

Research Article

Isolation, optimization and estimation of probiotic *Lactobacillus* from different natural sources

Durga Devi N. K^{*}, Reshma B, Hari Priya D, Santhosh Kumar D, Nagamani A. S. S.

KVSR Siddhartha College of Pharmaceutical Sciences, Vijayawada, Andhra Pradesh, India.

*Corresponding author: nelluriss@rediffmail.com

Revised: 2 June 2019; Accepted: 9 October 2019

ABSTRACT

Purpose: In the field of nutrition, probiotics are widely in use and lactic acid from probiotics acts as the best nourishing agent. Production of lactic acid from probiotics requires a large quantity of De Man, Rogosa and Sharpe agar (MRS) media that doubles the cost of production and may also affect vegan choice. So there arises a need to develop a cost-effective new media that replaces the animal sources. This study is about the optimizing and improving a cost-effective growth medium by replacing animal products with high nutritional cheap materials obtained from plants to produce lactic acid by isolated *Lactobacilli* strains from pure sources.

Method: This work is a comparative analytical study following use of different media for determination of best carbon sources (dates, pineapple, molasses, sucrose, fructose, galactose) and nitrogen sources (peanut, chickpea, wheat, lentils, Bengal gram, mung beans) among the different sources available to constitute the best media which is cost effective for the production of lactic acid. **Results:** In the optimization of nitrogen source, the highest growth was observed with the lentil seed powder. In the optimization of the carbon source, the highest increase was observed with molasses. Molasses a by-product of the sugar industry, incorporated as a carbon source, lentils as the nitrogen source in the media showed best results in terms of lactic acid production. **Conclusion:** The low-cost media is obtained by using plant sources, making it suitable for vegan choice. Animal products can be replaced with plant sources for the cost-effective production of probiotic *lactobacilli* and lactic acid.

Keywords

MRS media; Probiotics; Solid-state fermentation; Lactic acid; Vegetable-based media

INTRODUCTION

Lactobacillus's or lactic acid bacteria (LAB) are an order of gram positive, acid-tolerant, generally non-sporulating, non-respiring, either rod- or coccus-shaped bacteria that have similar physiological and metabolic characteristics. Milk products and decomposing plants contain these bacteria and produce lactic acid as the primary metabolic end product of carbohydrate

fermentation. This trait has, throughout history, linked LAB with food fermentations, as acidification inhibits the growth of other spoiling organisms. Annually, of the total lactic acid produced worldwide, 90% of the lactic acid production is from bacterial fermentation and remaining is produced synthetically by the hydrolysis of lactonitrile.(1-2)



This article is published under the Creative Commons Attribution CCBY License (<https://creativecommons.org/licenses/by/4.0/>). This license permits use, distribution and reproduction in any medium, provided the original work is properly cited.

The World Health Organization's (WHO), 2001 definition of probiotics, is "live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host.(3) During administration, probiotics have to be alive. Few concerns of the scientific literature are the reproducibility and viability on a large scale of the observed results, the sustainability and stability during use and storage, and finally the ability to survive in stomach acids and then in the intestinal ecosystem. Probiotics must have undergone a controlled evaluation to document health benefits in the target host. Only products containing live organisms shown in reproducible human studies to confer a health benefit can claim to be a probiotic.

Prebiotics serves as the food for the probiotics as they do not get digested in the human body but provides the fermentable substances to support the selected bacteria in the human gut. Most commonly used probiotics are strains of LAB.

Current research proves that human gut microbiota composition and activity is a crucial factor in assessing a person's risk for obesity and other associated diseases such as atherosclerosis, dyslipidemia, insulin resistance, diabetes, hepatic steatosis and steatohepatitis. There is growth in the market of probiotics due to awareness of their health benefits in conjunction with scientific data to prove this phenomenon. Some benefits of probiotics include immune stimulation, enhancement of bowel mobility, and reduction of inflammatory or allergic reactions. Recent research on the molecular biology and genomics of the probiotic bacteria *Lactobacillus* has focused on its interaction with the immune system, anti-cancer potential, its effect on adipocyte cell

size and body fat, and its potential as a biotherapeutic agent in cases of antibiotic-associated diarrhea, travelers' diarrhea, pediatric diarrhea, inflammatory bowel diseases and irritable bowel syndrome.

Specifically, interest has increased in lactic acid bacteria because of its potential to improve cholesterol as well as its anticarcinogenic, antipathogenic, and antidiabetic properties. To date, the largest segment of the digestive health market - particularly within the functional food category - is taken up by products made with probiotics, or 'friendly bacteria'. *Lactobacilli* can even be found in sensitive household products such as infant foods and a variety of pharmaceutical formulations.

This study aims to modify De Man Rogosa Sharpe (MRS) culture medium for the selective cultivation of probiotics strains for consumption by the strictly vegetarian human population. Vegetarian probiotic foods by definition must be free from all animal-derived ingredients. These not only include the product ingredients but the probiotic inoculum as well.

Solid state fermentation (SSF) is a biomolecule manufacturing process used in the food, pharmaceutical, cosmetic, fuel and textile industries. These biomolecules are mostly metabolites generated by microorganisms grown on reliable support selected for this purpose. This technology for the culture of microorganisms is an alternative to liquid or submerged fermentation, used predominantly for industrial purposes.(3-4) Solid state fermentation has existed for several centuries. In Asia and Japan, it is known as "Koji" fermentation.

MATERIALS AND METHODS

All the chemicals used in the production were of analytical grade available in our laboratory. The substances used belonged to the Hi-Media Laboratories Pvt Ltd. and Loba Chemie Pvt. Ltd.

Isolation of Lactobacilli

Isolate *Lactobacillus spp.* strains were obtained from natural sources like curd, milk, whey and cheese by spread plate method, followed by quaternary streaking. Stored the isolated bacteria in 20% glycerol at -40°C . The media used in production was MRS media.

Nitrogen sources

Various plant seed powders replaced the beef extract and peptone as the nitrogen source in the MRS media. Six different plant seeds were used that included mung beans, lentils, chickpea, peanut, Bengal gram and wheat. All the seeds were soaked separately for 8 hours. Germination of these seeds took 72 hours. The germinated seeds were dried at 45°C and then powdered.

Carbon sources

The carbon sources used were dates, pineapple waste, molasses, sucrose and galactose. Sucrose and galactose were procured from Loba Chemie Pvt Ltd.

Dates powder

The seeds were removed from the dates and cut into pieces, dried and powdered. This powder was then sterilized.

Pineapple

The pineapple is rich in carbohydrates and fibre. Used pineapple waste as a carbon source. This aids to reduce the cost of the medium. Pineapple waste was autoclaved for 15 min at 121°C resulting in the flocculation of particulates that settled rapidly upon

cooling them to room temperature. Separated the particulates by centrifugation for 10 min at 5000 rpm. Filtered the clear supernatant using Whatman filter paper under vacuum and store at -18°C .

Molasses

Harvested the sugar cane and striped their leaves to make molasses. Obtained their juice by extracting, cutting, crushing, or mashing. Boiled the juice to concentrate and promote the crystallization of sugar. The result of this first boiling is called first syrup and it has the highest sugar content. The first syrup is usually referred to in the Southern states of the U.S. as cane syrup, as opposed to molasses. Second molasses is created from a second boiling and sugar extraction and has a slightly bitter taste.

Optimization of nitrogen source

In the MRS media, the nitrogen sources are peptone, beef extract and yeast extract. As beef extract and peptone are animal-derived products, they were replaced with plant sources. According to the composition of the MRS media, 2.4% (1% peptone, 1% beef extract, 0.4% yeast extract) of nitrogen source is the requirement. Replaced this 2.4% with the respective plant seed powder.

Added all the components into the beaker and adjusted the volume with distilled water. Six different mediums were prepared using the various nitrogen sources such as mung beans, lentils, chickpea, peanut, Bengal gram and wheat.(5) The pH of the medium was adjusted to acidic with HCl or NaOH. Transferred the media into the boiling tubes and was autoclaved at 121°C at 15lbs pressure for 15-20 minutes. When the media reached room temperature, inoculated the organisms isolated from the four sources as described above into the media. After

inoculation, incubated the tubes at 37 °C for three days and experiment was repeated in triplicate.

Later the organism was streaked on to the fresh medium for identification of the organism. The organism was identified by performing biochemical tests such as gram staining test, catalase test and motility test.

After incubation, samples were collected, and suitable dilution were performed. The absorbance of the sample was determined using U.V-Visible spectrophotometer at 600 nm.

Optimization of carbon source

In the MRS media, the carbon source constitutes about 2%. These carbon sources were obtained from three natural sources (pineapple waste, dates solution and molasses) and purified natural sugars (sucrose, fructose and galactose). (5-9) All the components were added into the beaker, and the volume was adjusted with distilled water. Six different mediums were separately prepared by using six various carbon sources and aligned the pH of the medium with HCl or NaOH. Transferred the media into the boiling tubes, and was autoclaved at 121⁰C at 15lbs pressure for 15-20 minutes. When the media reached room temperature, inoculated the *Lactobacillus spp.* strains into the media. After inoculation, incubated the tubes at 37 °C for three days. The experiment was repeated in triplicate. Later the organism was streaked on to the fresh medium for identification of the organism. The organism was identified as described above. After incubation, samples were collected, proper dilution were performed and the absorbance of the sample was determined using U.V-Visible spectrophotometer at 600 nm.

Optimization of media

The best carbon source and the best nitrogen source based on maximum absorbance were identified in the above two processes. The new media together with yeast extract 0.4% was prepared by incorporating this particular carbon (molasses) and nitrogen (lentils) sources. The media was prepared by adding all the components into the beaker and adjusting the volume with distilled water. The prepared media was transferred into three conical flasks, and then was autoclaved at 121⁰C at 15lbs pressure for 15-20 minutes. The flask-I consisted of the standard MRS medium, flask-II consisted of the optimized media (1% lentils, 1% molasses) and flask-III consisted of higher strength 1.5% nitrogen source (lentils) and 1.5% carbon source (molasses). When the media reached room temperature, the media was inoculated with the organism. After inoculation, incubated the tubes at 37⁰C for three days. Later, streaked the organism on to the fresh medium for identification of the organism. The organism identification was done by the biochemical tests such as gram staining test, catalase test and motility test. After incubation, samples were collected and suitable dilutions were performed. Determine the absorbance of the sample using U.V-Visible spectrophotometer at 600nm.

Solid State Fermentation (SSF)

Bacterial strain and inoculum

Isolated the *Lactobacillus spp.* strains producing lactic acid, characterized as above and maintained them in the laboratory. The organisms grow on the MRS media. These organisms were streaked on freshly prepared agar slant and was incubated at 37⁰C for three days. To the test tube containing the slant 10 ml of saline water was added. With the help of a loop carefully, removed the organism without the agar. This solution was

transferred into a sterile test tube and mixed well with the help of a glass rod. This solution was used as the inoculum.

Production of lactic acid

To make it cost-effective and economically feasible, the basic need of any fermentation process is a regular screening of the medium ingredients. The superior productivity, use of inexpensive substrates and more straightforward downstream processing are some of the advantages offered by SSF.

Six solid state fermentation media were prepared. To the 250 ml Erlenmeyer flasks, ten grams of different solid substrates such as mung beans, lentils, chickpea, peanut, Bengal gram and wheat and 1.5 ml of a salt solution consisting of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ -0.02g/100ml; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ -0.05g/100ml; MnSO_4 -0.0001g/100ml were taken and used distilled water to moist them until the moisture content was 70%. Then the contents were mixed thoroughly, autoclaved at 121°C for 30 min and was cooled to room temperature. After cooling, flasks were inoculated with 100 μl of culture, incubated in a rotary shaker at 37°C for 72 hours and their residual activity was measured.

Extraction and recovery of lactic acid

Lactic acid was extracted from the media by using water as the solvent. Ten volumes of distilled water per gram of substrate were added to flasks and lactic acid was extracted by agitation at room temperature and centrifugation at 10,000 rpm at 4°C for 15 min. The clear supernatant was used as the enzyme source. Kimberley and Tailor's method was employed to estimate the total lactic acid, and the amount of lactic acid produced was expressed as g/g of bran.

RESULTS AND DISCUSSION

Optimization of nitrogen source

The estimation of growth by optimizing the nitrogen source is shown in Table 1. Figure 1 shows the lactobacilli growth in different medium after optimizing for nitrogen source. The highest growth was observed in the medium containing the lentils seed powder (Figure 1).

Table 1: Estimation of *Lactobacilli* growth by optimizing the nitrogen source

Nitrogen source	Absorbance (Nm)			Average
	Trial I	Trial II	Trial III	
Peanut	0.079	0.081	0.078	0.079
Bengal gram	0.086	0.085	0.082	0.084
Chick pea	0.110	0.115	0.109	0.111
Mung beans	0.065	0.062	0.067	0.064
Lentils	0.160	0.165	0.168	0.164
Wheat	0.081	0.079	0.085	0.081

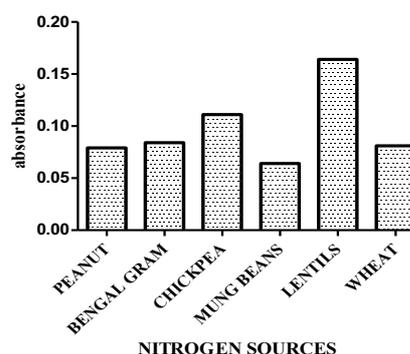


Figure 1: Estimation of *Lactobacilli* growth by optimizing the nitrogen source

Optimization of carbon source

Table 2 shows the estimation of growth by optimizing the carbon source. Figure 2 shows the lactobacilli growth in different medium after optimizing for carbon source. The highest growth was observed in the medium containing molasses.

Table 2: Estimation of *Lactobacilli* growth by optimizing the carbon source

Carbon source	Absorbance (Nm)			Average
	Trial-I	Trial-II	Trial-III	
Dry Dates	0.099	0.093	0.086	0.093
Pineapple	0.088	0.084	0.085	0.084
Molasses	0.179	0.179	0.181	0.179
Sucrose	0.089	0.089	0.086	0.089
Fructose	0.068	0.067	0.068	0.067
Galactose	0.150	0.151	0.149	0.151

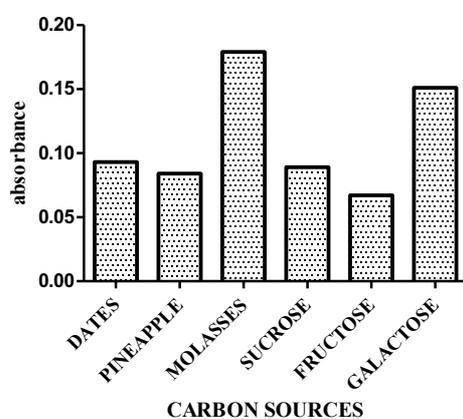


Figure 2: Estimation of *Lactobacilli* growth by optimizing the carbon source

Optimization of media

The results of lactobacilli growth by optimizing the media is shown in Table 3. The optimized medium-I (Flask II) was identified to have nearly double the absorbance value when compared with the standard medium (Flask I), and the optimized

medium-II (Flask III) was found to have a slightly higher absorbance value when compared with the standard (Table 3). Figure 3, a bar chart representing media on x-axis and absorbance on the y-axis, shows that both the optimized media resulted in a higher lactic acid production, the highest being in optimized medium I.

Table 3: Estimation of *Lactobacilli* growth by optimizing the media

Media	Absorbance (Nm)
Standard media (MRS)	0.272
Optimised media-I (Test-I or flask II)	0.500
Optimised media-II (Test-II or flask III)	0.316

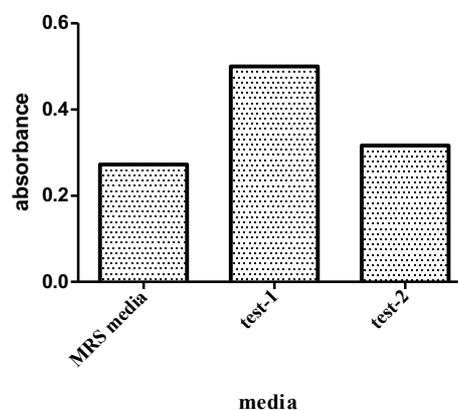


Figure 3: Estimation of *Lactobacilli* growth by optimizing the media

Solid state fermentation

The amount of lactic acid produced after fermentation for three days is shown in Table 4. Figure 4 bar chart, representing substrates on the x-axis and lactic acid produced on the y-axis, indicated that lentils used as the nitrogen source promoted the highest

production of lactic acid by solid-state fermentation.

Table 4: Estimation of lactic acid production by changing the substrates

Substrates	Lactic acid (g/g bran)
Peanut	0.071
Bengalgram	0.089
Chickpea	0.097
Mung beans	0.062
Lentils	0.179
Wheat	0.082

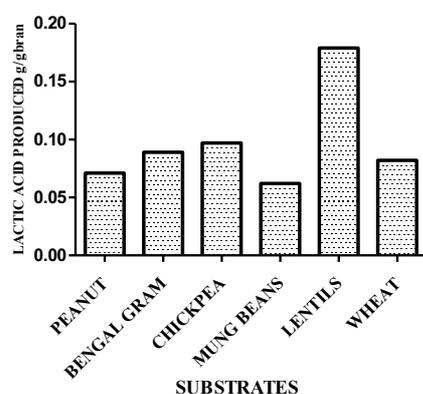


Figure 4: Estimation of lactic acid production by changing the substrates

CONCLUSION

The best source of the organism will always give the best yield of the product, which is vital in commercial production. In the optimization of nitrogen source, the highest growth was observed with the lentil seed powder. In the optimization of the carbon source, the highest increase was observed with molasses. Low-cost media is obtained by using plant sources, making it suitable for vegan choice. Animal products can be replaced with the plant sources for cost-effective production of probiotic lactobacilli and lactic acid.

Author's Declaration

The authors declare that all persons listed as authors have read and given approval for the submission of this manuscript.

Acknowledgements

This research was supported by K.V.S.R Siddhartha College of Pharmaceutical sciences, Vijayawada, India. We are thankful to Dr. N. Kanaka Durga Devi who provided expertise that greatly assisted the research.

Competing Interests

The authors declare that they have no competing interests to disclose.

REFERENCES

1. König, Helmut; Fröhlich, Jürgen. Lactic Acid Bacteria in Biology of Microorganisms on Grapes, In Must and Wine. Springer-Verlag. 2009; ISBN 978-3-540-85462-3; P. 3.
2. Benninga, H. A History of Lactic Acid Making, Kluwer Academic Publishers, Dordrecht, Netherlands (1990).
3. Food and Agriculture Organization and World Health Organization Expert Consultation. Evaluation of health and nutritional properties of powder milk and live lactic acid bacteria. Córdoba, Argentina: Food and Agriculture Organization of the United Nations and World Health Organization; 2001.
4. Hassan Pya. Potentials of Pineapple Waste as Growth Medium for Lactobacillus Species International Journal of Pharmacy and Pharmaceutical Sciences.
5. Kanakadurgadevi N, Saimrudula B. Comparison Between Guar Gum and Locust Bean Gum as Barrier Layers in Montelukast Press-Coated Tablets. The Indian Pharmacist. 2012 Dec; 53-63.
6. A. Shahravy. Optimization of Probiotic Lactobacillus casei ATCC 334 Production

- using Date Powder as Carbon Source. *Chemical Industry & Chemical Engineering Quarterly*. 2012; 18 (2) 273–282.
7. Umar Farooq. Optimization of Lactic Acid Production from Cheap Raw material: Sugarcane Molasses, *Pak. J. Bot.* 2012; 44(1): 333-338.
 8. Manju Pathak. Optimization of An Effective Growth Medium for Culturing Probiotic Bacteria for Applications in Strict Vegetarian Food Products. *Functional Foods in Health and Disease*. 2012; 2(10):369-378.
 9. Pandey, A. "Solid-State Fermentation". *Biochem. Eng. J.* March 2003; 13 (2–3): 81–84.
 10. N. Kanaka Durga Devi, Shaheena Shaik. Evaluation of Adaptogenic Activity of Plant Extracts by Microorganisms. *Der Pharmacia Lettre*. 2014; 6(4):184-189.