International Journal of Bio-pharmacology, Biotechnology and Allied Sciences

IJBBAS, September, 2020, 1(6): 627-638 Research Article www.ijbbas.com

ISOLATION AND IDENTIFICATION OF LACTOBACILLUS FROM CHOCOLATES

[1]Saxena S, [2]Saxena P

[1,2]Department of Biotechnology, SR Institute of Technology, Lucknow, UP, India.

*Corresponding Author: Sakshi Saxena

Email ID: ssaxena01@gmail.com

Available online at: www.ijbbas.com.

Received 2rd April. 2020; Revised 28th June. 2020; Accepted 1st August. 2020; Available online September.

ABSTRACT

From five different samples of chocolates, 20 lactic acid bacterial strains were isolated. The strains were termed as lactobacillus by performing various biochemical tests. Further antibacterial analysis of the secondary metabolites produced from these isolates was performed and found that culture C2 shows potent activity against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Escherichia coli*. The effect of pH and temperature on the growth of lactobacillus isolates was carried out and effective results were obtained at 37^0C and pH 7.

Key words: Lactic Acid, Secondary Metabolites, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*.

INTRODUCTION

Chocolate is a composition of fine solid matters of cocoa, sugar and milk powder in a constant fat phase. Chocolates remains solid at ambient (20°C - 25°C) and starts melting at body temperature (37°C) by giving a smooth solution of particulate solids with a pleasing cooling sensation in the mouth **[1]**. The regular phase influences the sensory characteristics such as mouth feel or melt in the mouth **[2]**. In spite of high sugar and fat content, consuming chocolates makes a positive contribution to human nourishment by the provision of antioxidants, including polyphenols such as flavonoids for example the procyanidins, epicatechin, and catechin **[2, 3]**.

It also contains minerals, specifically iron, magnesium, potassium, copper, and iron **[4, 5]**.

Due to the presence of cocoa, it is enrich in naturally occurred antioxidants expressing the health benefits. Milk solids added full cream milk powder and spray-dried skimmed milk powder which shows contribution into texture, liquid flow or texture properties **[5, 6]**.

Numbers of functional foods are taken as a part of a regular diet and provide the consumers with well-documented and physiological assistances such as probiotic bacteria **[7, 8, 9]**. Probiotics are live microorganisms and multiply in the human bowels that confer a health benefit by changing the enteric microflora. The main sources of these organisms are fermented dairy products, for example, yogurts.

However, the useful dairy product must comprise a defined number of live probiotic bacteria (usually at least 10^6 CFU/g). Furthermore, their number at the end of the own life is the most essential criterion when the health-promoting value of a given foodstuff is calculated **[14, 15, 16, 17]**.

Probiotic bacteria constructively affect human health by refining the gut micro-biota balance and the resistances against pathogens. Additional health benefits attributed to probiotics are the stimulation of the immune system, blood cholesterol reduction, vitamin synthesis, anti-carcinogenesis, and anti-bacterial activities.

MATERIALS AND METHODOLOGY

Sample collection:

The chocolate samples of different brands were collected from local shops near to Gomti Nagar Lucknow. The samples were collected in sterilized sample collection bags and bring to the laboratory.

Isolation of bacteria:

The 1 gram of samples were serially diluted in 0.85% NaCl solution and then spread over sterilized nutrient agar media. Further the cultures were selected on the basis of their morphological parameters further streaked on nutrient agar media to prepare pure cultures.

Strain identification:

Various staining's, biochemical tests, were carried out to identify the isolated strains.

Effect of pH and temperature on bacterial growth:

The growths of isolated strains were checked on two physiochemical factors, pH and temperature.

Growth curve study:

The cultures were inoculated in sterilized media and then absorbance were taken fixed time interval of 1 hour on UV-Visible Spectrophotometer at 620 nm.

RESULTS

Collection of chocolate samples:

Table 1: Chocolate samples

S.No	Sample Name
1.	 Dairy Milk
2.	 Milky Bar
3.	 5 - Star

4.	 Snickers
5.	 Munch

The chocolate samples of different brands were collected from local shops.

Bacterial isolation by serial dilution method:

A total of 20 bacterial cultures were isolated from all five collected chocolate product samples using serial dilution and spread plate method. These cultures were selected based on their different morphological characteristics.

Six cultures were obtained from each sample 1 & 3, four from each sample 2 & 4.These cultures were named as C1, C2….. C20.

Figure 1: Bacterial cultures on agar plates after the serial dilution and spreading.

Bacterial Purification:

Bacterial purification was done using the streak plate method by streaking the selected cultures in Petri plates.

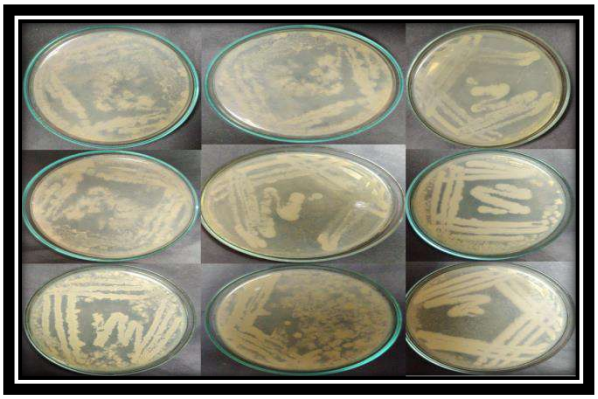


Figure 2: Few pure bacterial culture in agar plates after streaking.

Colony morphology:

Table 3: The morphology of all the selected colonies.

Culture name	Shape	Margin	Elevation	Pigmentation	Surface	Texture	Opacity
C1	Spindle	Discrete	Raised	White	Rough	Hard	Opaque
C2	Irregular	Lobate	Raised	Off white	Convex	Hard	Opaque
C3	Circular	Curled	Raised	Off white	Convex	Hard	Opaque
C4	Circular	Lobate	Flat	Yellowish	Smooth	Soft	Opaque
C5	Spindle	Discrete	Raised	White	Rough	Hard	Opaque
C6	Filamentous	Curled	Convex	Off-white	Smooth	Soft	Translucent
C7	Punctiform	Lobate	Pulmonate	Green	Smooth	Soft	Opaque
C8	Circular	Discrete	Flat	White	Rough	Hard	Opaque
C9	Irregular	Entire	Flat	Off white	Rough	Soft	Opaque
C10	Circular	Lobate	Convex	Yellowish	Convex	Soft	Opaque
C11	Circular	Lobate	Flat	Yellowish	Smooth	Soft	Opaque
C12	Spindle	Discrete	Raised	White	Rough	Hard	Opaque
C13	Filamentous	Curled	Convex	Off-white	Smooth	Soft	Translucent
C14	Punctiform	Lobate	Pulmonate	Green	Smooth	Soft	Opaque
C15	Circular	Discrete	Flat	White	Rough	Hard	Opaque
C16	Irregular	Entire	Flat	Off white	Rough	Soft	Opaque
C17	Circular	Lobate	Convex	Yellowish	Convex	Soft	Opaque
C18	Circular	Lobate	Flat	Off white	Smooth	Gummy	Opaque
C19	Irregular	Lobate	Raised	Off white	Convex	Hard	Opaque
C20	Circular	Curled	Raised	Off white	Convex	Hard	Opaque

The cultures obtained from different samples were differentiated based on their morphology. The morphology of all the selected colonies is given in the table below.

Strain identification:

After isolation of bacteria, Gram Staining was done to distinguish the isolated bacteria between Gram-positive and Gram-Negative groups.

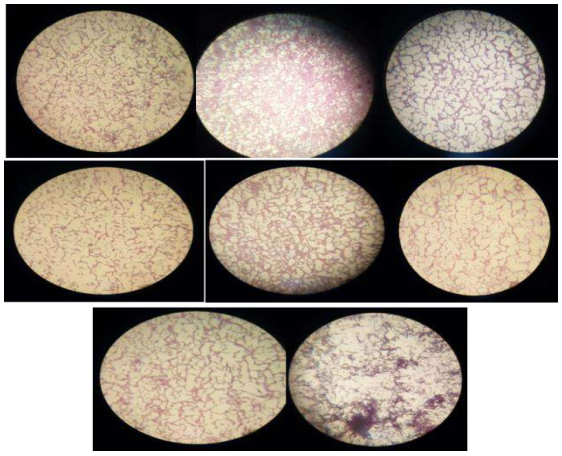


Figure 3: Gram's staining of a few cultures.

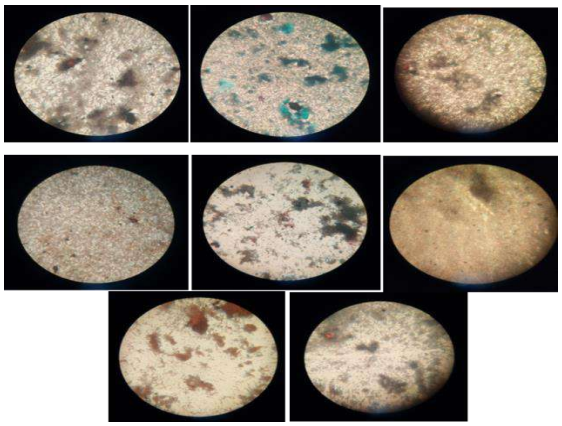


Figure 4: Endospores staining of a few cultures.

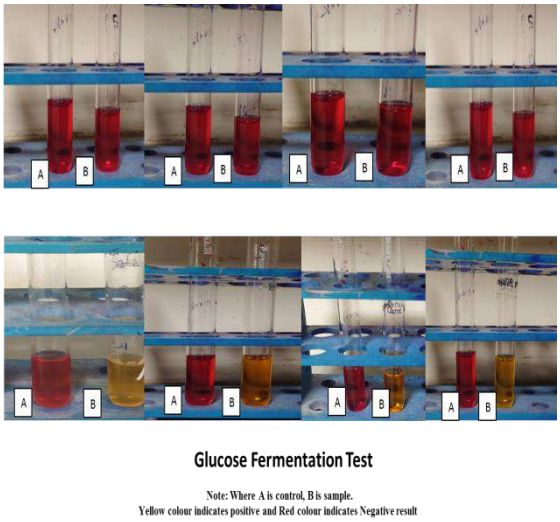


Figure 7: Glucose fermentation tests of a few cultures.

Table 4: Showing results of Gram's Staining, Endospores staining.

Culture	Gram's staining	Shape	Endospores staining
C1	Gram Negative	Rod	Negative
C2	**Gram Positive**	**Rod**	**Negative**
C3	Gram Positive	Rod	Positive
C4	Gram Positive	Coccus	Negative
C5	Gram Negative	Rod	Negative
C6	Gram Negative	Rod	Negative
C7	Gram Negative	Coccus	Negative
C8	Gram Negative	Rod	Negative
C9	Gram Positive	Rod	Positive
C10	Gram Positive	Rod	Positive
C11	Gram Positive	Coccus	Negative
C12	Gram Negative	Rod	Negative

Culture	Gram's staining	Shape	Endospores staining
C13	Gram Negative	Rod	Negative
C14	Gram Negative	Rod	Negative
C15	Gram Positive	Rod	Positive
C16	Gram Positive	Coccus	Negative
C17	Gram Positive	Coccus	Negative
C18	Gram Positive	Coccus	Negative
C19	Gram Positive	Coccus	Negative
C20	Gram Positive	Coccus	Negative

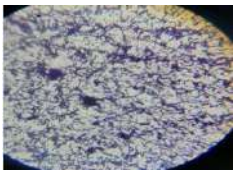
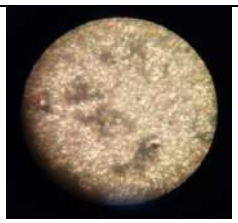
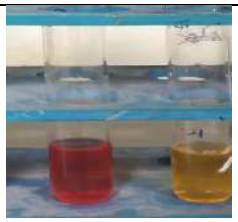
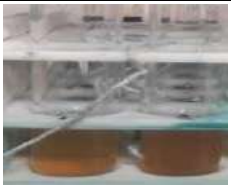
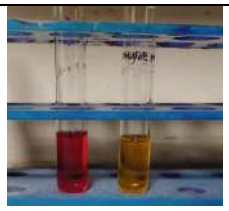
Table 5: Showing results of Catalase test, Mannitol Test, Starch hydrolysis test.

Culture	Mannitol test	Catalase test	Starch hydrolysis test
C1	Negative	Negative	Negative
C2	**Positive**	**Negative**	**Positive**
C3	Positive	Positive	Positive
C4	Positive	Negative	Negative
C5	Positive	Negative	Negative
C6	Positive	Negative	Negative
C7	Positive	Negative	Negative
C8	Positive	Negative	Negative
C9	Positive	Negative	Positive
C10	Positive	Positive	Negative
C11	Positive	Positive	Negative
C12C	Positive	Negative	Negative
C13	Positive	Positive	Negative
C14	Positive	Negative	Negative
C15	Negative	Negative	Positive
C16	Positive	Positive	Negative
C17	Positive	Positive	Negative
C18	Negative	Negative	Negative
C19	Positive	Negative	Negative
C20	Positive	Positive	Negative

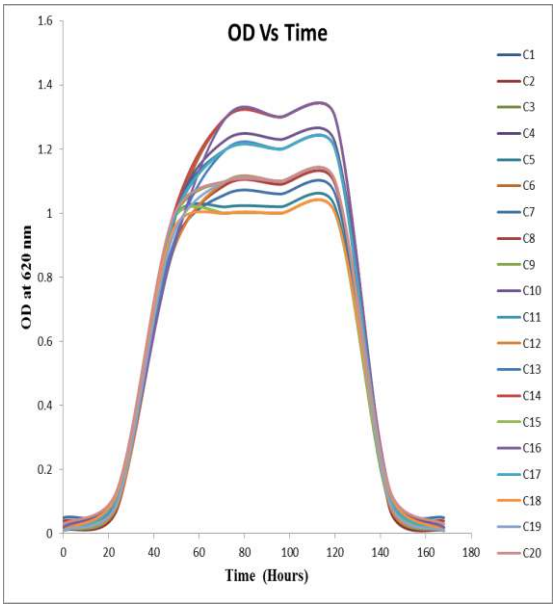
Table 6: Showing results of Methyl red, Voges Prausker, Glucose Fermentation test, Citrate test.

Culture	Methyl red test	Voges Prausker test	Glucose Fermentation test	Citrate test
C1	Negative	Negative	Negative	Negative
C2	**Positive**	**Negative**	**Positive**	**Negative**
C3	Positive	Positive	Positive	Negative
C4	Positive	Negative	Negative	Negative
C5	Negative	Negative	Negative	Negative
C6	Negative	Negative	Negative	Negative
C7	Negative	Negative	Negative	Negative
C8	Negative	Negative	Negative	Negative
C9	Positive	Negative	Positive	Positive
C10	Positive	Positive	Positive	Positive
C11	Positive	Positive	Negative	Positive
C12C	Negative	Negative	Negative	Negative
C13	Negative	Positive	Negative	Negative
C14	Negative	Negative	Negative	Negative
C15	Positive	Negative	Positive	Positive
C16	Positive	Positive	Negative	Positive
C17	Positive	Positive	Negative	Negative
C18	Positive	Negative	Negative	Negative
C19	Positive	Negative	Negative	Negative
C20	Positive	Positive	Negative	Negative

Table 7 : Biochemical properties of *Lactobacillus spp. given by* C2

S no.	Tests	Results
1	Gram's staining	 **Positive**
2	Shape	Rod, *Bacillus*
3	Catalase test	 Negative
4	Spore forming test	 Negative
5	Methyl red test	 Positive
6	Voges Prauskeur test	 Negative
7	Citrate test	Negative
8	Starch hydrolysis test	 Positive
9	Glucose fermentation test	 Positive

Growth curve study of isolates:



Graph 4: Growth kinetics analysis of isolates.

Growth kinetics of Bacterial strains was studied by taking the absorbance reading of the culture broth at 600nm after every 24 hrs. The stationary phase was observed on the third day of the reading.

Effect of temperature on isolates:

The lactobacillus isolates growth at different temperature range on solid media plates to check the effect of temperature on the growth. The selected temperatures were 4°C, 37°C and 60°C. The maximum growth was found at 37°C. The results are shown below.

Table 11: Effect of temperature on isolates

Culture	Temperature		
	37°C	**4°C**	60 **°C**
C1	++++	-	-
C2	++++	+	-
C3	+++	-	-
C4	++++	-	-
C5	++++	-	-
C6	++++	-	-
C7	++++	-	-
C8	++++	-	-
C9	+++	-	-
C10	++++	+	-
C11	++++	-	-
C12C	++++	-	-
C13	++++	-	-
C14	++++	-	-
C15	++++	+	-
C16	++++	-	-
C17	++++	-	-
C18	++++	-	-
C19	++++	-	-
C20	++++	-	-

Effect of pH on isolates:

Culture	pH		
	4	**7**	**11**
C1	++	++	++
C2	++	++	++
C3	++	++	++
C4	+	+	+
C5	+	+	+
C6	+	+	+
C7	+	+	+
C8	+	+	+
C9	-	-	-
C10	-	-	-
C11	-	-	-
C12C	-	-	-
C13	-	-	-
C14	-	-	-
C15	++	++	++
C16	++	++	++
C17	+	+	+
C18	+	+	+
C19	+	+	+
C20	+	+	+

DISCUSSION

The present study was carried out to isolate and identify the chocolate microflora and to study their ability for the production of antimicrobial metabolites active against pathogens. Chocolate product sample was collected from different locations. 20 bacterial isolations were done by serial dilution method on MRS media and further purification was done by continuous quadrant streaking.

The pure culture broths were prepared from the streak plates, and various biochemical tests were carried out. Biochemical tests such as Gram's staining, endospore staining, catalase test, etc. were done for strain identification given by Bergey's manual. Further, these isolated Lactobacillus strains were analyzed for their antimicrobial activity against the Gram-positive and Gram-negative bacteria (*E. coli, S. aureus, P. aeruginosa.)* by performing antibiogram analysis of crude metabolites by Agar Well Diffusion method of **Kerby Buer.** After performing the antibacterial test it was found that all strains do not show effective results, but culture 2 has shown the best potential antibacterial screening against all three pathogens.

The growth curve study of all 20 strains was also performed. The effect of pH and Temperature on the growth of all Lactobacillus strains were also performed and found the pH 7 and temperature 37^0C is best for the maximum growth.

CONCLUSION

The bacterial isolates identified as *Lactobacillus* a potent and rich source of antimicrobial metabolites active against various pathogens such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Escherichia coli*. The clear zone of inhibition with maximum diameter were observed during antibiogram analysis of extracellular antimicrobial component against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Escherichia coli*.

We concluded that these isolated strains can be used as antibiotics, and also very helpful for curing diseases.

The antimicrobial components from isolates found active against various pathogens can be used in combination with pre-existing antibiotics to enhance the activity. Production of antimicrobial components from lactobacillus C2 can be enhanced by further optimized of physicochemical parameters including media, incubation media, pH, etc.

Microflora of curd or microflora of chocolate products can inhibit the growth of pathogenic bacteria. Further studies can also focus on the characterization of amino acid and nucleotide sequences of these antimicrobial compounds in addition to the evaluation of the promising isolates for their probiotic usage.

REFERENCES

[1] Beckett, S. T. (Ed.). (2011). *Industrial chocolate manufacture and use*. John Wiley & Sons.

[2] Hii, C. L., Law, C. L., Suzannah, S., & Cloke, M. (2009). Polyphenols in cocoa (Theobroma cacao L.). *Asian Journal of Food and agro-industry*, *2*(4), 702-722.

[3] German, B., Schiffrin, E. J., Reniero, R., Mollet, B., Pfeifer, A., & Neeser, J. R. (1999). The development of functional foods: lessons from the gut. *Trends in Biotechnology*, *17*(12), 492-499.

[4] Belem, M. A. (1999). Application of biotechnology in the product development of nutraceuticals in Canada. *Trends in food science & technology*, *10*(3), 101-106.

[5] Childs MN. Nutraceutical Industry Trends. Journal of Nutraceutical, Functional and Medicinal Foods. 1999; 2(1):73-85.

[6] Dillard, C. J., & German, J. B. (2000). Phytochemicals: nutraceuticals and human health. *Journal of the Science of Food and Agriculture*, *80*(12), 1744-1756.

[7] Heenan, C. N., Adams, M. C., Hosken, R. W., & Fleet, G. H. (2004). Survival and sensory acceptability of probiotic microorganisms in a nonfermented frozen vegetarian dessert. *LWT-Food Science and Technology*, *37*(4), 461-466.

[8] Boylston, T. D., Vinderola, C. G., Ghoddusi, H. B., & Reinheimer, J. A. (2004). Incorporation of bifidobacteria into cheeses: challenges and rewards. *International Dairy Journal*, *14*(5), 375-387.

[9] Xanthopoulos, V., Petridis, D., & Tzanetakis, N. (2001). Characterization and classification of Streptococcus thermophilus and Lactobacillus delbrueckii subsp. bulgaricus strains isolated from traditional Greek yogurts. *Journal of food science*, *66*(5), 747-752.

[10] Dubey, N., & Shrivastava, S. (2019). Screening of in vitro Antimicrobial Potential of Fermentation Extracts Prepared from Indigenous Lactobacillus Isolates. *Journal of Drug Delivery and Therapeutics*, *9*(2-s), 184-187.

[11] Kamruzzaman, M., Islam, M. N., & Rahman, M. M. (2002). Shelf life of different types of dahi at room and refrigeration temperature. *Pakistan Journal of Nutrition*.

[12] Nebesny, E., Żyżelewicz, D., Motyl, I., & Libudzisz, Z. (2005). Properties of sucrose-free chocolates enriched with viable lactic acid bacteria. *European Food Research and Technology*, *220*(3-4), 358-362.

[13] Possemiers, S., Marzorati, M., Verstraete, W., & Van de Wiele, T. (2010). Bacteria and chocolate: a successful combination for probiotic delivery. *International journal of food microbiology*, *141*(1-2), 97-103.

[14] De, S., Pramanik, A., Das, A. K., Paul, S., Jana, S., & Pramanik, P. (2017). Isolation and characterization of Lactobacillus spp. from curd and its pharmacological application in probiotic chocolate. *The Journal of Phytopharmacology*, *6*(6), 335-339.

[15] De Man, J. C., Rogosa, D., & Sharpe, M. E. (1960). A medium for the cultivation of lactobacilli. *Journal of applied Bacteriology*, *23*(1), 130-135.

[16] De, S., Pramanik, A., Das, A. K., Paul, S., Jana, S., & Pramanik, P. (2017). Isolation and characterization of Lactobacillus spp. from curd and its pharmacological application in probiotic chocolate. *The Journal of Phytopharmacology*, *6*(6), 335-339.

[17] Hoque, M. Z., Akter, F., Hossain, K. M., Rahman, M. S. M., Billah, M. M., & Islam, K. M. D. (2010). Isolation, identification and analysis of probiotic properties of Lactobacillus spp. from selective regional yoghurts. *World J Dairy Food Sci*, *5*(1), 39-46.

[18] Pundir, R. K., Rana, S., Kashyap, N., & Kaur, A. (2013). Probiotic potential of lactic acid bacteria isolated from food samples: an in vitro study. *Journal of Applied Pharmaceutical Science*, *3*(3), 85.

[19] De, S., Sena, S., Bhowmik, I., Maity, S., & Bhowmik, S. (2016). Isolation, Characterisation and Identification of Lactobacilli spp. And Study of Its Pharmacological Activity In Vitro. *Int J Recent Sci Res*, *7*(11), 14296-14298.

[20] Foong, Y. J., Lee, S. T., Ramli, N., Tan, Y. N., & Ayob, M. K. (2013). Incorporation of Potential Probiotic L actobacillus plantarum Isolated from Fermented Cocoa Beans into Dark Chocolate: Bacterial Viability and Physicochemical Properties Analysis. *Journal of Food Quality*, *36*(3), 164-171.