Optimization of MRS (De Man, Rogosa and Sharpe) Media with an organic waste substitute to assess the potential of *Lactobacillus* in Ink degradation

Dolly Pandit*, Soma Bhunia, Gauthami Bitla, Hajra Gupta

Department of Biotechnology, University of Mumbai, Kishinchand Chellaram College, Mumbai, Maharashtra, India

ABSTRACT

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Lactobacillus species are normally found in human digestive and urinary tracts. In this study, the capability of probiotic strains to grow in media using banana peel waste as an alternative carbohydrate source was investigated. Lactic acid bacteria were isolated using homemade curd, banana peel powder was made using an alternate drum drier technique and used as a fermentation media on which strain of probiotic, *Lactobacillus* sp. were grown. The result showed that bacterial growth using banana peel powder obtained at 37°C under anaerobic condition (2- 3 days), was comparable to growth seen in MRS medium. It can be concluded that the banana peel powder can be utilized to produce culture medium for cultivation of probiotic bacteria as a substitute to MRS medium which is expensive for cultivating probiotics. This will not only result in cost reduction of probiotic from cheap, inexpensive Agro-waste material, but also helps in solving the environmental pollution problem caused by their extensive disposal. The capability of *Lactobacillus* sp. to degrade dye was also investigated. *Lactobacilli* was incorporated under anaerobic condition in the M9 media containing the dye. The dye degrading capability of *Lactobacillus* sp was estimated colorimetrically at 520nm. The result showed a decrease in the dye concentration obtained at 37°C under anaerobic condition. So, it can be concluded that the *Lactobacillus* strain can be used for the bioremediation of dyes.

Keywords: *Lactobacillus*, Probiotic, Banana peel powder

I. INTRODUCTION

Lactic acid bacteria (LAB) constitute a diverse group of gram-positive, non-sporulating, catalase negative

organism found in number of habitats. They are the known constituent of human gut and also occur widely in dairy, meat, plant and fermented product of commercial value.

There are three important features for screening probiotic potential of bacteria are antimicrobial activity, bile salt, and acid tolerance that can be used as medical. LAB produces hydrogen peroxide, diacetyl, bacteriocins and antifungal compound such as fatty acid during lactic acid fermentation. Bacteriocins are protein compounds which inhibit the growth of sensitive pathogenic bacteria and different degradation system in digestive system compared with antibiotics. Antimicrobial system is also prepared from them to compete and inhibit pathogenic microorganisms. LAB is also resistant to gastrointestinal juices, bile salt, gastric acid, lysozymes.

Lactobacilli colonies on agar media usually appear small(2-3mm), with entire margins, convex, smooth, glistening and opaque without pigment. Metabolically, *Lactobacilli* are at the threshold of anaerobic to aerobic life. *Lactobacilli* are extremely fastidious organisms, adapted to complex organic substrate, they require not only carbohydrate as energy and carbon source, but also nucleotide, vitamin and amino acid. *Lactobacilli* grow best in slightly acidic media with a pH of 6.4 - 4.5. *Lactobacilli* from dairy product is basically isolated on the MRS media, T streak method is usually done to isolate *Lactobacilli* from dairy product and then for incubation, an anaerobic environment is created by keeping it into the anaerobic glass chamber and then the growth is obtained after 2-3 days, which can be further used for research work. For short term preservation, culture is inoculated into MRS agar slant and then stored at 4-7°C and it is sub cultured weekly. Some species or strain however, die out quite rapidly with a series of transfer. For long term preservation, techniques used are lyophilization, vacuum or spray drying.

After isolation *Lactobacilli* is detected by a catalase test and a gram staining method. Catalase is a common enzyme found in most aerobic organism. It catalyses the decomposition of hydrogen peroxide to water and oxygen. It is a crucial enzyme which protect the cell

from oxidative damage by reactive oxygen. On gram staining, *Lactobacilli* turn blue in colour which indicate that the given organism is gram positive. Gram positive bacteria retain the colour of the crystal violet stain in the gram stain. This is the characteristic of bacteria that have a cell wall composed of thick layer of peptidoglycan.

Banana (Musaceae) is one of the most important fruit crops that is widely cultivated in tropical countries for its valuable applications in food industry. Banana peel constitute about 30 percent of fresh banana by weight. Ripe banana peel contains up to 8% crude protein and 13.8 percent soluble sugars and 4.8 percent total phenolics. Banana peels are rich in trace element, the ripened peel has approximately 30 percent free sugars. Green plantain peels contain 40 percent starch. A commercial medium such as de Man, Rogosa and Sharpe (MRS) or corn steep liquor is usually too costly for commercial production of probiotics. Therefore, exploring locally available sources as culture media for probiotics from various agro-industrial wastes could be a better alternative for reducing the cost of production. Hence the purpose of the study was to investigate the applicability of banana peel as a nutritional source and promoting the growth of lactic acid bacteria.

II. METHODS AND MATERIAL

Isolation of *Lactobacillus* on MRS Media: Lactic acid bacteria was isolated using homemade curd on MRS Media. Growth from MRS Broth cultures was used to streak on MRS agar plate. *Lactobacillus* species of these isolates were identified by comparing their sugar fermentation patterns with the scheme described in Bergey's Manual of Systematic Bacteriology.

Growth of Bacteria: *Lactobacilli* were grown on MRS agar plate under anaerobic conditions using an anaerobic chamber. The plates were incubated for 3-4 days, till we observe transparent pin point LAB

colonies. Preserve the *Lactobacilli* using saline solution or MRS agar butts by paraffin tape sealing.

Identification of Lactic Acid Bacteria (LAB):

Lactobacillus sp are a group of rod-shaped, gram-positive, non-spore-forming bacteria of the family Lactobacillaceae.

Gram Staining- Fix the smear using 95% methanol for 1- 2 mins, Gram Crystal Violet (45sec), Gram Iodine (45sec), Gram Decolourizer (10sec), Safranin (30sec), Examine microscopically under an oil immersion lens (100X).

Catalase Test- Catalase test is used to differentiate aerotolerant strains of catalase negative from species of catalase positive. 3% H₂O₂ solution is used for the identification of anaerobic bacteria. *Lactobacillus sp* shows catalase negative results.

Green Banana peel powder: It was made using the alternate drum drier technique in the lab. The indirect and rotating heat principle of the drum drier apparatus was redone in the lab using the large petri dish, burner, vessel, sand, small pieces of the peel, etc. The filter papers were used to remove the excess water from the pieces. The pieces were initially kept in sodium thiosulfate solution so that the oxidation doesn't take place. The pieces were heated in the petri dish for 5-6 hours till they were crisp. The pieces were heat dried in the hot air oven to eliminate any presence of moisture. Later, they were grinded for the formation of powder.

Fig.1: Alternate drum drier

Fig.2: Dried pieces of banana peel

Glucose estimation in the banana peel powder: Take 1gm of the powder in a test tube, add Fehling's A and B reagent and heat the test tube. Formation of red colour indicates the presence of glucose in the peel powder.

Fig.3: Banana peel powder

Isolation of *Lactobacillus* using Modified MRS Media: The glucose content in the MRS media is substituted by the peel powder. Using the modified MRS Media, the growth of *Lactobacillus* was seen in 2- 3 days, under anaerobic conditions.

Fig.4: Colonies of *Lactobacilli*

Fig.5: Anaerobic chamber

Growth Curve: The dynamics of the bacterial growth can be studied by plotting the cell growth (absorbance) versus the incubation time. The curve thus obtained is a sigmoid curve and is known as a standard growth curve. The increase in the cell mass of the organism is measured by using the Colorimeter.

Growth of *Lactobacilli* was observed in MRS broth, and Modified MRS broth for comparison at 540nm. A standard graph was plotted for better understanding.

Bioremediation of Dye using *Lactobacilli*: M9 (Minimal) Media (Glucose, $MgSO_4$, $CaCl_2$, H_2O) was used for this process, the dye used was Camlin Ink. 1:10 Dilution of the ink was made and added to the media. To check the efficacy 5 flasks were prepared- Blank, Negative (M9), M9+Ink, Positive (M9+LAB), M9+Ink+LAB. Incubation under an anaerobic chamber was done for a month. Colorimetric analysis of the flasks was done.

Fig.6: Ink Degradation

Colorimetric analysis for Ink degradation: The flasks were incubated for a month under anaerobic conditions. *Lactobacillus* degrades the dye using it as a nitrogen or carbon source. The degradation that had taken place was measured colorimetrically at 520nm. The absorbance (O.D) was measured after every 10 days of incubation.

III. RESULTS AND DISCUSSION

Glucose estimation in the banana peel powder:

Take 1gm of powder in a test tube add Fehling's A and Fehling's B and heat the test tube, the red coloured product formed indicates the presence of glucose in the green banana peel powder.

Fig.7: Fehling's Test

Isolation of *Lactobacillus* using Modified MRS Media:

Lactobacilli were isolated from homemade curd, under anaerobic conditions, by substituting the glucose content of MRS Media with banana peel powder. Growth was observed under 2 – 3 days, which shows that the optimized media has reduced the time duration of *Lactobacilli* from 4-5 days to 2 – 3 days.

Fig.8: Pinpoint *Lactobacilli* colonies, circled with black marker

Confirmation of *Lactobacilli* species:

After the isolation, two biochemical tests were performed.

Gram Staining- was performed using a grease free slide with the culture smear. Crystal violet and the mordant gram's iodine were used to stain the *Lactobacilli* culture. *Lactobacilli* being gram positive shows purple bacilli rod shape.

Fig.9: Gram staining (100X)

Catalase test- Catalase mediates the breakdown of hydrogen peroxide H_2O_2 into oxygen and water. To find out if a particular bacterial isolate is able to produce catalase enzyme, a small inoculum of a bacterial isolate is mixed into hydrogen peroxide solution (3%) and is observed for the rapid elaboration of oxygen bubbles. The lack of catalase is evident by a lack of or weak bubble production.

Catalase-positive bacteria- immediate effervescence (bubble formation).

Catalase-negative- no bubble formation.

Lactobacilli shows Catalase negative results.

Fig.10: Catalase Test

Microscopic observation- The *Lactobacilli* sp are gram positive organisms, slides were observed under 100X oil immersion lens and the purple rod shape bacilli was observed. Hence, confirming the identity of our isolated bacilli.

Growth Curve:

Growth curve for MRS Broth and MRS modified broth was compared by taking readings at 540nm, and plotting a graph. The graph showed maximal growth under MRS modified broth.

Banana peel contains multiple sugars which act as a continuous source of carbohydrate. The *Lactobacilli* utilize one form of sugar and grow at a faster rate giving rise to a log phase. Upon depletion of these sugar *Lactobacilli* enters into a lag phase and produces an enzyme and other necessary component that can utilize other form of sugar present in the banana peel and initiate its growth and again enters into an exponential phase and these goes on until all the sugar component in the media gets utilize.

Table.1: Absorbance at 540nm

<i>Days</i>	<i>MRS Broth (nm)</i>	<i>Modified MRS Broth (nm)</i>
1	0.14	0.42
2	0.15	0.50
3	0.17	0.56
4	0.18	0.57
5	0.16	0.55

Graph.1 Comparative study for growth curve of *Lactobacilli*

Bioremediation of dye using *Lactobacilli*:

Lactobacillus degrade the dye using it as a nitrogen or carbon source. The degradation that has taken place was measured calorimetrically at 520nm. The absorbance (O.D) was measured for every 10th day of incubation, for a month. To check the efficacy 5 flasks were prepared- Blank, Negative (M9), M9+Ink, Positive (M9+LAB), M9+Ink+LAB. Incubation under an anaerobic chamber was done.

Table.2: Absorbance at 520nm for Ink Bioremediation

Flasks	Initial O. D (520nm)	Final O. D (520nm)
Blank	0.0	0.0
Negative (M9)	0.56	0.56
M9+Ink	0.42	0.42
Positive (M9+LAB)	0.38	0.35
M9+Ink+ LAB	0.45	0.40

IV. DISCUSSION

Lactobacilli are a species of facultative anaerobic, gram-positive bacteria. They have been used for the

production of fermented foods, and as probiotics due to their health- promoting effects in human.

MRS media is the standardized media used to propagate *Lactobacillus*, it works very well but with the disadvantage of being quite expensive, therefore the aim of this study was to create a cheaper alternative with the help of organic waste material i.e., banana peel as an alternative carbohydrate source, all the other components of MRS media were kept same.

Lactobacilli was isolated using homemade curd, under anaerobic conditions. For confirmation of the species two biochemical tests like- Catalase test, Gram staining were done. Usually it takes 4- 5 days for the growth of *Lactobacillus* on MRS media, but with our optimized MRS media growth was seen under 2-3 days.

For the incorporation of banana peel as an alternative source of glucose in MRS media, it was converted into a powder form with the help of alternate drum drier technique. In order to prove the presence of glucose in the peel powder, a biochemical test with Fehling's reagent A and B was carried out.

Growth curve for both MRS media and Modified MRS media was done, to check the efficacy of growth. The dynamics of the bacterial growth can be studied by plotting the cell growth (absorbance) versus the incubation time. Growth curve for MRS Broth and MRS modified broth was compared by taking readings at 540nm, and plotting a graph. The graph showed maximal growth under MRS modified broth. Banana peel contains multiple sugars which act as a continuous source of carbohydrate. The *Lactobacilli* utilize one form of sugar and grow at a faster rate. Thus, the Modified MRS media shows greater efficacy for the growth of *Lactobacillus* i.e., under 2- 3 days.

An application study was done to check the role of *Lactobacilli* in Dye/ Ink degradation. For this process, use of M9 media was done. *Lactobacillus* degrade the dye using it as a nitrogen or carbon source. The degradation that has taken place was measured calorimetrically at 520nm. The absorbance (O.D) was measured for every 10th day of incubation, for a month. To check the efficacy 5 flasks were prepared- Blank,

Negative (M9), M9+Ink, Positive (M9+LAB), M9+Ink+LAB. Incubation under an anaerobic chamber was done. *Lactobacilli* showed some extent of degradation; hence it can be used for the bioremediation of dyes. Huge amounts of dyes in the waterbodies affect not only the aquatic life but also the health of the humans. It also increases the biochemical and chemical oxygen demand (BOD and COD), impair photosynthesis, inhibit plant growth, enter the food chain, provide recalcitrant and bioaccumulation, and may promote toxicity.

Interest in lactic acid bacteria is growing. It produces lactase, which breaks down sugars in dairy industry, functions as probiotic hence can be used to improve the gut health of humans, by balancing intestinal bacterial population, The toxic dye which is released from the industries can also be degraded by the *Lactobacilli*, also bacteriocins produced by lactic acid bacteria are great interest to the food fermentation industry because they may inhibit the growth of many food spoilage and pathogenic bacteria.

V. CONCLUSION

Lactic acid bacteria (LAB) constitute a diverse group of gram-positive, non-sporulating, catalase negative organism found in number of habitats. They are the known constituent of human gut and also occur widely in dairy, meat, plant and fermented product of commercial value. *Lactobacilli* from dairy product is basically isolated on the MRS media, T streak method is usually done to isolate *Lactobacilli* from dairy product and then for incubation an anaerobic environment is created by keeping it into the anaerobic glass chamber and then the growth is obtained after 2- 3 days and then it is further used for research work. After isolation, to confirm the species two biochemical tests- Catalase and Gram Staining are done.

Banana (Musaceae) is one of the most important fruit crops that is widely cultivated in tropical countries for its valuable applications in food industry. Banana peels

are rich in trace element, the ripened peel has approximately 30 percent free sugars. A commercial medium such as de Man, Rogosa and Sharpe (MRS) or corn steep liquor is usually too costly for commercial production of probiotics. Therefore, exploring locally available sources as culture media for probiotics from various agro-industrial wastes could be a better alternative for reducing the cost of production. Hence the purpose of the study was to investigate the applicability of banana peel as a nutritional source and promoting the growth of lactic acid bacteria. Banana peel powder was made using Alternate drum drier technique.

Growth curve for both MRS and Modified MRS media was done at 540nm, Modified MRS media showed faster growth of *Lactobacilli*, a standard graph was plotted.

An application study of dye degradation by *Lactobacilli* was done using M9 media, by measuring the absorbance at 520nm, some extent of degradation was seen after a month of anaerobic incubation.

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