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Probiotic potential of lactic acid bacteria strain LD/4

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Abstract

Lactic acid bacteria (LAB) are a group of phylogenetically diverse Gram-positive bacteria. Strain LD/4 showed sigmoidal growth responses in the modified TGYE medium. The kinetics of antimicrobial production followed typically a primary metabolite and was detected after 3h of growth in modified TGYE medium, in the mid- log phase. Antimicrobial production increased continuously during the growth of the bacterium and maximum activity was observed at the beginning of the stationary phase (8h). Thereafter, the antimicrobial compound remained stable on further incubation. During bacterial growth another important feature was observed, the pH of the medium that dropped from neutral to acidic range, which is a characteristic feature of lactic acid bacteria. The antimicrobial activity was assayed in the culture filtrate using qualitative method i.e. agar well diffusion assay (AWDA), showed inhibition zone, which indicated the presence of antimicrobial activity. After the initial identification, probiotic potential of the strain LD/4 has been assessed *in vitro* by studying several properties such as cell surface hydrophobicity assays [spontaneous aggregation assay (SA), auto-aggregation assay (AA), salt aggregation test (SAT), surface accumulation assay (SAA), cell adherence to the glass surface, microbial adhesion to hydrocarbon (MATH)], and survival in the simulated condition. From all the conducted tests, strain LD/4 exhibited a moderate level of cell surface hydrophobicity and also showed low pH tolerance (pH 2.5).

Key-Words: Probiotics, Lactic acid bacteria, Antimicrobial compound, Cell surface hydrophobicity

Introduction

Probiotics are live microorganisms (e.g., bacteria) that are either the same as or similar to microorganisms found naturally in the human body and may be beneficial to health (Gismondo, et al., 1999). Now, the definition of probiotics by the "Food and Agriculture Organization of the United Nations/World Health Organization" is "Live microorganisms, which when administered in adequate amounts, confer a health benefit on the host" (FAO/WHO, 2001). These microorganisms contribute to intestinal microbial balance and play an important role in maintaining health.

According to "Shah (2007) and Chow (2002)" the most popular strains are represented by the following genera: *Lactobacillus*, *Streptococcus*, and *Bifidobacterium*, but other organisms including enterococci and yeasts have also been used as probiotics. Some of these strains have been chosen based on selection criteria (Havenaar, et al., 1992) that are believed to be important for their efficacy such as origin of strain, *in vitro* adherence to intestinal cells (Coconnier, et al., 1992; Bernet, et al., 1993) and survival during passage through the gastrointestinal tract (Kullen, et al., 1997).

Probiotics can be found in dairy and non-dairy products. They are usually consumed after the antibiotic therapy (for some illnesses), which destroys the microbial flora present in the digestive tract (both the useful and the targeted harmful microbes). Regular consumption of food containing probiotic microorganisms is recommended to establish a positive balance of the population of useful or beneficial microbes in the intestinal flora (Soccol, et al., 2010).

However, it is now clear that different strains undoubtedly vary in their efficiency and probiotic potentials (Ng, et al., 2009). The probiotic strains are mainly isolated from natural microflora of the body. Little work has been done on the environmental strains, which could not only be used to extend the list of beneficial microflora, but many may also possess greater variability in these properties. Hence the present study is an effort to show some of the important properties of strain LD/4 that could be assessed *in vitro* for fulfilling the requirement of an effective probiotic organism.

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Material and Methods

Bacterial strains, culture media, growth conditions and chemicals

Lactic acid bacteria strain LD/4 was isolated from dosa batter and available in the laboratory culture stock. Strain LD/4 was propagated in modified TGYE medium (Tryptone, 15.0 g/l; Glucose, 20.0 g/l; Yeast Extract, 20.0 g/l, $MnSO_4$, 0.1 g/l, and pH 6.8) at 37°C and static condition. *Micrococcus luteus* MTCC 106 used as an indicator organism was grown in nutrient broth (peptone, 5.0 g/l; beef extract, 3.0 g/l; NaCl, 5.0 g/l, and pH 7.0) at 37°C and 200 rpm in an incubator shaker (Kumar, at al., 2010). Growth was generally monitored turbidometrically in terms of optical density (A_{600}) as well as viable cell counts. All the chemicals were obtained from Sigma-Aldrich, USA, and media components were purchased from Hi-media, Mumbai, India.

Identification of the strain LD/4

Cells of strain LD/4 were examined by light microscopy to determine their morphology and tested for Gram-staining reaction and catalase activity.

Growth Response, change in pH, and production of antimicrobial compound

Growth Response, change in pH, and production of antimicrobial compound was monitored in the culture grown at 37ºC in static condition over 24 h. The cells were subcultured to an initial OD_{600} of ~0.02, and these parameters were monitored at an interval of 1h till 8th sample then after overnight. Cell free supernatant was collected by centrifugation (Tarson minicentrifuge, 10,000 rpm for 10 min), filter-sterilized and the antimicrobial activity was determined in terms of inhibition of indicator strain *Micococcus luteus*.

Probiotic potential:

The main aim of this work was to identify and characterize strain LD/4 for the probiotic potential. After initial identification, some essential characters such as hydrophobicity, survival in simulated conditions, antimicrobial action (assayed in terms of AWDA method).

1. Cell surface hydrophobicity assays:

Various cell surface hydrophobicity assays were done with strain LD/4 as described by Krepsky, et al., (2003). For this, cells of exponential phase were collected by centrifugation at 7000 rpm for 10 min, washed with 0.01M phosphate buffer-0.15 M NaCl (PBS), and resuspended in 5 ml of the same buffer.

1.1. Spontaneous aggregation assay (SA):

For Spontaneous aggregation (SA) assay, a drop of an overnight grown culture in modified TGYE medium was placed on a clean glass slide and rotated manually. A positive test result (SA-positive) is recorded when the cells show clumping, and a negative test result (SA-negative) show a smooth turbid suspension (Ghosh, et al., 2008).

1.2. Auto-aggregation assay (AA):

The auto-aggregation assay was done by dispensing 50 µl of a bacterial suspension (~10^9cfu/ml), prepared in phosphate buffer saline (pH 6.8) on a clean glass slide. Auto-aggregation (AA) of cells was observed by manually rotating the droplet for 1 min at room temperature. Bacterial suspension that remained homogeneously turbid was considered AA-negative, whereas when clumped cells were observed, it was considered to be AA-positive (Ghosh, et al., 2008).

1.3. Salt aggregation test (SAT):

Two-fold serial dilutions of ammonium sulphate $(NH_4)SO_4$ in PBS (pH 6.8) ranging from 0.007M to 4.0M were prepared. Fifty microliters aliquots of each of these concentrations were placed on a clean glass slide and mixed thoroughly of ammonium sulphate giving visible bacterial cell clumping served as the numerical value of the level of salt aggregation test (SAT value). A positive test was considered for a strain with a SAT value of $\leq$ 2.0 M (Krepsky, et al., 2003).

1.4. Surface accumulation assay (SAA):

Surface accumulation assay is evaluated by quantitative polystyrene tubes. Aliquots of 5 ml of bacterial suspension with initial OD 0.02 at 600nm, in modified TGYE medium were dispensed in polystyrene tubes. The tubes were incubated for overnight at 37ºC. Then OD were taken of the decanted supernatant as well as homogeneously mixed culture. A fresh medium was poured into the tube of decanted supernatant and again incubated at the same conditions. The process was similarly repeated for three days and finally the OD was compared between the decanted supernatant as well as homogeneously mixed culture in terms of percentage by the formula given below:

$$\%\text{Adherence} = (A\text{-}A_0/A) \times 100$$

Where A_0 is the OD_{600} of the decanted supernatant and A is the OD_{600} of homogeneously mixed culture.

1.5. Cell adherence to the glass surface:

The cells were grown in 100 ml Erlenmeyer flask in the modified TGYE medium under static condition at 37^0C for 24 h. The initial OD was maintained 0.02 at 600nm and flasks were incubated for overnight at 37ºC. Then OD were taken of the decanted supernatant as well as homogeneously mixed culture. A fresh medium was poured into the flask of decanted supernatant and again incubated at the same conditions. The process was similarly repeated for three days and finally the OD was compared between the decanted supernatant as well as homogeneously mixed culture in terms of percentage by the formula as given above.

1.6. Microbial adhesion to hydrocarbon (MATH):
Cell surface hydrophobicity was assayed by MATH test. The cells were washed three times in ice –cold Milli-Q water and resuspended in phosphate buffer (pH 6.8) to an OD_{600} of 0.5. A 4.8 ml volume of cell suspension was mixed with 0.8 ml of n-hexadecane in a glass tube and vigorously shaken for 1 min. After the preparation rested for 30 min, the OD_{600} values of the aqueous phase were determined. The affinity of bacteria for the organic solvent was evaluated by the formula:

$$\%Adherence = (1-A/A_0) \times 100$$

Where A_0 is the OD_{600} of the bacterial suspension before mixing and A is the OD_{600} after mixing with the solvent.

2. Survival in the simulated conditions:
For probiotic potential, the survival of strain LD/4 is essential in acidic environment of stomach. Experiments were designed to see the cells survival under similar conditions along with the combination of orange juice (fresh) and whey (fresh). Electrolyte solution was adjusted to pH 2.5. Four sets: control electrolyte solution (pH 7.0), electrolyte solution (pH 2.5), orange juice (pH 4.5), whey (pH 3.5) were inoculated with approximately $2x10^9$ cfu/ml of an overnight grown culture and incubated at 37^0C for 2 h. One milliliter aliquot was taken from each that was serially diluted in normal saline, plated on TGYE agar plate and incubated for overnight at 37°C to get the colony count.

Results and Discussion

Identification of strain LD/4
Cells of strain LD/4 was identified to be Gram-positive, coccoid in shape and showed catalase-negative activity.

Growth Response, change in pH, and production of antimicrobial compound
As shown in Figure 1, **s**train LD/4 showed typical sigmoidal growth responses consisting of a short lag phase of ~ 2h and reaching stationary phase by 8h. During bacterial growth the change in pH of the medium was observed that dropped from neutral to acidic (pH 3.5). Antimicrobial activity followed a growth-associated pattern. The kinetics of antimicrobial compound was monitored along with growth and activity could be detected after 3h during the growth and antimicrobial compound was produced continuously and maximum activity was observed at the beginning of the stationary phase (8h).

Probiotic potential
As LAB are generally recognized as safe (GRAS) organisms, these have been considered and employed for their probiotic potential. After initial identification, some of the important properties of strain LD/4 that could be assessed *in vitro* for fulfilling the requirement of an effective probiotic organism.

Cell surface hydrophobicity assays :
Different tests were carried out to assess the cell surface hydrophobicity of the strain LD/4. These comprised spontaneous aggregation (SA), auto-aggregation (AA), salt aggregation test (SAT), surface accumulation assay (SAA), and microbial adhesion to hydrocarbon (MATH). For example, strain LD/4 shows a low level response to SA and AA- tests, while SAT-test value was observed < 1M, which showed it to carry strongly hydrophobic cell surface. Moreover, in SAA-test, cells of strain LD/4 exhibited 21.11% adherence with the polystyrene tube on the first day whereas maximum adherence was recorded on the third day which was 33.65% as shown in Table 1. Similarly, adherence to the glass surface, 33.69% on the first day and maximum 50.15% on the third day were exhibited by the strain LD/4 (Table 2). The results of microbial adhesion to hydrocarbon (n-hexadecane), the strain showed ~18% of hydrophobicity. From above these results it could be concluded that strain LD/4 has a moderate hydrophobic potential.

Survival in the simulated conditions:
The effect of acidic environment on cells of strain LD/4 showed approximately 50% survival in an electrolyte solution (pH 2.5), 60% of orange juice and 47% in whey as compared to control (electrolyte solution pH 7.0) (Figure 2).

Probiotics are living micro-organisms, which upon ingestion in certain numbers exert health benefits beyond inherent basic nutrition (Tannock, 2002). A variety of probiotic bacteria have been targeted as potential therapeutic agents (Czerucka, et al., 2007; Meurman and Stamafova, 2007). Potential probiotic species differ in terms of their bioavailability, metabolic activity, and mode of action. Antimicrobial activity is thought to be an important means for probiotics to competitively exclude or inhibit invading bacteria (Carr, et al., 2002; Rose and Holm, 2002).

The strain LD/4 showed a sigmoidal growth response and the kinetic pattern of antimicrobial compound production typically following a primary metabolite, confirmed the trend previously reported (Ferreira, et al., 2007; Ghrairi, et al., 2008) in particular and LAB bacteriocins in general (Bendali, et al., 2008; Tiwari and Srivastava, 2008, Kumar, et al., 2010). However, unlike lacticin NK 24 (Lee and Paik, 2001), plantaricin TF711 (Hernandez, et al., 2005), and bacteriocin from *P. pentosaceus* K23-2 (Shin, et al., 2008), which followed the same pattern but showed a significant drop after the late stationary phase, in strain LD/4,

production level remained stable. A little decrease in antimicrobial yield has also been observed from *E. faecium* F58 (Achemchem, et al., 2005). During bacterial growth another important change was observed, the pH of the medium that dropped from neutral to acidic range.

Prolonged bacteriocin activity suggests its possible resistance to proteolytic enzymes or else due to the low pH (Torri Tarelli, et al., 1994), created either by the producer itself or other members of the microflora in the natural environment. This resistance could also be assigned to the hydrophobic nature of the antimicrobial compound (De Vuyst and Vandamme, 1994; Thomas, et al., 2000).

Several methods used for the detection of antimicrobial activity of bacteriocin known in the literature (Parente and Hill, 1992; Zezza, et al., 1993). Of these methods, AWDA method (Tagg and McGiven, 1971) was found feasible and quick to assess the presence of bacteriocin in culture supernatant could also be applied to strain LD/4.

The host-specific adhesion of probiotic bacteria to mucosal surfaces is crucial in the competitive exclusion of pathogenic microorganisms and merits special attention (Bengmark, 1998; Ouwehand, et al., 1999). The hydrophobicity of the bacterial cell surface is an important determinant in the adherence of the bacteria to both living and non-living surface. From all the conducted tests, strain LD/4 exhibited a moderate level of cell surface hydrophobicity, and thus strain LD/4 could perhaps moderately adhere to the gut wall. In contrast, *Lb. rhamnosus* L60 (Liliana, et al., 2008), and *Lb. plantarum* (Ghosh, et al., 2008) showed high cell surface hydrophobicity.

Probiotic bacteria are mostly delivered through the food system and must be acid tolerant to survive in the human gastro-intestinal tract. It has been reported that many bacteria are subjected to stresses in the stomach, where pH between 2.0 and 3.0 is encountered (Liong and Shah, 2005). Strain LD/4 showed tolerance to pH 2.5 and like many probiotic strains showed a slight drop in viable counts (Shin, et al., 2008). Strain LD/4 showed acidifying activity during the growth as described earlier, which is perhaps the reason, that these cells could sustain themselves at low pH (2.5). These results are in accordance with those shown by Mourad and Nour-Eddine (2006), that most strains of lactic acid bacteria show fast or medium acidifying activity.

Conclusion

Strain LD/4 was isolated from dosa batter, identified to be Gram-positive, coccoid in shape and showed catalase-negative activity. The bacterium showed a typical sigmoidal growth response and the antimicrobial production followed the kinetic pattern typical of primary metabolite synthesis. The antimicrobial activity was assayed by using qualitative method i.e. agar well diffusion assay (AWDA). During bacterial growth the pH of the medium that dropped from neutral to acidic range, which is a characteristic feature of lactic acid bacteria. Strain LD/4 was also assessed *in vitro* by studying certain probiotic properties such as cell surface hydrophobicity assays and survival in the simulated condition. From all the conducted tests, strain LD/4 exhibited a moderate level of cell surface hydrophobicity and also showed low pH tolerance (pH 2.5). The probiotic potential demonstrated by strain LD/4 are unique and could be a characteristic feature of strain. These properties of strain LD/4 may be applied at industrial scale for the use of probiotic strain. There is a great demand of natural product for therapeutic purposes in clinical application for the treatment of water borne diseases and strain LD/4 could be a better alternative to clinically available probiotic strains.

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Table 1: Surface accumulation assay of strain LD/4

Days	Initial OD_{600nm}	Final OD_{600nm}	Net Adherence OD_{600nm}	% of Adherence
1.	1.259	1.596	0.337	21.11
2.	1.383	1.925	0.542	28.16
3.	1.414	2.131	0.717	33.65

Table 2: Adherence of strain LD/4 on the glass surface

Days	Initial OD_{600nm}	Final OD_{600nm}	Net Adherence OD_{600nm}	% of Adherence
1.	1.417	2.137	0.72	33.69
2.	1.378	2.385	1.007	42.22
3.	1.342	2.692	1.35	50.15

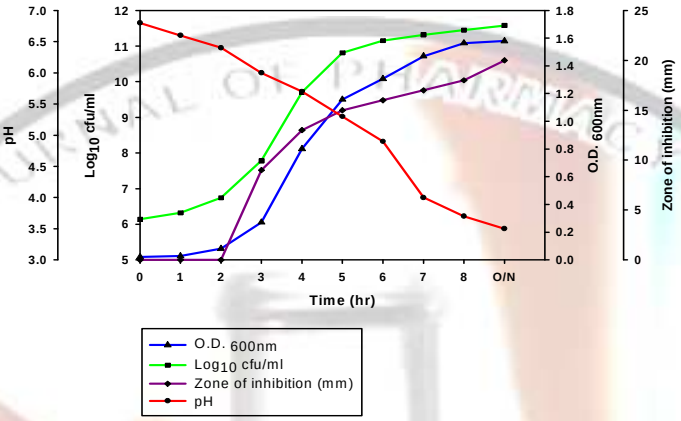

Fig. 1: Growth Response, change in pH and production of antimicrobial compound in modified TGYE medium

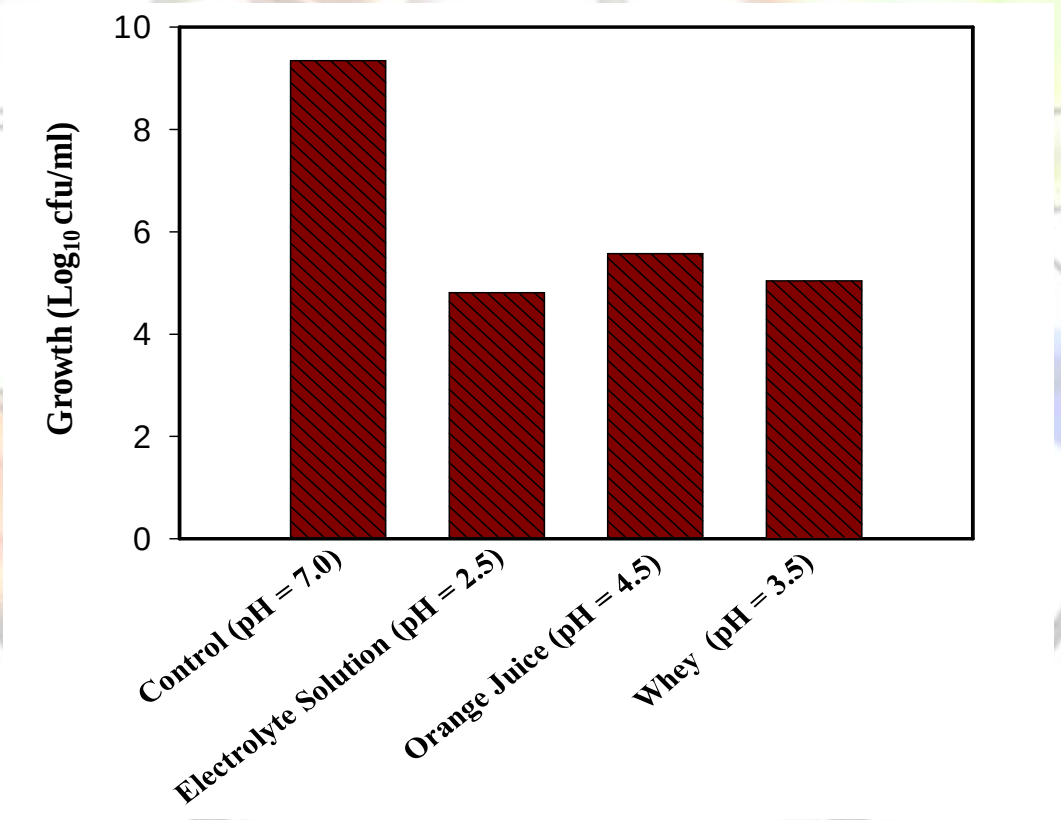

Fig. 2: Survival of cells of strain LD/4 in simulated condition