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ANTIBACTERIAL ACTIVITY OF A PROBIOTIC *LACTOBACILLUS PLANTARUM* AGAINST URINARY TRACT INFECTION CAUSING PATHOGENS

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ABSTRACT

The main objective of the present study is to investigate antibacterial activity of a probiotic *Lactobacillus plantarum* against urinary tract infection causing pathogens. The proteinaceous substance produced by the probiotic strain is highly effective against all the urinary tract infecting pathogens used in the study. The cell free supernatant of the *L.plantarum* bacteria is able to inhibit the growth of *Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. The recorded inhibitory zone was ranged from 16 mm to 26 mm against urinary tract infecting pathogens. Results from this study concluded that the compound bacteriocin produced by *L. plantarum* have the potential to inhibit urinary tract infecting pathogens.

KEYWORDS: *L.plantarum, E.coli, Staphylococcus aureus, Klebsiella pneumoniae* and *Pseudomonas aeruginosa*.

INTRODUCTION

Urinary tract infection is a condition where one or more parts of the urinary system become infected. Urinary tract infections are the most common of all bacterial infections and can occur at any time in the life of an individual. Almost 95% of cases are caused by bacteria that typically multiply at the opening of the urethera and travel upto the bladder. Urinary tract infection is one of the most common bacterial infections encountered in clinical practice in Europe and North America. It is estimated that 150 million cases of urinary tract infections are common, painful and disruptive, the recurrent nature of urogenital infection, emergence of multidrug resistant bacteria and patient dissatisfaction with side effects of drugs need

better ways to diagnose, treat and prevent infections.[1] Alternative strategies like probiotics would be a beneficial treatment option to tackle this kind of problems.[2]

In recent years, there has been increased focus on the use of probiotic such as *Lactobacillus* sp. for prophylaxis and treatment of urinary tract infections.[3] Probiotics have been defined as "Live microorganisms which when administered in adequate amounts confer a health benefit on the host".[4] Lactobacilli are an important part of the normal flora commonly found in the mouth, gastrointestinal tract and female genitourinary tract. They also protect the female urogenital tract from pathogen colonization by competitive exclusion of pathogens from the cell surface, co-aggregation with certain pathogenic bacteria; adhere to epithelial cells and biofilm formation based on the autoaggregation and surface hydrophobicity.[5] The uses of antimicrobial agents are not only select resistance bacteria but it can disturb the balance of body by killing friendly bacterial strains. When this happens, bacteria and yeast can move in and flourish leading to urogenital tract infection.[6] There is a need to develop longtime alternative therapy against urinary tract infections by the application of probiotic's as antimicrobial agents.

MATERIALS AND METHODS

Isolation and Identification of bacteriocin producing *L. plantarum*

The present experimental strain *L. plantarum* was isolated from the grass silage according to the method.[7] The isolated strain was identified based on the biochemical profile and fermentation pattern studies. The biochemical profiles were carried out according to Bergey's manual of Systemic Bacteriology.[8] The API 50CHL test (Himedia (P) Ltd., Mumbai) was applied for identification by fermentation patterns. The *L. plantarum* strain was grown on MRS agar (Himedia, Mumbai) and stored at 4°C. Liquid culture was grown in MRS broth (Himedia) and stored at 4°C.

Test Organisms

The urinary tract infection causing pathogens such as *E.coli, Staphylococcus aureus, Klebsiellla pneumonia* and *Pseudomonas aeruginosa* used in the present experiment were procured from Microbial Culture Collection Centre (MTCC), Chandigarh, India. All cultures were maintained in nutrient agar and stored at 4^{o}C in the refrigerator.

Optimization of culture conditions

Effect of temperature on bacteriocin production

To determine the optimum temperature for bacteriocin production, the *L.plantarum* strain was grown in MRS broth at different incubation temperatures such as 25, 30, 35, 40 and 45^{o}C for 12 hrs. After incubation, the culture supernatant was assayed for bacteriocin activity (Arbitrary Unit; AU/ml). The optical density at 600nm and changes of medium pH were also recorded.

Effect of pH on bacteriocin production

MRS broth was prepared and adjusted at different pH values such as 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, and 7.5 using 0.1N NaOH solution. The bacteriocin producing organism was inoculated and incubated at 37^{0}C for 12 hrs. The culture supernatant was assayed for bacteriocin activity. The OD at 600nm and pH change was recorded.

Effect of incubation time on bacteriocin production:

To determine the efficient incubation time for bacteriocin production, the bacterial strain was grown in MRS broth at different incubation times such as 12, 24, 36, 48, 60 and 72 hours at 37˚C. After incubation, the culture supernatant was assayed for bacteriocin activity. The optical density at 600nm and changes of medium pH were also recorded.

Antibacterial activity

The inhibitory assay was carried out by agar well diffusion assay method.[9] The bacteriocin producing strain *L.plantarum* was grown in MRS broth overnight and centrifuged at 10,000 rpm for 10minutes and then the resulting supernatant was purified by membrane filtration (0.45mm pore size). This bacteriocin solution (supernatant fluid) was serially diluted up to 1: 200. Then 50 µl of two fold diluted sample was transferred to the wells of 5mm diameter in the MRS agar plate which were already inoculated with indicator strains. The plates were incubated at 37^{0}C to allow colonies development. After incubation at 37^{0}C for 24 hours, the inhibition zone was observed. The antibacterial activity was calculated with the inhibitory effect against indicator strains, One arbitrary unit was (AU) defined as reciprocal of the highest dilution showing a minimum inhibition zone.

Assessment of Activity Index

The activity index of bacteriocin produced by *L. plantarum* was assessed by the method.[10] The activity index was calculated by comparing the resultant inhibition zones of bacteriocin with standard antibiotics using the formula,

$$\text{Activity Index (AI)} = \frac{\text{Inhibition zone of the sample}}{\text{Inhibition zone of the standard}}$$

Assessment of increased zone area

Increased zone area was assessed by the method.[11] It was calculated by the mean surface area of the inhibition zone generated by the standard antibiotic and bacteriocin produced by *L.plantarum*. The increased zone area was calculated by using the following formula,

$$\text{Increased zone area} = (b^2-a^2)/a^2$$

Where, a and b refer to the zones of inhibition for antibiotic and bacteriocin respectively.

Data analysis

The obtained data for bacteriocin activity in the experiment was analyzed by mean, standard deviation and one-way analysis of variance (ANOVA) according to the method.[12]

RESULTS AND DISCUSSION

Isolation and Identification of bacteriocin producing *L. plantarum*

The isolated colony was identified with the information available in Bergey's manual of Systemic Bacteriology and the API 50 CHL test kit supplied by Himedia (P) Ltd, Mumbai. The biochemical properties and fermentation pattern of the isolated *L. plantarum* strain is provided in Table 1. The API 50 CHL system is a one of the methods for identification of lactic acid bacteria isolated from Kimchi by physiological reactions.[13]

Table 1: Biochemical properties of *L.plantarum* strain and results of API test

Biochemical test	*L.plantarum* strain
Gram staining	+
Catalase	+
Oxidase	+
Citrate	+
Fermentation patterns (API results)	
Arabinose	+
Cellobiose	+
Fructose	+
Galactose	+
Glucose	+

Lactose	+
Maltose	+
Mannitol	+
Mannose	+
Melibiose	+
Raffinose	+
Rhamnose	_
Salicin	+
Sorbitol	+
Sucrose	+
Trehalose	+
Xylose	+
Adonitol	+
Inulin	+
Inositol	+

Optimization of culture conditions

Effect of temperature, pH and incubation time on bacteriocin production

The influence of temperature on bacteriocin revealed that the maximum bacteriocin production of 3400 AU/ml was recorded at 35^{o}C (Table 2). The optical density value at 600nm was also high (0.9 OD) at 35^{o}C after 12 hrs of incubation. The statistical one-way ANOVA test for the data on bacteriocin production revealed that there was significant difference between temperatures (F=59.48; P>0.05). The effect of pH on the production showed the highest optical density recorded at 600 nm was 1.2 at pH 6.5 using *L.plantarum* strain. The final pH value was changed to 3.9 with the respective strain. The increased bacteriocin activity of 3200 AU/ml was observed at pH 6.5 using *L.plantarum* strain (Table 3). Statistical analysis of the pH was performed to evaluate the bacteriocin activity by analysis of variance (ANOVA) which showed highly significant difference (F=53.020; P<0.05). Earlier reports revealed that the maximum bacteriocin activity was recorded at 30^{o}C temperature and the optimum pH for bacteriocin production was 6.5 then the pH was declined to 4.[14] More or less similar findings are observed in the present study.

Table 2: Effect of temperature(^{O}C) on bacteriocin production by *Lactobacillus plantarum* (Each data represents an average of five individual replicates)

Temperature (^{o}C)	O.D at 600nm	Final pH	Activity of bacteriocin (AU/ml)
25	0.4	4.2	1600
30	0.6	4.1	1800
35	0.9	3.9	3400
40	0.8	5.5	800
45	0.8	5.5	400

Table 3: Effect of pH on bacteriocin production by *Lactobacillus plantarum* (Each data represents an average of five individual replicates)

pH	O.D at 600nm	Final pH	Activity of bacteriocin (AU/ml)
4.5	0.4	4.2	800
5.0	0.6	4.1	1600
5.5	0.9	4.5	200
6.0	1.0	4.1	600
6.5	1.2	3.9	3200
7.0	1.1	4.6	400
7.5	1.0	4.8	200

The effect of incubation time on bacteriocin production is depicted in Table 4. The results revealed that the raised level of bacteriocin production (3800 AU/ml) was recorded at 48 hrs incubation period. The optical density value at 600nm was also high (0.9 OD) at 48 hrs of incubation in 35^{o}C. The final pH value was reduced from 7 to 3.9. Statistical analysis of the incubation time against bacteriocin activity was evaluated by analysis of variance (ANOVA) it showed highly significant difference ($F=52.80$; $P<0.05$).

Table 4: Effect of incubation time(hrs) on bacteriocin production by *Lactobacillus plantarum* (Each data represents an average of five individual replicates)

Incubation time (hours)	O.D at 600nm	Final pH	Activity of bacteriocin (AU/ml)
12	0.4	0.0	800
24	0.6	5.2	1800
36	0.8	5.0	3200
48	0.9	3.9	3800
60	1.2	4.0	1600
72	1.0	4.2	1200

Inhibition assay (Well diffusion method)

Table 5 revealed that the maximum zone of inhibition (26mm) was recorded against *E.coli* as indicator strain. Followed by this, 24mm, 18mm, and 16mm were recorded against *Staphylococcus aureus, Klebsiella pneumoniae* and *Pseudomonas aeruginosa* respectively (Fig.1).

Table 5: Inhibitory activity of *Lactobacillus plantarum* against indicator strains in well diffusion method at 24 hours.

S.No.	Indicator strains	Size of zone of inhibition (mm)
1.	*E.coli*	26
2.	*Staphylococcus aureus*	24
3.	*Klebsiella pneumoniae*	18
4.	*Pseudomonas aeruginosa*	16

The statistical one-way ANOVA test for the data on inhibitory activity of *L.plantarum* against indicator strain revealed that there was significant difference (F=3.85; P>0.05) between bacterial strains. The bacteriocin obtained from *Lactobacillus sp.* were tested against *Enterobacter cloacea, Proteus vulgaris, Pseudomonas aeruginosa, Gardnerella vaginalis* and it showed inhibitory activity against *Gardnerella vaginalis* and *Pseudomonas aeruginosa.*[15, 16] *Staphylococcus aureus, Micrococcus luteus, Klebsiella* sp., *Salmonella typhi, Vibrio cholerae, E.coli* and *Shigella* sp. strains were sensitive to bacteriocin.[17] The *L.plantarum* strain produced bacteriocin which was active against water borne pathogens such as *Salmonella typhi, Vibrio cholera, E.coli* and *Shigella dysenteriae.*[18,19] Similarly in the present experiment, bacteriocin from *L.plantarum* was tested for its *invitro* antibacterial activity against urinary tract infection causing pathogens.

The activity index and increased zone area was calculated based on the data of inhibition zones for antibiotics and bacteriocin was recorded and provided in Tables 6-8. It revealed that all the tested urinary tract infection causing pathogens exhibited an excellent antibacterial activity than the standard antibiotics (rifampicin, penicillin and ampicillin). Among the tested UTI pathogens, the highest activity index (4.33) and increased zone area (17.38) were observed against *E.coli* and *Staphylococcus aureus* respectively (Table 7).

Table 6: Activity index (AI) and increased zone area of bacteriocin and standard antibiotic (rifampicin) against urinary tract infection causing pathogens

Urinary tract infection causing pathogens	Zone of inhibition (mm)		Increased zone size (mm)	Activity index (b/a)	Increased zone area $(b^2-a^2)/a^2$
	Antibiotic (a)	Bacteriocin (b)			
E.coli	10	26	16	2.6	5.76
Staphylococcus aureus	8	24	16	3.0	8.00
Klebsiella pneumoniae	11	18	7	1.64	1.68
Pseudomonas aeruginosa	15	16	1	1.07	0.14

Table 7: Activity index (AI) and increased zone area of bacteriocin and standard antibiotic (penicillin) against urinary tract infection causing pathogens

Urinary tract infection causing pathogens	Zone of inhibition (mm)		Increased zone size (mm)	Activity index (b/a)	Increased zone area $(b^2-a^2)/a^2$
	Antibiotic (a)	Bacteriocin (b)			
E.coli	6	26	20	4.33	17.78
Staphylococcus aureus	6	24	18	4.00	15.00
Klebsiella pneumoniae	10	18	08	1.80	2.24
Pseudomonas aeruginosa	7	16	09	2.29	4.22

Fig.1: Antibacterial activity of *Lactobacillus plantarum* against urinary tract infection causing pathogens

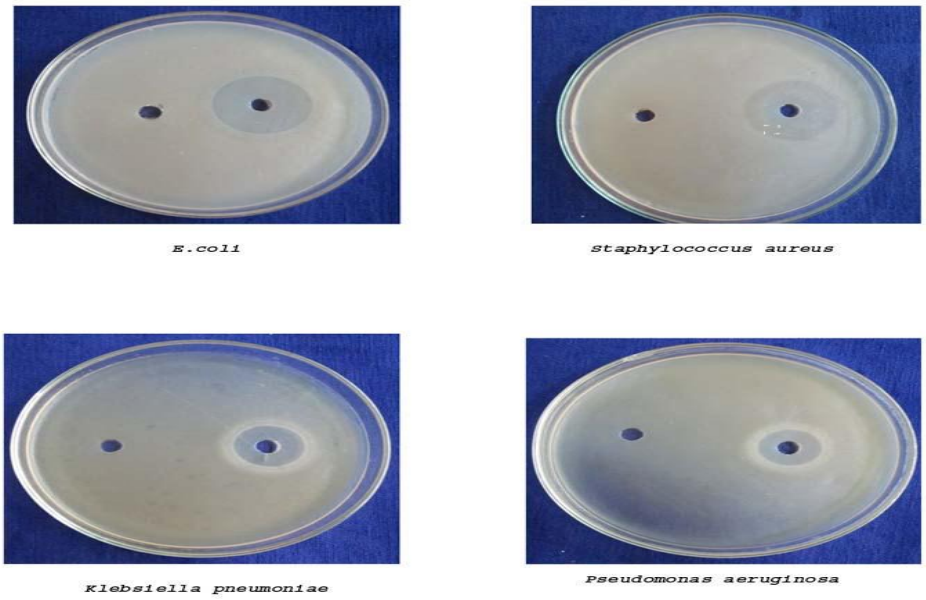

Table 8: Activity index (AI) and increased zone area of bacteriocin and standard antibiotic (Ampicillin) against urinary tract infection causing pathogens

Urinary tract infection causing pathogens	Zone of inhibition (mm)		Increased zone size (mm)	Activity index (b/a)	Increased zone area $(b^2-a^2)/a^2$
	Antibiotic (a)	Bacteriocin (b)			
E.coli	7	26	19	3.71	12.80
Staphylococcus aureus	8	24	16	3.00	8.00
Klebsiella pneumoniae	13	18	05	1.38	0.92
Pseudomonas aeruginosa	8	16	08	2.00	3.00

CONCLUSION

Based on the results obtained in the present attempt, it can be concluded that the bacteriocin producing *Lactobacillus plantarum* has wide range of antibacterial spectrum against urinary tract infection causing pathogens. The bactericidal activity exerted by this strain makes it as a topical therapeutic agent to combat many infections and could provide medicinally value added advantages to the human beings.

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