Liladhar Shivram, Pandav Parag Vishwanath (2012) Assessment of probiotic potential of Lactobacillusspp. Form cheese and preparation of probiotic ice-cream.IJRAP3(4):532-536

[8] Poonam B Chauhan and Divya Daru (2016) isolation and characterization of lactobacillus isolated from milk, curd and fecal sample and assigning their probiotic value. International Journal of Pharma and Bio science 7(3):(B) 1070-1075.

[9] Shivram, P. L.and P. P. Vishwanath (2012).Assessment of probiotic potential of lactobaciilussp isolated from cheese and preparation of probiotic ice-cream. IJRAP,3(4):532-536.

[10] Soliman A. H. S., Sharoba A. M., Bahlol H. E. M., Soliman A. S. and Radi O. M. M. (2015) Evaluation of Lactobacillus acidophilus, Lactobacillus casel and Lactobacillus plantarum for probiotic characteristics. Middle East Journal of Applied Sciences 05:94-101.