Probiotic and Techno-Functional Traits of *Lactobacillus pentosus* DS2 Isolated from Naturally Fermented Plant Beverage

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**Article History:**
Received on: 30 Oct 2020
Revised on: 03 Nov 2020
Accepted on: 09 Dec 2020

**Keywords:**
Probiotics, Autoaggregation, Co-aggregation, Cell hydrophobicity, Antibiotic susceptibility

**ABSTRACT**

The research on the isolation of novel lactic acid bacteria (LAB) strains from different fermented plant beverages is receiving immense attention for their significant health benefits towards human health. The present study aimed to isolate and categorize various functional attributes of *Lactobacillus pentosus* DS2 isolated from fermented black carrot beverage. The isolated *L. pentosus* DS2 strain exhibited resistance to acid and higher salt concentrations. The isolated strain was identified by using 16S rRNA gene sequences. *L. pentosus* DS2 showed high survivability of about 6.75 to 7.02 log CFU/ml from pH (2-8) and at a different salt concentration (1-10%) log CFU/ml ranged from 7.92 to 6.41 log CFU/ml. According to the obtained results, auto-aggregation as well as cell surface hydrophobicity was about 16.2 ± 0.35 and 90 ± 0.21 % respectively, while co-aggregation value was 72.5 ± 2.12 and 82 ± 1.41% with *Escherichia coli* and *Staphylococcus aureus* respectively. The enzymatic screening was performed and estimated as 0.54 ± 0.01, 103 ± 1.41, and 80.5 ± 2.89 U/mL of amylase, protease, and phytase. Cholesterol removal by *L. pentosus* DS2 was 47.15 ± 0.41%. The adherence levels by *L. pentosus* DS2 to different cell lines such as Caco-2 and HT-29 ranged from 17.65 ± 0.25 to 19.79 ± 0.31 % respectively. Antibiotic susceptibility pattern obtained showed a different degree of antibiotics sensitivity, such as resistance to ampicillin. Thus, the isolated *L. pentosus* DS2 has all the desired properties to be used as a potential probiotics strain.

**INTRODUCTION**

Fermented plant beverages (FPB) are a kind of potential functional food that is very common in Asian countries, particularly in China, Japan, and India (Kabak and Dobson, 2011). Plants which include vegetables, fruits, cereals, legumes are materials used to produce fermented plant beverages. FPB is dissimilar from other fermented products such as wine, vinegar, yogurt. The difference is there is no alcohol in fermented plant beverages, or very less amount of alcohol is found in them. LAB are the most prominent group of probiotics which have a beneficiary effect on their host by enhancing intestinal balance, and these are usually integrated with plant beverages. LAB is naturally associated with various dairy and non-dairy fermented food formulations. With the increasing trend of veganism, cholesterol contents, lactose intolerance demand of plant-based probiotic bev-
has increased particularly fruits and vegetable origin beverages as they contain bioactive nutritive metabolites such as vitamins, minerals, fibers, and non-nutritive constituents which include phenolic substances, flavonoids, bioactive peptides which possess potential health effects along with metabolic and functional versatility of LAB.

LAB contains a group of enzymes and also some metabolic traits which are involved in the biotransformation of natural compounds present in plants by incorporating various metabolic and functional pathways. LAB fermentation affects the nutritional quality of plant-based material by the degradation of toxic or antinutritional factors. LAB isolated from autochthonous microbiota show potential as a starter to ferment plant-based material and ensures the preservation of various attributes: natural color, firmness, antioxidant potential, and other health-promoting metabolites. This preservation effect may be the result of the modification in organic acid profiles such as the production of lactic acid, acetic acid, and the metabolic breakdown of amino acids. FPB harbor a large population of LAB. Besides increasing the shelf life, these isolated LAB strains have various beneficial properties on fermented products which include improved functional, sensory, and organoleptic properties. Isolation of LAB strains from fermented fruits and vegetables such as cucumber, radish, sauerkraut, table olives, carrot, etc. hold a great promise as probiotics and contribute fermented vegetables with enormous health benefits like healthy gut microbiota, enhanced immune system, and to increase in functional and nutritional properties via formation of novel bioactive substances or by increasing bioavailability of prevailing one. They have shown great tolerance towards gastrointestinal stresses like subjection to gastric juices, bile salts, antibacterial activity towards Bacillus cereus, Listeria monocytogenes, S.aureus and E.coli, Salmonella enterica. Hence, LAB probiotic strains are obtained from fermented foods as capable probiotics for human utilization. Therefore, the current study aimed to isolate and identify indigenous LAB from fermented black carrot beverage and to determine techno-functional traits.

MATERIALS AND METHODS

Isolation and maintenance of strains

Different batches of spontaneously fermented BC juice samples were withdrawn for the isolation of LAB strains. De Man, Rogasa, and Sharpe (MRS) agar (Titan Biotech, India) comprising 6% NaCl was utilized to obtain LAB in the present study (Kingston et al., 2010). To differentiate LAB from inhabitant bacteria diversity, 1% of CaCO3 was incorporated into MRS agar. Fermented BC juice samples were drawn at different time intervals during the fermentation process and were suitably diluted, followed by spreading over the MRS agar plates, which comprised 6% NaCl and 1% CaCO3. MRS plates were kept under incubation at 37°C for 2 days. Colonies were selected based on their morphology such as small greyish or white colonies, either flat or raised, whether smooth or rough surrounded with clear zone were selected and purified by repeated subculture on MRS agar plates. LAB strain was maintained in MRS broth comprising 20% (v/v) glycerol at -20°C till further use.

Identification of lab

Rapid colony PCR and high throughput PCR methods were done for the taxonomic proof of identity (Sharma et al., 2015). Other bacterial sequences were obtained from NCBI mega BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to analyse the 16S rRNA gene sequence obtained from the isolate for their pairwise identities. DNA baser (http://www.dnabaser.com/) software was used to determine the nucleotide constituents of each amplified sequence, and the 16S rRNA entry was submitted to GenBank.

Techno-functional and probiotic attributes

pH tolerance

The pH resistance of isolated strain was done according to (Pundir et al., 2013) with little variation. Briefly, cells were cultivated in MRS broth at 37°C and kept for overnight incubation, and further sub-cultured in fresh MRS media adjusted to pH 1.0-8.0 with hydrochloric acid (5.0 M) and sodium hydroxide (5.0 M), following which 1% (v/v) of pre-culture 16 hr old LAB was inoculated and cultivated at 37°C for 4h under shaking conditions at 200 RPM. The survival population of the LAB was determined by the following equation,

\[ \text{Survival rate} \% = \frac{\text{viable cells survived} (\log \text{CFU})}{\text{initial viable cells transferred} (\log \text{CFU})} \times 100 \]

Salt tolerance

The NaCl tolerance of LAB isolates was done according to (Jampaphaeng et al., 2017) with some variation. The MRS broth was adjusted at different concentrations of NaCl ranging from 1-10% (w/v), and then 1% (v/v) of 16 hr old pre-culture was inoculated into MRS broth and grown at 37°C for 24 h. The survival population of the LAB was determined by the following equation:

\[ \text{Survival rate} \% = \frac{\text{viable cells survived} (\log \text{CFU})}{\text{initial viable cells transferred} (\log \text{CFU})} \times 100 \]
Cell surface hydrophobicity

The adhesion of LAB cells to hydrocarbon such as hexadecane was estimated. Cells were recovered by centrifugation (5000 rpm for 15 min at 4°C), washed repeatedly with PBS (pH 7.0), and resuspended in PBS to get roughly $10^8$ CFU/mL. Afterwards, 2 mL of LAB culture was placed in close contact with 0.6 mL of xylene. LAB suspension was thoroughly mixed and kept for incubation at 37°C for 10 mins. OD ($A_t$) of the aqueous phase was analysed at 600nm.

The hydrophobicity (%) was analysed as,
\[ H(\%) = \left[ \frac{A - A_{O}}{A} \right] \times 100 \]

Cell adhesion

The different cell lines, such as HT-29 and Caco-2, were obtained from NCCS, Pune, India. Both the cell lines were regularly cultured in Dulbecco’s modified Eagle’s minimal essential medium (DMEM) added with 10-20% (v/v) sterilized foetal bovine serum. The cells were also augmented by 1% (v/v) Gentamycin sulfate solution in DMEM. The cell lines were also augmented by 1%(v/v) Gen- poicin solution in DMEM. The cell line was kept at 37°C in the presence of 10% CO₂. The cell lines were fed with DMEM medium regularly until the cells reached 80% confluency.

Adhesion of LAB strain to HT-29 and Caco-2 cells was considered using a modified protocol (née Lehto and Salminen, 1998). Monolayer cell lines of HT-29 and Caco-2 cells were washed with PBS (pH 7.0) and inoculated with LAB and incubated at 10% CO₂ at 37°C.

To count the viable adhered LAB, the monolayer cells were removed by trypsinization for 15 min at 37°C. The viable cells were counted on MRS agar at 37°C.

Cholesterol assimilation

The cholesterol assimilation by the isolated Lactobacillus strain was studied as per the method given by (Walker and Gilliland, 1993). The percentage of cholesterol assimilation in supernatant broth as compared to the uninoculated broth was recorded by ($C_o - C_i$) according to the following equation,
\[ \text{% cholesterol assimilation} = \left[ \frac{C_o - C_i}{C_o} \right] \times 100 \]

where, $C_o$ represents OD₅₅₀nm of MRS broth with LAB, $C_i$ represents OD₅₅₀nm of MRS cell-free supernatant.

Auto-aggregation assay

Aggregation capabilities of isolated LAB strain were estimated by the protocol given by (Collado et al., 2008) with few modifications. The 16 h old bacterial pre-culture was harvested by centrifugation at 5000×g (10 min, 4°C) and washed twice with PBS (pH 7.1). The washed LAB cells were suspended again in PBS. Absorbance was determined at a wavelength of about 600 nm ($A_{600}$) at two different time intervals (0 or 2h).

Aggregation (%) was expressed as,
\[ \text{Aggregation} = \left[ 1 - \left( \frac{A_t}{A_0} \right) \right] \times 100 \]

Co-aggregation assay

Co-aggregation ability of isolated LAB strain with pathogenic bacteria was done according to (Ekmekci et al., 2009) with some modification. 2ml of cells from both LAB and pathogenic strains were mixed and kept under incubation at 37°C. The absorbance of a suspension was determined at a wavelength of 600nm ($A_{600nm}$) at different time intervals (0 or 2h) and co-aggregation was calculated as,
\[ \text{Co-aggregation} = \left[ \left( \frac{A_{pot} + A_{LAB}}{A_{mix}} \right) \times 2 \right] - 1 \times 100 \]

Enzyme production

Screening for degradative enzymes

The enzymatic screening of the LAB was performed by spot/well inoculation on agar containing a particular substrate. LAB for amylase production was spotted on modified MRS media containing 0.5% peptone, 0.7% yeast extract, 0.2% NaCl, 0.75% starch and 1.5% agar was used. A zone of clearance was observed around the bacterial colonies after incubating plates for 24-48 hours followed by flooding with iodine solutions. For protease, LAB was spotted on the medium comprising of skim milk (1%) and agar (1.5%). Phytate hydrolysis by LAB was observed by using modified Sperber’s media containing 10% CaCl₂ and 10% KH₂PO₄. After cultivation at 37°C for 48h, the zone of clearance (mm) adjacent to the colony was observed and recorded using a digital micrometer.

Antibiotic sensitivity

Antibiotic susceptibility of isolated LAB was estimated by the protocol given by (Wang et al., 2019) with little variations by using the antibiotic disc diffusion method on Mueller Hinton agar plates. Antibiotic discs (Himedia) containing ampicillin (2mcg), amoxyclav (30mcg), ampicillin/sulbactam (10mcg), gentamycin (10mcg), erythromycin (10mcg), ofloxacin (2mcg), piperacillin (75mcg) were used to assess the susceptibility. Overnight bacterial culture of L. pentosusBC2 was spread over the Mueller Hinton agar by three-way swabs, and different antibiotic discs were placed. The plates were kept for incu-
bation at 37°C for 12 to 18 hours and observed for zone formation. This study was conducted in two replications. Results were documented as an average from duplicates. Results for Inhibitory zones were classified as resistant R (≤ 14 mm), intermediate susceptible I S (15–19 mm), and susceptible S (≥ 20 mm) towards antibiotic discs. Clinical and Laboratory Standard Institute (Abby and Deak, 2019) was referred for the interpretation of antibiotic sensitivity.

RESULTS AND DISCUSSION

Isolation of autochthonous LAB

LAB isolated from different fermented plant products can be utilized for food preservation, food enrichment, and probiotic supplements along with food for human consumption. Isolated colonies were initially Gram-stained and confirmed to produce catalase. Taxonomic identification confirmed that ASC isolated in the present study was Lactobacillus pentosus DS2. In the present study, LAB strain isolated from fermented black carrot juice was identified as _L. pentosus_ DS2 with accession number MT197323 by using 16S rDNA sequencing. A phylogenetic tree was constructed to find out the genetic relationship between the _L. pentosus_ DS2 with other strains (Figure 1). The obtained results were consistent with the previous studies suggesting LAB are members of fermented plant products. The other LAB species associated with fermented plant products are _Lactobacillus Plantarum_, _Lactobacillus casei_, and _Lactobacillus brevis_. In a similar study, _Pediococcus acidilactici_ Ch-2 was isolated from fermented apricot called chuli has shown potential probiotic characteristics with many functional properties and novel compounds. In another study, strains of _Lb. pentosus_, _Lb. Plantarum_ and _Lb. paracasei subsp_. _Paracasei_ isolated from naturally fermented olives has shown desired in vitro probiotic attributes such as resistance towards low pH, high levels of bile salts, Variable efficiency were observed for adherence towards cell lines like Caco-2 cells which were same regarding strain’s susceptibility towards different antibiotics (Argyri et al., 2013).

**Techno-functional and probiotic attributes**

**pH and salt tolerance**

The selection of probiotic LAB based on pH tolerance is a significant criterion because before reaching the intestinal tract where pH lies between 6 to 7.5, LAB should be able to withstand acidic gastric conditions (pH nearly 1-2). In the present study, at pH 1 survival rate of viable counts as zero, and from pH 2-8 survival rate of viable counts increased gradually from 6.75 to 7.02 log CFU/ml and reaching max at pH 7 (Figure 2 A). Other studies have also confirmed that there will be a marked reduction in viable bacterial cells on prolonged exposure to gastric acidic conditions. Incubation time could be another reason for the relatively less rate of survival under acidic conditions (low pH) which was extended by 8 times than that of normal GIT passage in vivo. These results were following previous studies that showed LAB isolated from fermented olives possess the ability to survive at low pH (Zago et al., 2013).

NaCl tolerance of lactic acid bacteria is a vital criterion to find out the effect of increasing salt concentration on LAB. NaCl can act as an inhibitory substance that may have deleterious effects on the growth of some bacteria. LAB’s are unprotected to osmotic stress when higher concentrations of salts are required to be added to a product. The present experimental results showed _L. pentosus_ DS2 was able to grow in the incidence of different NaCl concentrations ranging from 1-10%. Bacterial growth reduction was observed with increasing concentration of NaCl. The survival rate of viable LAB count decreased from 7.92 to 6.41 log CFU/ml (Figure 2 B). These findings were similar to a previous study that showed the growth of LAB isolates from fermented olives gradually showed a decreasing trend with the increase in NaCl concentration, attaining the lowest count when NaCl was 10% Zago et al. (2013)

**Cell adherence property (CSH)**

Adherence is an important property which enables the LAB to bind with intestinal epithelial cell lining and thus help in inducing cell barrier. LAB shows auto-aggregation property through which the advantageous impact of colonization takes place inside the gastrointestinal tract. Therefore indicating aggregation of lactobacilli a desirable characteristic, especially in the human gut. The CSH is another important anticipated property of probiotic strains which designates significant adherence behavior of suitable probiotic candidates. In the present study, _L. pentosus_ DS2 showed a significant level of hydrophobicity (90%), and the autoaggregation level was about 16 ± 0.35%. Another probiotic property that is of significant importance is co-aggregation in which LAB can interact closely with other pathogens and prevents their colonization. The current study demonstrated significant co-aggregation property with _E. coli_ (72.5 ± 2.12) and _S. aureus_ (82 ± 1.41) (Table 1). The present results were similar to the findings previously observed by other studies. According to (Montoro et al., 2016),
Figure 1: Phylogenetic tree of *L. pentosus DS2*. The tree was constructed using software MEGA X by neighbor-joining method based on 16S rDNA gene sequences with 100 replications in the bootstrap test.

Figure 2: Effect of pH range (A) and salt concentrations (B) on the survival of the *L. pentosus DS2*

Table 1: Cell adherence property of *L. pentosus DS2* isolated from fermented black carrot

| S.No. | Strain       | Hydrophobicity (%) | Auto-aggregation | Co-aggregation |
|-------|--------------|--------------------|------------------|---------------|
|       |              |                    | E.coli           | S.aureus      |
| 1     | *L. pentosus DS2* | 9                  | 16.2±.35         | 72.5±2.12     | 82±1.41       |

Table 2: Quantitative and Qualitative enzyme production by *L. pentosus DS2*

| Enzyme Production | Zone of hydrolysis (mm) | Quantitative estimation (U/mL) |
|-------------------|-------------------------|-------------------------------|
| Amylase           | 8.43 ± 0.18             | 0.54 ± 0.01                   |
| Phytase           | 11.9 ± 0.68             | 80.5 ± 2.89                   |
| Protease          | 14.65 ± 0.21            | 103 ± 1.41                    |
both auto and co-aggregation of LAB were strain-specific which may involves adhesion, aggregation and mucus binding properties.

One of the prerequisite measures for the selection of promising probiotics is their potential to bind to epithelial cells and mucosal lining for their colonization in the intestine. LAB adherence to the gastrointestinal surface increases their residing time which has a direct impact on the host well-being by modulating their intestinal immune system and is also important for the removal of potential pathogens. Various reports have been published with human epithelial cell lines such as HT29 and Caco-2 to determine the adherence potential of probiotic starters (Grover et al., 2011). In the current study, the adherence level of *L. pentosus DS2* to Caco-2 and HT-29 cell lines ranged from 17.65 ± 0.25 to 19.79 ± 0.31 % respectively which agrees with the adherence extent reported previously to see the potential of LAB strain in reinstating the gut microbiome composition (Sharma and Kanwar, 2017). LAB displaying variation in adherence potential towards the intestinal cell lines was demonstrated by many workers thus, representing that adherence potential is extremely strain-specific (Figure 3)

### Enzyme production

Enzyme production by the probiotic strains is important for their selection, and it also provides information about the enzymatic ability of a strain
to their specific performance and their effect on the development of organoleptic characteristics of the final product. In the current case, *Lactobacillus pentosus* DS2 was screened qualitatively and quantitatively for commonly known degradative enzymes, i.e., amylase, proteases, and phytase. The production of all above mentioned dietary enzymes was examined by inoculating the *Lactobacillus pentosus* DS2 with specific media contains the respective substrate. In qualitative screening, the strain was observed for the zone of starch hydrolysis (8.43 ± 0.18mm) when flooded with iodine solution for extracellular production amylase. Zone of clearance (14.65 ± 0.21mm) for protease production around the colonies on skim milk agar and extracellular phytase production (11.9 ± 0.68mm) on modified Sperbers media. In continuation, quantitative estimation of all the dietary enzymes has been examined, and it was established that 0.54±0.01, 103±1.41 and 80.5±2.89 U/mL of extracellular production of amylase, protease, and phytase of the L. pentosus DS2 in MRS medium (Table 2). These findings were similar as obtained by previous studies (Pailin et al., 2001).

**Cholesterol assimilation**

Cholesterol assimilation by probiotic bacteria in the gut through different mechanisms such as assimilation, enzymatic degradation, or by any other mechanisms allows the reduced absorption of cholesterol by enterocytes and more excretion or removal of the cholesterol from the host. Thus, aids in lowering the serum cholesterol level, and thereby helps in the management of hypercholesterolemia related CVDs. In the present study, a significant rate of cholesterol removal by *Lactobacillus pentosus* DS2 (47.15 ± 0.41%) was obtained and agreed with different reports (Benitez-Cabello et al., 2019).

**Antibiotic sensitivity**

Probiotic bacteria possess a large number of biotechnological values and have been explicitly selected for the prevention of antibiotic resistance spread. The isolated LAB strain was screened against antibiotics with broad-spectrum and produced inhibiting effects on both protein synthesis as well as on cell wall synthesis (Table 3), our results demonstrated that the isolated strain was resistant to ampicillin but was inhibited by amoxiclav, ampicillin/sulbactam, gentamycin, ertapenem, ofloxacin, piperacillin. LAB resistance or intermediate resistance to ampicillin, amoxiclav, amp/sulbactam, ofloxacin, and gentamycin could be the innate properties of the strain species and even of the genus. Therefore, innate resistance cannot be transferred horizontally among microorganisms. LAB belonging to the genera *Lactobacillus, Pediococcus,* and *Leuconostoc* possess strong inherent resistance towards the antibiotics used in the present study (Nawaz et al., 2011). This result agreed with (Halami et al., 2000), who reported that the LAB shows more resistance to the principal types of antibiotics.

**CONCLUSIONS**

The present study investigated *Lactobacillus pentosus* DS2 isolated from naturally fermented black carrot for their potentiality as a potent probiotic. Findings divulged that the *Lactobacillus pentosus* DS2 has a great ability to withstand low pH, higher salt concentration, and has shown higher survival rates. Although, the isolated strain presented numerous cell surface properties, in the case of, hydrophobicity, auto- and co-aggregation. Thus, the cell surface-binding properties of isolated *Lactobacillus pentosus* DS2 will help in attachment and colonizing the gastrointestinal tract, which is an important aspect of antimicrobial effects and other disease treatments. Furthermore, this strain has shown the great ability of cholesterol removal. Also, the isolated strain was able to produce a significant amount of enzymes. A significant level of adherence was shown on caco2 and HT-29 cell lines. Antibiotic susceptibility shown by isolated strain was satisfactory. LAB strain exhibited complete resistance against ampicillin. LAB isolated from fermented black carrot under *in vitro* conditions demonstrated promising probiotic characteristics with functional merits and thereby expressing their abilities to be used as probiotics in food and feed formulations. Further *in vivo* studies are required to be done to determine their health benefits. Safety properties need to be done before their use as probiotics in food industries.

**Conflict of Interest**

The authors declare that they have no conflict of interest for this study.

**Funding support**

We hereby acknowledge the funding and institutional support provided by the Amity University Rajasthan.

**ACKNOWLEDGEMENT**

We hereby acknowledge the support and facilities provided by the Amity University Rajasthan.

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