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EXPLORING PROBIOTIC POTENTIAL, ANTI-OXIDANT AND ANTIDIABETIC PROPERTIES OF LACTIPLANTIBACILLUS PLANTARUM IN VITRO.

Microbiology

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ABSTRACT

Diabetes is a chronic metabolic condition marked by sustained hyperglycemia resulting from impaired insulin action. This study sought to assess the hypoglycemic and antioxidant characteristics of lactic acid bacteria (LAB) strain and investigate their probiotic potential, antioxidant efficacy, and antidiabetic effects. The hypoglycemic activity of the isolate was evaluated by examining their inhibitory effects on four particular enzymes: α-glucosidase, α-amylase, lipase, and urease. The antioxidant activity was evaluated using four assays: DPPH, ABTS, superoxide dismutase (SOD), and reducing power assays. *Lactiplantibacillus plantarum* exhibited significant suppression of α-glucosidase (>75%) and α-amylase (>85%). Its antioxidant and radical scavenging properties were analogous to those of ascorbic acid at a concentration of 50 µg/mL. The capacity of the *Lactiplantibacillus plantarum* strain to their resistance to bile salts and acidic environments were used to evaluate the probiotic potential. Research indicates that the strain, possessing hypoglycemic, antioxidant, and probiotic properties, may benefit diabetes prevention.

KEYWORDS

Lactiplantibacillus plantarum, antidiabetic, superoxide dismutase, hypoglycemic, α-glucosidase

INTRODUCTION

Microbiology is the scientific discipline focused on the examination of tiny organisms, such as bacteria, viruses, fungi, algae, archaea, and prions, which are imperceptible without magnification. These bacteria are essential in nutrient cycling, biodegradation, climate dynamics, food deterioration, and illness, impacting various natural and industrial processes.[1]. They have significance for a variety of uses, including food processing, bioremediation, biofuel production, and medicine. Important microbiological pioneers including Jenner, Fleming, Marshall, and Zur Hausen generated ground-breaking discoveries like vaccinations and antibiotics that still influence microbial study and modern healthcare [2] & [3]. Microbiological studies are essential for addressing global challenges in food production, water quality, and energy security [4]. Recent findings highlight the capacity of specific microorganisms to scavenge reactive oxygen species (ROS) and demonstrate antioxidant properties, indicating their potential utility in health and environmental contexts [5]. Probiotics, live microorganisms administered in adequate amounts, are a prominent example; they support health by enhancing the gut microbiota, bolstering immune function, reducing infection risks, and sustaining mucosal integrity [6]. Probiotics are live microorganisms that provide health benefits when consumed in adequate amounts. First formally studied in the 1990s, probiotics remain central in microbiological research for their potential therapeutic benefits.

Probiotics are widely known for their ability to restore intestinal balance as well as their anti-inflammatory, antioxidant, antibacterial, and anticancer effects [7]. The acidic environment of the stomach is a difficulty for probiotic survival; yet, the gut microbiota is essential for sustaining host health by contributing to nutrition, immunological function, and physiological activities. The composition of the gut microbiota profoundly affects the host's overall health [8]. Lactic acid bacteria (LAB) have been used for natural bio-preservation of raw materials since ancient times [9]. The objective of this study is to examine the probiotic features and antioxidant properties of the bacteria *Lactiplantibacillus plantarum* MTCC 2621.

METHODS

Culture and maintenance of probiotics bacteria

The Microbial Type Culture Collection and Gene Bank (MTCC) in India provided with the *Lactiplantibacillus plantarum* MTCC 2621 probiotic strain, underwent overnight incubation in de Man Rogosa and Sharpe (MRS) broth at 37°C with agitation set at 150 rpm.

Bile acid and pH tolerance of probiotic bacteria

Probiotic overnight bacterial culture was introduced into freshly prepared MRS broth containing 0.3%, 0.7%, and 1% bile salts (Himedia Pvt. Ltd). Also, as a control, test bacterial strain was introduced in MRS broth devoid of bile. The test solutions were incubated at 37 ºC for 3 hours. Growth was monitored at various time intervals, and the absorbance was noted at 600 nm. [10]

The pH of the MRS broth was changed to 2, 4, 6, 7 (control) to assess the tolerance capacity under various pH settings. One hundred microliters of overnight-cultivated LAB were introduced into five milliliters of MRS broth and incubated for four hours at room temperature. Optical density at 600 nm (OD600nm) was assessed to monitor growth kinetics. [11]

Haemolytic Property

The LAB culture was inoculated over sheep blood agar and maintained for 48 hours at 37 °C. The blood agar plate was scrutinized for indications of β-haemolysis (clear zones surrounding the colonies), α-haemolysis (greenish zones around the colonies), or γ-haemolysis (absence of zones around the colonies). [12]

Evaluation of anti-oxidant activity - DPPH assay

The DPPH radical-scavenging ability of LAB strain has been examined according to the established protocol. [13] [14]. One milliliter of LAB from the mother culture (10^8 CFU mL^-1) was combined with three milliliters of DPPH solution (0.1 mM) and incubated at ambient temperature in darkness for 30 minutes. The activity of DPPH in scavenging radicals was assessed by the absorbance at 517 nm using a spectrophotometer.

$$DPPH\ radical\ scavenging\ (\%) = \left(1 - \frac{A_1}{A_2}\right) \times 100$$

To estimate the antioxidant activity of LAB strain, superoxide anion scavenging, β-carotene bleaching inhibition, and ascorbate autoxidation inhibition experiments were the samples (A1) and control (A2), Absorbance was evaluated for distilled water containing DPPH solution.

2-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt radical scavenging activity

The ABTS radical scavenging experiment was carried out based on the protocol described in [13]. Before being used, a 7 mM ABTS stock solution was mixed with 5.5 mM potassium persulfate and left in the dark condition for 6 hours to produce the ABTS cation. For optimal results, dilute the ABTS+ solution with distilled water until it reaches an absorbance of 0.8 at 734 nm. The experiment consisted of combining 150 µL of bacterial strain with 1.35 mL of ABTS+ solution, the mixture was kept at 37°C for 10 minutes, and the absorbance was measured at 734 nm.

$$ABTS\ radical\ scavenging\ activity\ (\%) == \left(1 - \frac{A_1}{A_2}\right) \times 100$$

After the samples reacted with the ABTS+ solution, absorbance values for the bacterial samples and the control (distilled water), were denoted by A1 and A2, respectively. Each biological sample was subjected to three distinct tests, with five technical replicates conducted for each experiment.

Superoxide anion scavenging assay

The anion superoxide scavenging power was determined by before established method. [15] A sodium phosphate buffer at a strength of 0.2 M, 15 µM phenazine methosulfate, 50 µM nitroblue tetrazolium, and 75 µM normal acetic acid were combined to create the reaction combination. After that, 50 microlitres of LAB strain were combined with 1 millilitre of this reaction mixture, and the combination was let to stand at 37 degrees Celsius for ten minutes. The optical density at 560 nm was noted.

$$\text{Superoxide anion scavenging activity (\%)} = \left(\frac{A_1 - A_2}{A_1}\right) \times 100$$

Where A1 and A2 denote the absorbance of the experimental sample and control, respectively. The biological sample was examined using five practical replicates in three independent tests.

β-Carotene bleaching inhibitory activity

A reaction solution for the β-carotene bleaching inhibition experiment was made by combining 3 mg of β-carotene, 66 µL of linoleic acid, 300 µL of Tween 80, and 10 mL of chloroform. Following the evaporation of the combination under reduced pressure, 75 milliliters of distilled water were incorporated for dilution. 200 µL of LAB strain were amalgamated with 4 mL of the prepared reaction mixture for the experiment, which was thereafter incubated for 2 hours at 37°C in the absence of light. Sample absorbance at 470 nm was noted. [16]

Percentage inhibition was estimated by

$$\beta\text{-Carotene bleaching inhibitory activity (\%)} = \left(\frac{A_{1,\ 2h} - A_{2,\ 2h}}{A_{2,\ 0h} - A_{2,\ 2h}}\right) \times 100$$

Sample and control (distilled water) absorbances are A1 and A2, respectively Three independent biological investigations analyzed each sample with five technical replicates.

Ascorbate autoxidation inhibition assay

A solution of 5 mmol/L ascorbate and 0.2 mol/L sodium phosphate buffer (pH 7.0) was combined with 9.8 mL of pure water or 0.1 mL of cell-free extract from LAB strains (at a concentration of 10^8 CFU/mL) as the control. The reaction solution was allowed to incubate at the ambient temperature for a period of ten minutes, after which the absorption reading was taken at 265 nanometres.[17]

Ascorbate autoxidation inhibition was calculated as,

$$\text{Inhibition rate (\%)} = \left(\frac{A_1 - A_2}{A_1}\right) \times 100$$

In this study, A1 and A2 denote the absorbance values for the control group and the experimental sample, respectively. The experiment was independently performed three times, with each biological sample evaluated using five technical replicates.

. α- Amylase inhibition activity

The α-amylase inhibition assay outlined by Iqbal et al [18]. Concisely, the microwells of a 96-well plate were filled with 15 µL of phosphate buffer with a pH of 6.8 and 25 µL of α-amylase enzyme with a concentration of 0.14 units per milliliter. Subsequently, the test sample (Lactic acid bacteria) and starch solution (40 µL) were added to the combination. A total of thirty minutes of incubation at a temperature of fifty degrees Celsius was followed by the addition of ninety microlitres of iodine reagent, which consisted of five millimolar iodine and five millimolar potassium iodide. The absence of any test sample in the negative control results in a level of enzymatic activity that is equal to one hundred percent. Acarbose was thought to be a positive control, while a blank was believed to be a solution that did not contain any enzymes and just contained the test sample.

Urease inhibitory activity

The urease inhibition capacity was calculated by 10 µL of the experimental sample was mixed to a reaction mix that contained 25 µL of urease, 100 mM urea, and 50 µL of phosphate buffer (3 mM, pH 4.5). The mixture was allocated into a 96-well plate and incubated at 30°C for 15 minutes and 50 µL of a phenol reagent (1% w/v phenol and 0.005% w/v sodium nitroprusside) was added to each well. Subsequently, 70 µL of an alkaline reagent was combined and kept at 30°C for an extra 50 minutes. The urease inhibition was evaluated by the absorption at 630 nm, as outlined by [18]. Thiourea served as the positive control, while a mixture without test sample performed as the blank.

Lipase inhibition assay.

Lipase inhibition was measured using a modified procedure [18]. Following a 5-minute centrifugation at 16,100 rpm with the lipase enzyme (10 mg/mL) suspended in water, the supernatant was collected. The reaction combination contained 150 µL lipase, 350 µL Tris buffer (100 mM, pH 8.2), and 50 µL test sample in an Eppendorf tube. The reaction began with 450 µL of olive oil as the substrate. The inhibitor was Orlistat, while the blank included 400 µL buffer, 150 µL lipase, and 450 µL substrate without the test sample. After 2 hours of the incubation process at 37 °C, the mixture underwent centrifugation, and 200 µL of the supernatant was aliquoted to a microtiter plate. Absorbance was quantified at 400 nm utilizing a microplate reader.

α-Glucosidase inhibitory activity

α-Glucosidase (0.05 g in 100 mL of ice-cold distilled water) was pre-incubated with different concentration of bacterial strain. To commence the enzymatic reaction, p-nitrophenyl-α-D-glucopyranoside (3 mM) was introduced as a substrate into the potassium phosphate buffer containing α-glucosidase and bacterial strain and incubated at 37 °C for 20 minutes, then terminated by adding 2 mL of 0.1 M Na_2CO_3. A control sample containing α-glucosidase devoid of bacterial sample was incorporated to establish baseline enzyme activity. [19].

RESULTS AND DISCUSSION

Bile Acid and pH tolerance of Lactic acid bacteria

The gallbladder stores the bile produced by the liver. It facilitates digestion. Once the bacteria enter the intestinal tract, their survival depends on their bile resistance, Bile salt resistance testing has been carried out to judge the capacity of LAB to endure the host's intestinal passage. Lactic Acid Bacteria was grown at 37°C for 48 hours. Bile salt solution (3% w/v) was the basic bile concentration in the intestine. [20] At every interval of time (0–3 h), the isolated strain showed viability at concentrations of 0.3%, 0.7%, and 1% of bile acid and showed significant exponential viability growth only at a concentration of 0.3% were presented in Fig.1.A

Most bacteria struggle to grow in low pH settings. Lactic acid bacteria, on the other hand, have acidophilic properties that allow them to survive and thrive in acidic environments. It is critical to separate this resistance from conditions characterized by excessive levels of free acids (H+), as they might stifle growth. The pH resistance test is used to examine lactic acid bacteria's resilience to both acidic and basic environments [21]. The presence of colonies was observed in all pH (pH 2, pH 4, pH 6, and pH 7). A significantly high difference in the viable colony of strain was observed in pH 4, pH 6 also shows high viable rates were shown in Fig.1.B

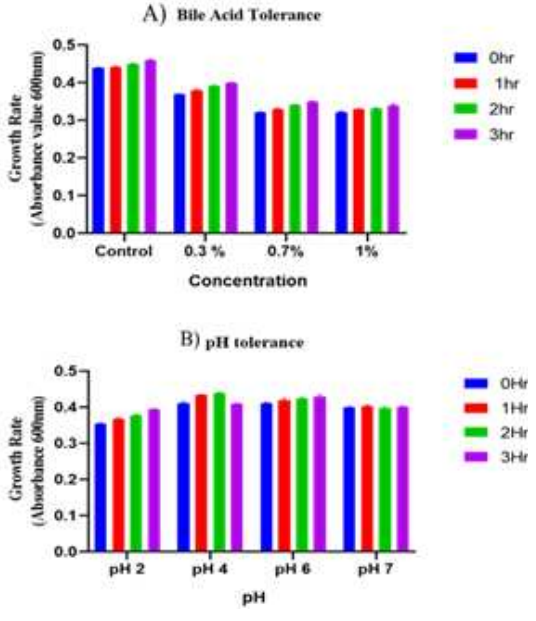

Figure 1: Bile tolerance (Fig.1.A) and pH tolerance (Fig.1.B) of *L.plantarum*, Data are expressed as mean ± SD (n = 3).

Safety assessment of bacterial strain

The haemolytic activity of *Lactiplantibacillus plantarum* assessed on sheep blood agar exhibits no zone surrounding the culture. The examined strain exhibited γ haemolysis, indicating the absence of haemolytic activity. The green zones surrounding colonies indicate α-hemolysis, clear zones denote β-hemolysis, and the absence of zones signifies γ-hemolysis on Columbia blood agar plates. Only strain exhibiting γ-hemolysis are deemed safe.

Antioxidant properties of lactic acid bacteria strain *Lactiplantibacillus Plantarum*

Antioxidants are chemical compounds that help to prevent or mitigate oxidative damage caused by free radicals [22]. Antioxidant activity is linked to a variety of components produced by LAB strains, including NADH, proteins, and antioxidant enzymes [23]. Antioxidant activities of lactic acid bacteria strains have previously been documented [15, 24–28]. The antioxidant system of probiotics can be attributed to the scavenging of reactive oxygen species (ROS), chelation of metal ions, inhibition of enzymes, reduction activity, and inhibition of ascorbate auto-oxidation, as well as the scavenging of oxidising substances or prevention of their generation in the intestine [29]. The DPPH radical scavenging property (40.4%), The β-carotene bleaching inhibition activity (56.7%), the reducing power activity (67.1%), The superoxide anion scavenging activity (56.51%), the ascorbate oxidation (78.1%), ABTS radical scavenging (35.46%), ABTS radical scavenging (35.46%) and Ascorbate autoxidation scavenging (63.2%) of lactic acid bacteria strain *L. plantarum* were presented in Fig.2. The overall result concluded that the lactic acid bacteria *L. plantarum* showing high antioxidant scavenging activity.

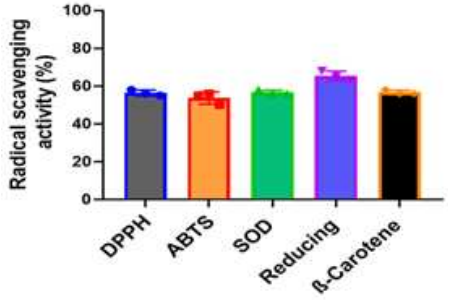

Figure 2: DPPH scavenging, β-Carotene bleaching inhibition, reducing power, Superoxide anion radical scavenging, Ascorbate autoxidation inhibition, ABTS radical scavenging, Data are expressed as mean ± SD (n = 3).

Enzyme inhibition potentials of lactic acid bacteria strain *Lactiplantibacillus plantarum*

The enzymes such as α-amylase, alpha-glucosidase, urease, and lipase inhibition potential of *L. plantarum* were evaluated as shown in Fig.3. α-glucosidase and α-amylase are the two digestive tract enzymes that catalyze the breakdown of starch to produce monosaccharides. α-amylase activity reduction causes the delay of starch hydrolysis, which is a crucial component in regulating postprandial hyperglycemia. Insulin resistance in the liver and unregulated gluconeogenesis caused by overexpression of glucose-6-phosphatase are characteristics of type II diabetes. The result demonstrated that the enzyme inhibitory activity of *L. plantarum* the α-amylase, glucose- 6 phosphatases, urease, and lipase activity was found to be 77%, 76%, 75%, 78%, and 79% of α-amylase, alpha-glucosidase, glucose- 6 phosphatase, urease, and lipase were observed. Thus, the result indicates that *L. plantarum* displayed comparable inhibitory activity against all enzymes.

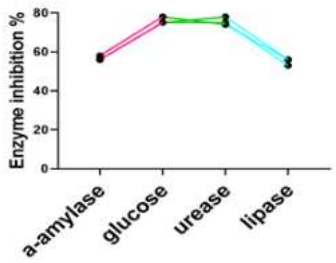

Figure.3. α-amylase, glucose- 6 phosphatase, urease and lipase Data are expressed as mean ± SD (n = 3).

Case Study

The current results showed that the isolated LAB, *L. plantarum* was safe and had important properties, such as the ability to tolerate bile salts and acidity, to be resilient in the gastrointestinal environment and have antioxidant and antidiabetic property.

CONCLUSIONS

This investigation demonstrated that the strain successfully inhibited α-glucosidase and α-amylase. Ingesting probiotic lactic acid bacteria can enhance gastrointestinal health and diminish the danger linked to diabetes. Nevertheless, LAB strain like Lactobacillus spp. may present benefits compared to pharmacological treatments, owing to their potential application as dietary supplements, medicinal foods, or biotherapeutics for diabetes. They may possess a broader range of action. Animal models should be employed for evaluation to enhance our understanding of the bioactivity sources and the associated bioavailability of the Lactobacillus strain discussed in this study.

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