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Abstract

Background: Lactic Acid Bacteria (LAB) produce various substances that exhibit antagonistic activity against other bacteria. These bacteria as well as the compounds secreted by them have increased attention since they have the potential to inhibit food related pathogens.

Methods: Mild growth inhibitors secreted by Lactobacillus casei in supernatant was used against other pathogenic microorganism. Aim of present study was to determine the antibacterial activity of Lactobacillus casei against enteropathogens. Antibacterial activity was performed with Culture supernatant of Lactobacillus casei against the Klebsiella pneumoniae, Escherichia coli, Salmonella enteritidis and Pseudomonas flourescens. L. casei was inoculated in peptone water for 24 hours incubated at 37 ?C and supernatant was used for the antibacterial activity. 25µl, 50µl, 75µl and 100µl supernatant were added and Optical density was determined with the help of spectrophotometer at 570 nm.

Results and Conclusions: Our result showed a strong in vitro antibacterial activity against various enteropathogenic bacteria which are the potent source of diarrheal sickness. Highest dose of supernatant shows antibacterial activity in all cases ($p < 0.05$). Further studies for its potential as an antibacterial agent may be confirmed after in vivo studies.

Introduction

The spread of drug resistant pathogens is one of the most serious threats to successful treatment of microbial diseases. Down the ages essential oils and other extracts of plants have evoked interest as sources of natural products. They have been screened for their potential uses as alternative remedies for the treatment of many infectious diseases [1]. According to World Health Organization (WHO) majority of the world's population depends on traditional medicine for primary healthcare [2]. Fungus and bacteria are the important microbes which are likely to secrete secondary metabolites as an antibiotic like substances which inhibits or stops the growth of other microorganisms [3].

Antimicrobial effect of Lactic Acid Bacteria is mainly due to lactic acid and other organic acid production, which results in decreasing the pH of the growth environment [4]. Bacteriocins produced by LAB can also affect more unrelated species like Bacillus subtilis, Listeria monocytogenes. Among the antimicrobial substances produced by microorganisms, bacteriocins have gained an increasing interest in the recent years. These are antimicrobial, small ribosomally synthesized proteins, which inhibit or even kill bacteria, including a number of potential food borne pathogens and food spoilage microorganisms [5]. They are usually inhibitory to strains closely related to the bacteriocin producing bacteria. These antimicrobial compounds doesn't harm the producer strains [3]. Bacteriocins are heat labile peptides having molecular weight of 3000 – 6000 [6]. Antimicrobial agents produced are Generally Recognized as Safe (GRAS). Lactic Acid Bacteria have arisen a great deal of attention as a novel approach to control pathogens in foodstuffs and also used in therapeutic approaches. These secrete mild antibiotics called as Lactobiotics [7]. Various methods have been use like Agar well diffusion Method [8], Well dilution assay [9] and Colony count assay [10]. However, much of these previous studies were focused on to Lactobacillus live cells. Studies have revealed that Lactobacillus sp. has an antimicrobial activity against various pathogens. On the other hand, literature reveals that the secondary metabolites are released by the bacterial cell in growth medium or broth. Keeping these views in fact, supernatant was used inspite of the whole bacterial cell.

Present study was focused to find the effect of supernatant of L. casei against various enteropathogens.

Materials and Methods

Reagents

All the inorganic and organic chemicals used in this study were of analytical grade.

Cultures

All the cultures of bacteria used in this study were purchased from Hi Media, Navi Mumbai, India. ATCC number L. casei ATCC 334, E. coli ATCC 8733, P. fluorescens ATCC 13525, S. enteritidis ATCC 13076 and K. pneumoniae ATCC 5510.

Revival of culture

All cultures were streaked over Nutrient agar media (Hi Media, Navi Media) and then used for experimental purpose.

Peptone water was use to standardized the test organism. Test organisms were grown in peptone water at 37 °C for 12 hours. With the help of Spectrophotometer (UV-Vi Shimadzu, Japan, 1601) the concentration of all organism was determined and standardized for all organisms at 104 CFU/ml.

The known lyophilized culture of L. casei (ATCC 334) was obtained from Hi Media, Navi Mumbai, India. Cultures were grown under anaerobic conditions in De Man-Rogosa-Sharpe (MRS) broth (Hi Media) for 24 hours at 37°C for maintenance.

Antibacterial activity assay against E. coli, P. fluorescens, S. enteritidis and K. pneumoniae

To access the antibacterial activity in broth, Peptone water was used to culture E. coli, P. fluorescens, S. enteritidis, K. pneumoniae and L. casei. A set of four tubes were prepared for each test organism containing 5 ml peptone water. Control was also used for all the enteropathogens in which no supernatant was added. Peptone water containing the culture of L. casei was centrifuged at 20,000 rpm for 15 minutes, and supernatant was collected. pH of the supernatant was 7. Supernatant pH was adjusted 6.5 with 1N HCl, and sterilized by filtering through a 0.45 μm-pore-size cellulose acetate filter membrane and stored at -20ºC until used [11].

Before inoculation optical density at 570 nm was observed for each tube. Test tubes were then inoculated with the test organism at 37°C and after 1 hour Optical Density was observed for the growth of test organism. Supernatant added was of different concentration in each test tube of 25μl, 50μl, 75μl and 100μl after interval of half an hour OD's were observed at 30 minutes, 60minutes, 90 minutes ant 120 minutes for time standardization.

Screeninig for the compound with antibacterial activity

Ammonium sulphate precipitation of crude extract

Crude extract was obtained by the method described by Muriana and Klaenhammer [12]. Precipitate was collected by centrifugation at 15000 x g for 2 hours and resuspended in sodium phosphate buffer (50 mM pH 7.0). This was a semipurified extract

High temperature treatment

Crude semipurified product was heated at 90 oC for 60 minutes in water bath and then antibacterial activity was performed.

Proteolytic enzymes sensitivity

Effect of Proteolytic enzymes on semipurified extarct was assessed by using proteolytic enzymes. Pronase, Trypsin, Substilin and α-Chymotrypsin were used. Enzymes and extract (1 mg/ml) were mixed and incubated at 60 minutes for 37 oC. For control, enzyme was preincubated for 10 minutes in hot boiling water. And then this was used for the antibacterial activity.

Statistical analysis

The experimental results were expressed as Mean ± Standard error of mean (SEM) of three replicates. Two way analysis of variance performed (ANNOVA). The level of significance was set at $P < 0.05$.

Results

The experimental results were expressed as Mean ± Standard error of mean (SEM) of three replicates. Two way analysis of variance performed (ANNOVA). The level of significance was set at $P < 0.05$.

Results

Antibacterial activity of L. casei was studied against the four enteropathogens with the help of Optical density. In which supernatant of L. casei at different concentration (25 μl, 50 μl, 75 μl and 100 μl) was added.

Antibacterial activity against P. fluorescens

After inoculation of P. fluorescens the OD after 1 hour at 570 nm were 0.660 in all the test tubes. After adding supernatant in each tube and OD's was observed after 30, 60, 90 and 120 minutes. There was a dose dependent reduction in OD. Inhibition of growth observed as the quantity of supernatant increases. After 30 minutes lowest growth was observed in the sample in which 100μl was added (0.487±0.210), while decrease in OD in 25 μl was lowest (0.567±0.021). OD in case of 100 μl was decreased significantly from 0.660±0.363 to 0.240±0.365. There was no significant difference in case of 50 μl (0.329±0.325) and 75 μl (0.326±0.521) respectively. In control the OD was found to increase from 0.660±0.126 to 0.785 at the end of two hours. [Table I]

Antibacterial activity against E. coli

Sterilized peptone water was used. 1 hour after inoculation of E. coli at 570 nm OD was 0.532 in all the four test tubes. Supernatant was added in each tube and OD's were observed after 30, 60, 90 and 120 minutes. Abrupt decreased in OD was observed in case in which 100 μl was added it was changed from 0.532±0.126 to 0.187±0.255. Samples in which 25μl,

50µl, 75µl of supernatant added were showing less difference in OD i.e 0.410±0.324, 0.285±0.843 and 0.240±0.725. In control the OD was 0.854 after 2 hours. [Table II]

Antibacterial activity against K. pneumoniae

After inoculation of K. pneumoniae the OD at 570 nm was 0.404 for all the four test tubes. After adding the supernatant OD's after 30, 60, 90 and 120 minutes were observed. Significant decrease in OD was observed in case of sample in which 25 µl of supernatant was added n it was found to be decreased from 0.404±0.001 to 0.299±0.321. Also, sample in which 100 µl was added there was a significant decrease in OD from 0.404±0.213 to 0.117±0.218. Sample with 75 µl supernatant was showing a significant decrease in OD (0.165±0.508) than of 50 µl in which OD was 0.210±0.626. In case of control 0.733 OD was observed after 2 hours. [Table III]

Antibacterial activity against S. enteritidis

OD of sterilized peptone water was same i,e 0.098. After inoculation of S. enteritidis OD after one hour was found to be 0.584 for all the test tubes. When supernatant was added no significant decreased in OD was observed in sample in which 25 µl and 50 µl. Whereas in case of 75 µl and 100 µl significant reduction in OD was found. It was 0.276±0.674and 0.120±0.870 respectively. In case of control 25 µl and 50µl it was also found to be significant in comparison with control. However OD of the supernatant has increased in regular manner .[Table IV]

High temperature treatment and proteolytic enzyme activity on supernatant

Extract was tested for high temperature activity. It was showing antibacterial activity after the heat treatment at 90 ?C at 60 minutes in waterbath. According to Axelsson L A et al. 1993 some bacteriocins produced by Lactobacillus strains have high temperature stability. Antibacterial activity was lost after the treatment of proteases. When supernatant treated with protein degrading enzymes it was not showing antibacterial activity against the test organisms. The control in which enzymes was preincubated with hot water was showing antibacterial activity against the test organisms.

In all the studied enteropathogens the amount of supernatant as well as the time was differ significantly $P < 0.05$. OD was decreasing dose and time dependently.

Discussion

Diarrhea is the severe cause of mortality in India. Present study was performed to determine in vitro antimicrobial activity. Antimicrobial activity of Lactobacillus species against various pathogens has earlier been observed [13], [14], [15], [16]. Various methods have been reported like agar well diffusion Method [8], well dilution assay [9] and colony count assay [10]. We have performed the agar well diffusion assay. No satisfactory result was observed i.e. no zone of inhibition was there. This might be possible that supernatant poured in the wells spilled out at the base of agar. Due to this no zone was observed. So, to get the satisfactory results we have used Optical density method in present study. We have also performed the contact time method as suggested by Godiosa et al. [10]. They used both the test organism and pathogen however contact time between two cultures varied and then spread the broth on solid media. After incubation they found that as the contact time increases between both cultures, number of colonies of enteropathogen decreased. As the contact time between L. casei and the pathogen increased number of colonies of the pathogen decreased (results are not shown). Antibacterial activity in broth is preferred because of diffusion of anti-microbial compounds and bioactive compounds with different indigenous bacteria along with pathogens [17].

Peptone water was used in this study since, it is a basal broth medium for the growth of various bacteria also growth in peptone water was very clearly visible. Deleterious activity of L. casei against potential pathogens was assessed by measuring the optical density as a function of time. Lactic acid is the main bioactive compound secreted by Lactic Acid bacteria. Lactic acid acts as a permeabilizer of the gram-negative bacterial outer membrane [18].

In each set of experiment when sterilized peptone water was taken and it was clear. One hour after the innoculation of enteropathogens. OD were increased likewise, 0.660 for Pseudomonas fluorescens, 0.532 for E. coli, O.404 for K. pneumoniae and 0.584 for S. enteritidis. When supernatant in varying concentration were added it has been observed that growth of enteropathogens decreased. In all the cases highest decrease in OD was observed in the sample in which 100 µl of supernatant was added. Maximum decreased in OD for 100 µl supernantant was observed in case of 0.464 in case of S. enteritidis while minimum decresed in this case was found to be in K. peumoniae. This may be possible that supernatant dose of 100 µl of L. casei is not enough to decrease the OD. In case when 25 µl of supernatant was added highest decrease in OD was observed in case of P. fluorescens. OD decreases from 0.660 to 0.430. This dose was satisfactory in case of P.

fluorescens. When 50 µl supernatant was added, highest decrease in OD was observed in P. fluorescens and it was 0.3310 (from 0.660 to 0.329) and lowest decreased was observed in K. pneumoniae (from 0.404 to 0.276). In case of 75 µl of supernatant highest decrease was observed in P. fluorescens which was fall from 0.660 to 0.384. Here, lowest decrease was found in K. pneumoniae (from 0.532 to 0.240).

The observed antibacterial activity was not due to the acidic pH because supernatant was maintained at pH 0 (neutral). This suggests that acid is not responsible for the antibacterial activity against the test organisms. This finding was same as previously performed by Zdravka Sholeva et al 1998. Antibacterial activity of supernatant was lost when treated with different proteases. However the control supenatant was showing antibacterial activity. This gives a reason to conclude that the protein present in supenatant is responsible for the antibacterial activity.

Our results have shown that the supernatant shows an effective antibacterial activity against all the enteropathogens included in this study. In all the samples OD decreases as the quantity of supernatant increases. But drastic change in OD was found in case of P. fluorescens. Some other study should also be performed on the comparative effects of the various constituents of the supernatant to assess the constituent which is giving highest antibacterial activity against pathogens. An in vivo study is recommended to observe its antibacterial and antagonistic property in host. Same study should be perform in gram positive bacteria such as Staphylococcus aureus and Clostridium botulinum which are potent cause of diarheal sickness .

Conclusion

Antibacterial activity shown by *L. casei* is beneficial both in food industries as well as in medical sector. With this study we want to conclude that *Lactobacillus casei* can be used against various enteropathogens which are the major cause of diarrhreal sickness in developing country like India. Lactic Acid Bacteria secrets mild antimicrobial compounds which are not harmful for the host. Further studies should be necessary to use Lactic Acid Bacteria as a therapeutic agent against the diarrheal sickness.

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Illustrations

Illustration 1

Table No 1:
OD of Pseudomonas fluorescens after addition of the supernatant. *P values along the column are significantly different than the control. 'P values along the row are significant different than the values in same row at T=0

Supernatant	T= 0 min	T=30 min	T=60min	T=90min	T=120 min
25 µl	0.660*±0.009	0.567*±0.021	0.498*±0.123	0.466*±0.029	0.430'*±0.125
50 µl	0.660*±0.032	0.490±0.011	0.428*±0.021	0.445*±0.009	0.329'*±0.325
75 µl	0.660±0.0128	0.579*±0.108	0.500*±0.251	0.465*±0.079	0.326'*±0.521
Control (No supernatant	0.660±0.126	0.692±0.221	0.720±0.245	0.752±0.546	0.785±.853

Illustration 2

Table No 2:
OD of Escherichia coli after addition of the supernatant. *P values along the column are significantly different than the control. 'P values along the row are significant different than the values in same row at T=0

Supernatant	T= 0	T=30 min	T=60 min	T=90 min	T=120 min
25µl	0.532*±0.001	0.473*±0.274	0.465*±0.412	0.434*±0.254	0.410*±0.324
50 µl	0.532*±0.210	0.364±0.753	0.340*±0.742	0.290*±0.670	0.285'*±0.843
75 µl	0.532*±0.520	0.343±0.129	0.309*±0.213	0.278*±0.560	0.240'*±0.725
100 µl	0.532±0.126	0.310±0.982	0.267*±0.287	0.223*±0.117	0.187'*±0.255

Control (No supernatant)	0.532±0.134	0.592±0.276	0.663±0.210	0.709±0.432	0.854'±0.129

Illustration 3

Table No 3:
OD of Klebsiella pneumoniae *P values along the column are significantly different than the control. 'P values along the row are significant different than the values in same row at T=0

Supernatant	T=0	T=30 min	T=60 min	T=90min	T=120min
25µl	0.404±0.001	0.368*±0.126	0.342±0.277	0.310±0.267	0.299*±0.321
50 µl	0.404*±0.043	0.289±0.843	0.276±0.123	0.252*±0.421	0.210'*±0.626
75 µl	0.404±±0.598	0.230±0.177	0.197±0.078	0.187*±0.573	0.165"±0.508
100 µl	0.404±0.213	0.190±0.532	0.175±0.300	0.140±0.227	0.117'±0.218
Control (No supernatant)	0.404±0.721	0.598±0.376	0.678±0.218	0.702±0.632	0.733'±0.129

Illustration 4

Table No 4:
OD of Salmonella enteritidis after addition of the supernatant. *P values along the column are significantly different than the control. 'P values along the row are significant different than the values in same row at T=0

Supernatant	**T=0**	**T=30 min**	**T=60 min**	**T=90 min**	**T=120 min**
25 µl	0.584±0.387	0.570*±0.076	0.545*±0.551	0.539*±0.178	0.516*±0.165
50 µl	0.584*±0.129	0.499*±0.218	0.467*±0.167	0.420*±0.438	0.387'*±0.213
75 µl	0.584±0.219	0.351*±0.166	0.326±0.108	0.308*±0.216	0.276'*±0.674
100 µl	0.584*±0.096	0.236±0.190	0.210±0.632	0.167±0.376	0.120'*±0.870
Control (No supernatant)	0.584±0.376	0.619±0.172	0.693±0.761	0.770±0.642	0.807'±0.111

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