

Original Research Article

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Isolation and Purification of Bacteriocin Produced by *Lactobacillus plantarum* Isolated from Honey Bees Gut and its Inhibitory action against Major Human Colon Pathogens

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ABSTRACT

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Lactobacillus plantarum plays an important role in probiotics as *Lactobacillus* species have been extensively used since decades against infectious diseases and is widely studied for their ability to protect against pathogens. This *Lactobacillus plantarum* is used regularly on various probiotics based food to maintain good digestion and pH of fluids secreted in colon of humans for healthy digestion. The present work aims to critically evaluate some central methods and procedure to know behaviour of *L. plantarum* at various ranges of pH and temperature. For this Honey bee was used for isolation of *Lactobacillus plantarum*. This *L. plantarum* was isolated from colon part of honey bee especially from rectum region of stomach. The bacteriocin isolated from *Lactobacillus plantarum* obtain from gut of honey bee was also used to study inhibitory effect against major human colon pathogens (*shigella*, *E.coli*, *Staph.aureus* and *Salmonella typhimurium*) and its inhibition was studied and result obtain on various basis was noted. As majority of pathogens shows inhibition against bacteriocin produced these plays a key role in treatment of various diseases caused by pathogenic bacteria and has serious effects on human's health.

Introduction

Lactic acid bacteria (LAB) are widely used in food industry as starter culture for fermentation. These organisms have been widely used as probiotics. Many of these lactic acid bacteria are known to produce antibacterial substances including bacteriocin which can inhibit the growth of several pathogenic bacteria. Bacteriocin from lactic acid bacteria are natural antimicrobial peptides or small proteins with bactericidal or

bacteriostatic activity against genetically closely related species. Bacteriocin is a protein or complex proteins biologically active with antimicrobial action against other bacteria, principally closely related species. They are produced by bacteria and are normally not termed antibiotics in order to avoid confusion and concern with therapeutic antibiotics, which can potentially illicit allergic reaction in humans and other medical problems. Bacteriocin differs from most therapeutic antibiotics in being proteinaceous

agents that are rapidly digested by proteases in the human digestive tract (Klaenhammer, 1998). Bacteriocin is ribosomally synthesized peptides; this fact creates the possibility of improving their characteristics to enhance their activity and spectra of action. Antibiotics are mainly considered to be secondary metabolites that act as inhibitory substances in small concentration, excluding the inhibition activity caused by metabolic by-products like ammonia, organic acids, and hydrogen peroxide. Some studies of characterization of bacteriocins show that these molecules can be active under certain ranges of temperature and pH (Daeschel *et al.*, 1990).

Bacteriocin production could be consider as an advantage for food and feed producers since, in sufficient amounts these peptides can kill and inhibit pathogenic bacteria that compete for same ecological niche or nutrient pool. Use of bacteriocin as potential bio-preservative in milk, cheese and apple juice has major function. This role is supported by the fact that many bacteriocins have a narrow host range, and is likely to be most effective against related bacteria with nutritive demands for the same scarce resources. Bacteriocin can be classified broadly as those synthesized by Gram-positive and those by Gram-negative organism's (Lash *et al.*, 2005). Among those synthesized by Gram-positive organisms, *Lactobacilli* bacteriocins are of commercial value.

Lactobacillus plantarum has been isolated from various habitats and several bacteriocins (antimicrobial peptides) have been described in strains from milk, cheese, sorghum beer, barley beer and fermented cucumber. In this we worked on the antimicrobial properties of bacteriocin produced from *Lactobacillus plantarum*, isolated from honey bees gut which has been purified and characterize. Because of increasing demand for more

natural and microbiologically safe food products, there is a need for bio preservation methods. Bacteriocin has considerable potential supplements or replacements for currently used antibiotics. Therefore, in this paper we reported on the antimicrobial properties of bacteriocin produced from *Lactobacillus plantarum*.

Materials and Methods

Collection of samples

Honey bees were collected in month of November. In the present study honey bees were collected from Mahatma Gandhi Mission College of Agricultural Biotechnology, They were collected from botanical garden present in the college premises.

Isolation and morphological identification of Bacteriocin Producing Bacteria

White creamy aqueous sample was collected from abdomen (rectum part) of honey bee with the help of sterile blade. The sample was collected in sterile tubes and was mixed properly using rotary shaker, further it was serially diluted in sterile distilled water up to 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , 10^{-6} .

The diluted sample were streaked using nichrome wire loop onto de Man Rogosa Sharpe (MRS) agar plates and incubated at 37°C for 48 hrs under anaerobic condition.(anaerobic conditions was maintained using anaerobic jar).

Morphological identification

Colony observation

The colony was observed optically after 48 hours of incubation and characters were studied (such as appearance, colony characterization etc.).

Grams staining

The loop full suspension was taken from culture obtained on Petri-plate after 48 hours of incubation. Obtained results were observed under microscope for colony characterization.

Motility Test

These tests determine the motility of obtain strain. Loop full suspension of obtained strain was streaked at middle of cover slip. Later cover slip was fixed to one well concave slide with help of grease.

Observation was done using microscope. If movement is observed it is positive to motility test and if no movement observed under microscope it is negative towards motility test.

Biochemical identification

pH Test

MRS broth at pH 3, 5, 7 and 9 were prepared by adjusted with 10N HCL and 1N NaOH. Further under controlled conditions the loop full suspension was picked from obtained colonies on Petri-plates and was inoculated on MRS broth in conical flask. The inoculated MRS broth was allowed to incubate for 48 hours at 37°C under anaerobic conditions.

Catalase Test

A loop full suspension was picked from colonies obtained on Petri-plates. It was diluted in sterile distilled water in sterile test tube and was mixed well using rotary shaker. 3% of H₂O₂ (Hydrogen peroxide) was added to suspension culture in test tube and obtained results were noted. Producing bubble or froth, indicated Catalase-positive and no bubble or froth indicated Catalase-negative.

Vitamin B-12 assay

Lactobacillus shows maximum growth in presences of vitamin B12 and show zone of exhibition in area of media were concentration of vitamin B12 is present (Madhu, 2010).

Three plates of MRS agar were prepared and 0.5 ml of obtained strain was spread on each Petri-plate. Simultaneously dilution of vitamin B12 was done up to 10⁻¹, 10⁻² and 10⁻³ aseptically. Each diluted vitamin B12 solution was added on each of the three plates with the help of sterile disc of Whatmans no.1 filter paper dipped in vitamin B-12 solution (Aseptically). Further the plates were kept in refrigerator for 10 minutes and regular incubation of 48 hours at 37°C under anaerobic condition was carried out.

Determination of sugar fermentation

Sugar fermentation test was performed using 1% (w/v) sugar in MRS broth. Mannitol, Maltose, Sucrose and Sorbitol were used in the test. 10 ml media was dispensed and Durham's tube was inserted invertably in each of test tubes. Fresh culture was inoculated at 37°C for 48 hrs. Only media was used as negative control. Results were observed by gas formation.

Growth of *Lactobacillus plantarum* at different temperature range

Obtained strain of *Lactobacillus plantarum* were kept at different range of temperature ranging from 4°C to 55°C. It is done to determine the growth activity of *Lactobacillus plantarum* at different ranges of temperatures.

Four plates with MRS broth were prepared and inoculated with obtained strain of *L. plantarum* under controlled conditions. They were further incubated at four different

temperatures i.e. at 4°C, 20°C, 37°C and 54°C for 48 hrs. Colonies were counted using colony counter.

Production of bacteriocin

The isolated species of *Lactobacillus plantarum* was propagated in MRS broth (500 ml) seeded with 10% inoculum of overnight conditions. Further it was incubated for 48 hrs at 37°C under anaerobic condition.

After 48 hours of incubation, the whole broth was centrifuged at 10,000g for 15 min and the cell-free supernatant was used as crude bacteriocin (centrifugation of broth was done by using tarson Polypropylene centrifugation tube of 50 ml each). Obtained supernatant after centrifugation was collected in centrifuge tubes.

Purification of bacteriocin

The cell-free culture supernatant (crude bacteriocin) obtained after centrifugation was saturated with 70% ammonium sulphate and stored at 4°C overnight to precipitate out the proteins (kept in centrifuge tubes). The pellet was collected after centrifugation at 10,000g at 4°C for 30 min using cooling centrifugation. Obtained pellet was dissolved in phosphate buffer (0.1M, pH-7) and dialyzed against phosphate buffer at 4°C overnight.

Conversion of g to r.p.m = (G force refers to Relative Centrifugal Force (RCF). $RCF = 1.118 * 10^{-5} * r * (rpm)^2$).

Dialysis

Pellet dissolved in phosphate buffer was dialyzed overnight by continuous stirring at 600 r.p.m using magnetic stirrer at 4°C (dialysis bags was activated by keeping them under warm water). The dialyzed samples were

collected in autoclaved centrifuge tubes with help of micro-pipette.

Column chromatography

The dialyzed protein obtained was applied to glass wool column chromatography that was re-equilibrated with phosphate buffer (pH-7). The flow rate was adjusted to 24 ml/h and fractions (10ml each) were collected. The samples were stored in autoclaved centrifuge tubes (1ml each). The fractions showing high bacteriocin activity were pooled and concentrated in lyophilizer.

Bacteriocin assay

The antimicrobial activity of the bacteriocin isolated from *L.plantarum* was determined using the well diffusion method as described by Ivanovo *et al.*, Pathogens used for antimicrobial test was *E.coli*, *Staph.aureus*, *shigella* and *Salmonella typhimurium*. Of the following pathogens used for antimicrobial test *Staph.aureus* is only pathogen that is gram positive, while all others gram negative in nature.

For this four agar plates were seeded with the indicator bacteria used (one plate for each pathogen indicating bacteria). 5-mm diameter wells were made in each of plate using cork-borer under controlled conditions.

50 µl of the bacteriocin were placed in 5-mm diameter wells that had been cut in agar plates previously seeded with the indicator bacteria.

Plates were kept in refrigerator for complete diffusion of added 50 µl bacteriocin in wells for about 10 minutes. After 10 min of diffusion process plates were allowed to incubate at 37°C for 24 hrs. Observation of zone of inhibition was noted for each pathogen after completion of incubation of 24 hrs (Daeschel *et al.*, 1990).

Results and Discussion

Morphological identification

Colony observation

Whitish colony with little elevation was observed after 48 hours of incubation at 37°C under anaerobic conditions. It also has shiny appearance with uniform growth.

Gram staining

The obtained strain after gram staining was observed as rod shaped, in form of chain and was gram positive when observed under microscope.

Motility test

After observation under microscope no movement was seen to either end of cover-slip where amount of oxygen is present to utilize. It also determines the obtain strain is anaerobic in nature, it clearly give positive result that the obtain strain is *Lactobacillus plantarum*.

Biochemical identification

Catalase test

With addition of 3% of hydrogen peroxide in the obtained strain minute bubbles was observed after 1 min. As some strains of *Lactobacillus plantarum* are micro-aerophilic they are able to degrade Hydrogen peroxide in minute amount.

Vitamin B12 assay for *Lactobacillus plantarum*

Zone of exhibition was observed in near the disc which was placed in middle of Petri-plate dipped in the 3 different concentration of vitamin B12 solution.

Zone was observed with uniform growth near the disc dipped in various concentration of vitamin B12, this indicates that the obtained strain shows maximum growth in presences of vitamin B12 which is same in case of *Lactobacillus plantarum*.

Determination of sugar fermentation

With 48 hours of incubation gaseous was formed from the obtained strain which utilizes the provided carbohydrates (maltose, Mannitol, sucrose and Sorbitol) (Table 1).

The gaseous formed was clearly seen as gaseous formed by the strain by utilizing provided carbohydrates was trapped in the Durham's tube that was seen floating on test tube. This shows positive result for obtain species as to be *lactobacillus plantarum*.

pH test

Obtained strain of *lactobacillus plantarum* was treated with different concentration of pH from acidic to alkaline. It is done to determine the growth activity of *lactobacillus plantarum* at different level of concentration (Table 2).

To determine effect of pH 50 ml of MRS broth was prepared which was seeded with different concentration of pH (3, 5, 7, and 9) and autoclaved in 100 ml of conical flask. 1% obtained strain of *lactobacillus plantarum* was sub-cultured in 50ml of MRS broth in controlled conditions and was allowed to incubate further at 37°C for 48 hours under anaerobic conditions.

Growth of *Lactobacillus plantarum* at different pH was calculated by taking Optical density (O.D) with MRS Broth as control measure. The obtained results were as below.

From above observation it can be concluded that *Lactobacillus plantarum* are able to grow

from pH- 3 to pH-11. But there growth varies as its pH changes that is *Lactobacillus plantarum* has maximum growth between pH- 6 to 8

And its growth slow down when pH moves towards acidic or towards alkaline medium and requires more time for complete growth than normal incubation period of 48 hours.

Table.1 Gaseous formed by *L. plantarum* by utilization of carbohydrates

Sr.No.	Carbohydrates	Gas formed
1	Mannitol	Positive
2	Sorbitol	Positive
3	Maltose	Positive
4	Sucrose	Positive

Table.2 O.D of Bacteriocin obtained at different pH

Sr.No	pH concentration	O.D obtained at 600 nm
1.	pH-3	0.4
2.	pH-5	1.37
3.	pH-7	2.65
4.	pH-9	1.73

Table.3

S.No	Temperature	No. of colonies obtained (using colony counter)
1	4°C	0
2	16°C	7
3	20°C	75
4	37°C	250
5	54°C	165

Table 4

S.No	Pathogens	Zone of Inhibition (mm)	Incubation Period
1.	<i>E.coli</i>	18	24 hrs
2.	<i>Staph.aureus</i>	21	24 hrs
3.	<i>Shigella</i>	19	48 hrs
4.	<i>Salmonella Typhimurium</i>	No zone of inhibition	-----

Fig.1 Isolated colonies of *Lactobacillus plantarum* from Honey Bees gut



Fig.2 Image of zone of exhibition

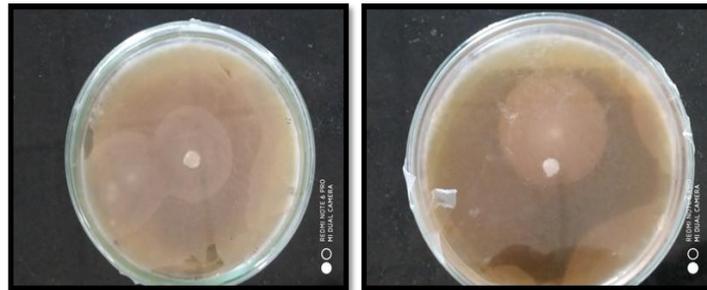
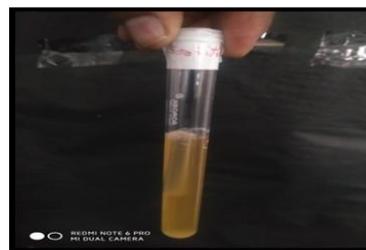


Fig.3



Mannitol



Sorbitol

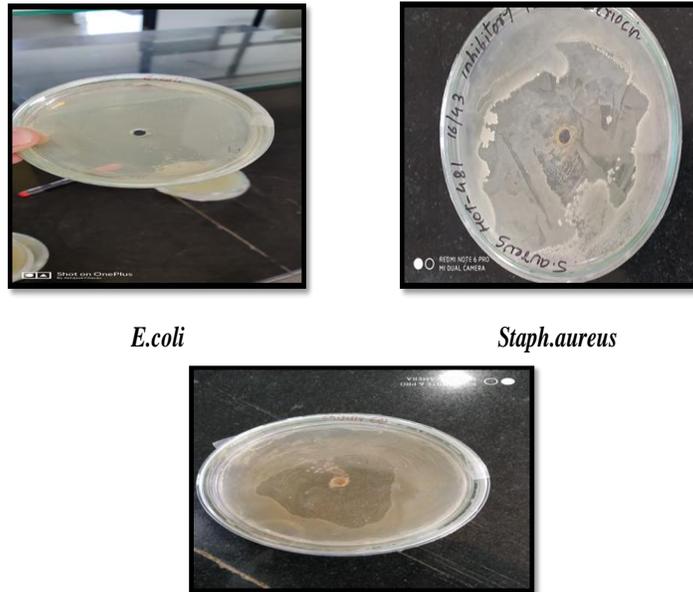


Maltose



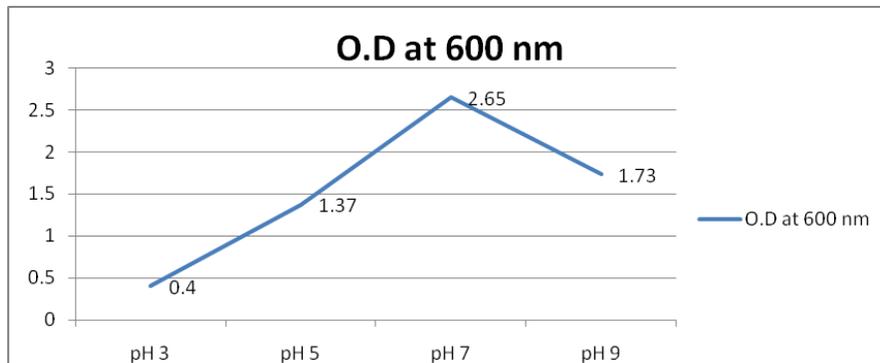
Sucrose

Fig.4

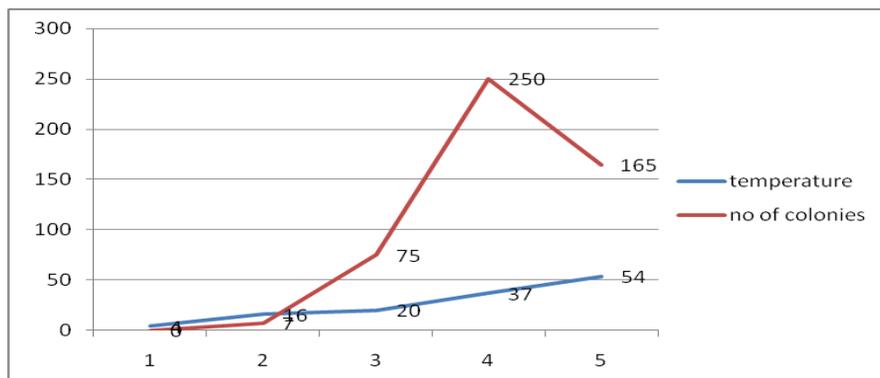


Note: shigella has incubation period from 12 to 96 hrs, so results in *shigella* were obtained after 48 hrs of incubation.

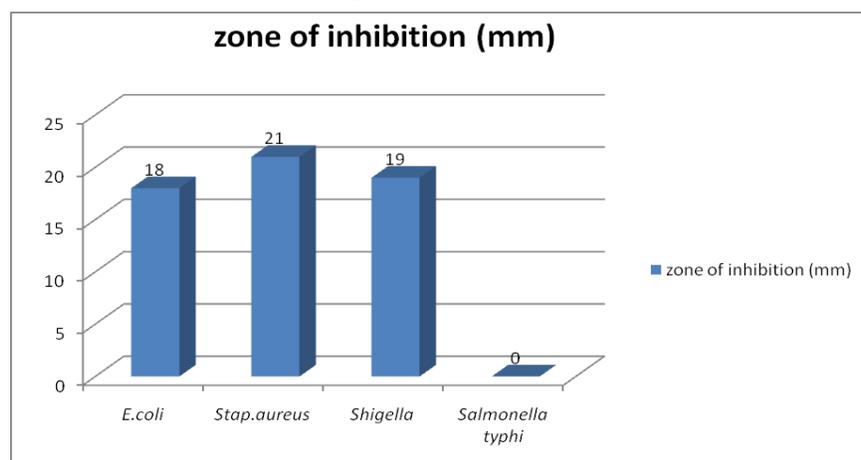
Graph.1 O.D at 600 nm showing amount of bacteriocin at different range of pH concentration



Graph.2 Showing relation between microbial growths at different level of temperature



Column graph.3 For zone of inhibition



Growth of *Lactobacillus plantarum* at different temperature range

No growth was observed below the temperature 16°C. Minute growth was obtained at 16°C and was in more number at 20°C as compared to 16°C with small spots of colonies at regular spacing on Petri-plate.

Maximum growth was seen at temperature range from 34°C to 45°C.

Growth was also observed at 54°C but was low as compared to temperature range from 34°C to 45°C (Table 3).

Bacteriocin assay (zone of inhibition)

Bacteriocin isolated from *Lactobacillus plantarum* was able to inhibit the activity of test colon pathogens like *E.coli*, *Staph.aureus* and *Shigella* and zone of inhibition was observed clearly in this test colon pathogens. But no zone of inhibition was observed in case of *Salmonella typhi* (Table 4 and Fig. 1–4).

Lactobacillus plantarum was successfully isolated from gut of honey bee. Many tests were conducted in order to confirm obtained strain as *lactobacillus plantarum* in nature. The tests were Grams staining, pH test, catalyses test, carbohydrate test, vitamin B12

assay test and motility test. After performing all the tests the result obtained in each category show that the isolated strain is for *L.plantarum*.

Later the bacteriocin was been isolated from the obtained species of *L.plantarum* and purification of bacteriocin was done to obtain crude bacteriocin. For purification methods like dialysis by using dialysis bags and chromatography process was used. Bacteriocin assay was studied on the colon pathogens like *E.coli*, *Staph.aureus*, and *shigella* and *salmonella typhi*. Crude bacteriocin was used to study inhibition activity on these test pathogens. Simultaneously the behavior of *lactobacillus plantarum* was studied at different temperature from 0°C to 54°C and their growth ability was observed at particular selected temperatures (0°C, 16°C, 20°C, 37°C and 54°C). At same time characterization of *L.plantarum* was done by growing them at different pH media and results were calculated by taking O.D of the all pH media in which *L.plantarum* was grown. Different pH concentrated media used were media of pH 3, 5, 7 and 9. Trials of page was done in order to determine the molecular weight and also to study the bands of obtained protein called bacteriocin from *L.plantarum*.

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