The Bioscan
AN INTERNATIONAL QUARTERLY JOURNAL OF LIFE SCIENCES
20(1): S.I (1), 93-103, 2025 www.thebioscan.com

Development and Evaluation of Probiotic Based Bioinoculant for Enhancing Soil Fertility and Growth Variables in *Triticum aestivum* (Wheat)

Asmita Shinde[1], Aishwarya Pawar[2], Prathmesh Bhosale[3], Manasi Raut[4], Manisha Shinde[5]*

[1,2,3,4,5]Department of Microbiology, Yashavantrao Chavan Institute of Science,

Karmaveer Bhaurao Patil University, Satara – 415 001, Maharashtra, India

*Corresponding author: msshinde@ycis.ac.in

DOI: 10.63001/tbs.2025.v20.i01.S.I(1).pp93-103

KEYWORDS
Probiotics
Nitrogen fixation
Phosphate solubilisatiossn
Lactic acid bacteria
Soil fertility
Triticum aestivum (Wheat)

Received on:

10-02-2025

Accepted on:

07-03-2025

Published on:

09-04-2025

ABSTRACT

Modern agriculture focuses on enhancing crop yield to fulfill the food demands of a growing global population, mostly depend on the intensive application of chemical fertilizers and pesticides. However, the excessive use of these agrochemicals has been found as a major cause of the decline in microbial biodiversity, leading to reduced soil fertility, contamination of soil and water resources, and significant hazards to human and environmental health. Sustainable agricultural practices promote the use of microorganisms to enhance crop yield and soil health. Plant growth-promoting microorganisms play a pivotal role in sustainable agriculture by enhancing soil fertility and promoting plant growth through beneficial microbial interactions. The present work focuses on the development of bioinoculants derived from probiotic organisms isolated from dairy products such as curd and buttermilk. The isolated probiotics microbial strains, including *Lactococcus cremoris* subsp. *tructae*, *Streptococcus lactaricus*, and *Lacticaseibacillus sharpeae* were studied to determine their nitrogen fixation and phosphate solubilization ability. The efficacy of these probiotic microorganisms was evaluated through in vitro and field trials, where agronomic parameters including plant height, spike height, number of spikes, and grain yield were measured. Statistical analysis using the Mann-Whitney U test in Python was employed to determine the significance of the observed effects. The results demonstrated a significant enhancement in plant growth, indicating that probiotic-based bioinoculants offer a sustainable, eco-friendly alternative to chemical fertilizers. This study highlights the potential of probiotic microorganisms isolated from fermented dairy products in improving crop productivity and soil health within the framework of sustainable agriculture.

INTRODUCTION

Agriculture is a major economic sector occupying approximately 37% of land area around the world, as per the report of the Food and Agriculture Organization (FAO) [11]. Good agricultural practices are mostly relied on utilization of environmentally and economically sustainable resources to achieve long-term sustainability goals [33]. However, modern agricultural practices are largely focused on high yield rather than sustainable agricultural approaches. The high agricultural productivity makes the modern agricultural practices more popular and seems to be the best solution to fulfill the food demand of the world's expanding population [44]. Modern agriculture emphasizes the use of chemical fertilizers to enhance agricultural yield and chemical pesticides to manage phytopathogens that are responsible for losing an estimated 9-16% of key cereal crops, including wheat, rice, and maize. However, the extensive use of chemicals in farming is the major cause of water and soil pollution, soil deterioration, and biodiversity loss. Chemical pesticides are also used in modern agriculture [38]. In contrast, sustainable agriculture focuses on ecological processes like plant-microbial interactions, the use of biological pest and disease control systems, organic matter, and nutrient cycling in farms. These sustainable approaches make the best use of natural resources to enhance soil fertility, soil water content (above- and below-ground), biodiversity, and genetic variation in plant features, and also limit the usage of external resources like synthetic chemicals, fossil fuels, etc. [37]. In a natural ecosystem, plants interact with a variety of microorganisms, which may be beneficial, harmful, and benign microbes. The beneficial plant-microbe interaction plays a significant role in the development of sustainable agriculture. The utilization of plant growth-promoting microbes (PGPM) as a bioinoculant in soil is considered an environmentally friendly alternative to chemical fertilizers [16]. PGPMs offer an environmentally sustainable alternative to chemical fertilizers by enhancing nutrient availability, regulating phytohormone production, and improving plant resilience to biotic and abiotic stresses [25]. The microorganisms known to enhance agricultural productivity are *Azotobacter, Azospirillum, Bacillus, Burkholderia, Enterobacter, Flavobacterium, Pseudomonas, Rhizobium, Frankia, Klebsiella, Clostridium, Trichoderma, Beauveria, Serratia and Streptomyces* [1][14][43]. In addition to these well-established PGPMs, probiotic microorganisms are also reported to have the potential to stimulate plant growth and thus crop yield. Although research on the role of probiotics in plant growth promotion is still emerging, the common probiotic microorganisms like lactic acid bacteria have been reported for

their plant growth-promoting properties and could serve as effective bioinoculants in sustainable agriculture [29].

Lactic acid bacteria as plant growth-promoting microorganisms

Probiotics are the microorganisms that offer health benefits when consumed, mainly improving or maintaining the gut microbiota. Lactic acid bacteria (LAB), popular probiotic microorganisms, are a genetically varied collection of Gram-positive, rod or spherical, non-spore-forming, catalase-negative, facultative anaerobic bacteria [18]. The primary genera of LAB include *Leuconostoc, Enterococcus, Lactobacillus, Lactococcus, Bifidobacterium, Pediococcus,* and *Streptococcus* [34]. Although LAB are well known for their human health benefits, emerging studies have demonstrated their role in plant growth promotion [38]. Metagenomics research of plant microbiomes showed the presence of *Rhizobacteria* along with other microorganisms, including LAB. However, their low abundance in the plant-soil ecosystem makes their identification and functional characterization more challenging [38]. Nevertheless, studies of plant-LAB interaction have reported their beneficial role to plants and in the enhancement of agricultural yield [26]. LAB promotes seed germination, improves soil fertility, aeration, and solubility, reduces abiotic stress, and neutralizes harmful gases [35]. Some bacteria produce organic acids or enzymes that solubilize phosphorus in the soil, making it accessible to plants and other microbes [32]. The presence of gene sequences encoding two kinds of alkaline phosphatase enzymes has been identified in *Lactococcus lactis*. These enzymes catalyse phosphate mineralization and permit the organism to solubilize a variety of phosphorus compounds [6]. In food crop systems, nitrogen also plays an important role because it is a major nutrient that determines yield [37]. Historically, food production relied on natural nitrogen sources like manure and soil organic matter. However, since the 1960s, chemical nitrogen fertilizer use has surged, resulting in significant environmental nitrogen loss, impacting water and ecosystems [15]. Aside from LAB's ability to dissolve phosphate, it has been claimed that some strains of LAB can fix atmospheric nitrogen for plant use through the process of Biological Nitrogen Fixation (BNF) [13]. Bacteria have the enzyme nitrogenase, which converts atmospheric nitrogen into ammonia, which may be assimilated by many species. These bacteria are known as nitrogen fixers [32]. It was reported that *Lactococcus lactis* isolated from the mucilage microbiota of Sierra Mixe maize has recently been identified as a diazotroph capable of BNF [17]. In addition to this, some findings suggested a function for LAB as a biocontrol agent against soil-borne phytopathogens [22]. Special features of some LAB species make them suitable candidates for biological control agents [21]. Screening of lactic acid bacteria (LAB) for activity against ten plant pathogens associated with potatoes, such as *Pectobacterium carotovorum, Fusarium oxysporum*, and *Rhizoctonia solani*, showed that *Lactiplantibacillus plantarum* KB2 LAB 03 could decrease the severity of disease by 40–90% [39]. *L. lactis subsp. diacetylactis* was shown to suppress the growth of *Fusarium* species, a major cause of tomato crown and root rot, up to 62.42% on de Man Rogosa Sharpe (MRS) agar medium [3]. PGPM, such as LAB, also boosts plants' capacity to tolerate stressful conditions by shielding plants from abiotic stressors or modifying the plant's stress response, therefore enhancing the survival of the entire phyto microbiome [23]. *Lactobacillus* strains have been proven to alleviate abiotic stress in plants. It was shown that medicinal plant clones infected with *L. plantarum* (ATCC 9019) were more resistant to salt stress. Plants treated with *L. plantarum* showed different metabolic responses to salt stress [26]. Apart from this, some microorganisms produce hormones that can stimulate plant growth, example, *L. acidophilus* has been found to generate cytokinins [26,27]. Certain *Lactobacillus* strains have been demonstrated to generate Indole-3-acetic acid (IAA) [30]. All these reports emphasize the role of LAB in the enhancement of crop yield and therefore in sustainable agriculture. Further research is required to elucidate the mechanisms underlying the plant growth-promoting effects of probiotic microbes and their potential applications in enhancing crop productivity while reducing reliance on chemical inputs.

Sustainable agriculture relies on eco-friendly alternatives to chemical fertilizers, with microbial bioinoculants playing a crucial role in enhancing soil fertility and crop productivity. The present study focuses on the isolation and characterization of nitrogen-fixing and phosphate-solubilizing lactic acid bacteria (LAB) to develop a potent bioinoculant to enhance soil fertility. The isolated strains were evaluated for their plant growth-promoting abilities, including nitrogen fixation and phosphate solubilization. Their efficacy was further assessed through in vitro and field experiments on wheat (Triticum aestivum L.), aiming to improve crop yield while promoting sustainable agricultural practices.

MATERIALS AND METHODS

Collection of samples

A curd and buttermilk sample was collected from Satara city in sterile glass bottles for isolation of lactic acid bacteria.

Isolation of lactic acid bacteria from curd and buttermilk samples

Lactic acid bacteria in curd and buttermilk samples were enriched in MRS broth (de Man, Rogosa, and Sharpe medium) (Hi Media M641-100G) for 48 hours at room temperature in a shaker incubator. From enriched MRS broth, 0.1 ml was inoculated on sterile MRS agar plates and incubated for 48 hrs in microaerophilic conditions at room temperature. Subsequently, the pure culture of isolated lactic acid bacteria was purified and preserved on MRS agar slants at 4^0andsub subcultured for every three weeks for preservation of culture [20].

Identification and characterization of isolates

Study of cultural and morphological characters

The cultural, colony characteristics, and morphological characteristics of LAB isolates were studied and recorded. The Gram nature and motility (by hanging drop methods) of isolates were studied using a compound light microscope [40].

Biochemical characterization of isolates

The LAB isolates were studied for their biochemical characters such as carbohydrate utilization (1% of glucose, fructose, sucrose, maltose, arabinose, mannitol, and lactose) [19], catalase test [4], oxidase test and indole test, methyl red test, Voges Prausker (VP) test, citrate utilisation test [19].

16S rRNA sequencing and phylogenetic analysis

Molecular identification techniques such as PCR (Polymerase Chain Reaction) amplification of 16s rRNA gene followed by sequencing were employed for accurate species identification. The phylogenetic tree was constructed from the sequenced 16S rRNA region of three bacterial isolates identified from curd and buttermilk samples[42].

Evaluation of plant growth-promoting characteristics of isolates

Phosphate solubilization

A loopful of suspension of isolates namely C1, C2, and B1 was spot inoculated on sterile Pikovasky's agar medium (pH 5.5, Hi Media M520-100G) and agar plates were incubated at microaerophilic condition for 48 hrs at room temperature and observed for development transparent zones of phosphate solubilization surrounding the colony of isolates [7].

Nitrogen fixation

A loopful of suspension of isolates namely C1, C2, and B1 was spot inoculated on sterile N_2- free Ashby's Mannitol Salt Agar Medium (Hi Media M706-500G) and plates were incubated at microaerophilic condition for 48 hrs at room and were examined for visible bacterial growth on the plates [2] that provide the evidence for atmospheric nitrogen fixation carried out by microorganisms. The bacterial isolates that were able to fix atmospheric nitrogen were selected and subjected to estimation of nitrogen fixed by the isolates to determine their efficiency by Kjeldahl's method [36]. This method was developed by Johan Kjeldahl in 1883 for the quantitative estimation of organic nitrogen present in chemical substances like ammonia. In this method, three control sets were prepared: Control 1 was sterile MRS broth not inoculated with isolates, Control 2 was MRS broth inoculated with isolates C1, C2, and B1 but kept at refrigeration temperature, and Control 3 was the same as Control 2 but centrifuged at 3000 rpm. The pellet was collected, dried at 105°C, and its weight was measured while the supernatant was used for nitrogen estimation. Test sets were prepared by

inoculating isolates C1, C2, B1, and their consortium in MRS broth and kept in a shaker incubator at room temperature for 48 hours. Nitrogen content in both control and test sets was estimated using Kjeldahl's method, where samples were digested with $CuSO_4$, H_2SO_4, and NaCl, distilled with NaOH, and titrated with 0.01 N HCl until a colour change was observed. The nitrogen content was calculated for all samples by using the formula [36].

$$\text{Percentage of nitrogen in the sample} = \frac{1.4\ V.N}{W}$$

On-field effect determination of isolates on wheat (*Triticum aestivum L.*)

Preparation of bacterial inoculums

The isolates were cultured on MRS agar plates and incubated for 48 hours. A suspension was prepared from a fresh log phase (Optical Density at 620 nm 0.86) culture of isolates [41]. The total viable count (TVC) of the suspension was determined [5]. The same suspension was inoculated into 100 ml conical flasks, each containing 50 ml of MRS broth, and incubated for 48 hours to prepare the bacterial inoculums [41].

Bioassay-based evaluation of plant growth promotion

The bioassay on the effect of plant growth promotion of isolates was examined with Wheat (*Triticum aestivum L.*) in a pot experiment. In this experiment, five treatments were carried out: (1) seeds sown without any inoculation of bacterial isolates (control), (2) seeds treated with the bacterial culture of isolate C1 (3) Seeds treated with the bacterial culture of isolate C2 (4) Seeds treated with the bacterial culture of isolate B1 (5) Seeds treated with the consortium of bacterial culture of all the three isolates.

Initially, wheat seeds were surface sterilized with a treatment of 95% ethanol for 5 min and rinsed with sterilized distilled water four times. Then wheat seeds were inoculated with four bacterial cultures separately at room temperature for 2 hrs. Control seeds were also treated in the same manner with MRS broth without inoculation of any isolates. Finally, seeds were grown in pots (20 cm diameter x 20 cm depth) filled with 1.5 kg of soil previously sterilized in a hot air oven at 1200C daily for 1 hour for 3 days (pH 6.0). The experimental design was laid in a Randomized Block Design (RBD). In each treatment, a block of 5 seeds planted in a pot was considered as a replicate. Five replications were conducted for all the treatments of all control and test sets [41]. The seedlings were inoculated with the respective cultures of C1, C2, B1, and mix, and one pot was treated only with MRS broth, which was uninoculated with any isolates to serve as a control. For growth evaluation, a total of 25 randomly selected plants (5 plants in each replication) from each treatment were uprooted after 2 months of inoculation, and data on plant length (cm), number of spikes, spike height, and number of grains were recorded [24].

Statistical analysis

The data obtained from Kjeldahl's method and plant growth bioassay, including parameters such as plant height, spike height, number of spikes, and number of grains, were statistically represented in graphical form and analyzed separately using the Mann-Whitney U Test (a non-parametric test) in Python software [10].

Results

Isolation and characterisation of bacteria

A total 3 isolates of lactic acid bacteria, tentatively labeled as C1, C2, and B1 were obtained from curd and buttermilk samples using a sterile MRS medium under microaerophilic conditions. The cultural and colony characteristics on MRS agar were studied and recorded (Table no.1, Fig. no.1). Isolates C1 and C2 were found to be gram positive, nonmotile cocci while B1 was Gram positive, nonmotile rod (Table No.2 and FigNo.2). Biochemical characters of all three isolates were studied.

Table No. 1: Colony characters of LAB isolates C1, C2 and B1 on MRS agar in microaerophilic condition.

LAB isolates	Size	Shape	Colour	Margin	Opacity	Elevation	Consistency
C1	1mm	Circular	White	Entire	Opaque	Raised	Mucoid
C2	2mm	Circular	White	Entire	Opaque	Raised	Moist
B1	1mm	Circular	White	Entire	Opaque	Convex	Moist

Table No.2: Gram nature and motility of LAB isolates C1, C2 and B1

LAB Isolate	C1	C2	B1
Gram nature	Gram-positive cocci	Gram-positive cocci	Gram-positive short rod
Motility	Non motile	Non motile	Non motile

Table No. 3: Biochemical tests of LAB isolates C1, C2, and B1

Sugar Fermentation Test			
Test	**C1**	**C2**	**B1**
Glucose	+++	+++	++
Sucrose	+++	++	+++
Lactose	+++	+++	+++
Fructose	+	++	+++
Mannitol	+	+	-
Maltose	+	+++	+++
Arabinose	-	+++	++

IMVIC Test			
Indole test	-	-	-
Methyl red test	+	-	+
Voges Proskauer test	+	+	+
Citrate utilization test	+	-	+
Catalase and Oxidase Test			
Catalase	-	-	-
Oxidase	-	-	-

Note: +++: High growth, ++ : Moderate growth, + : Growth, - : No growth

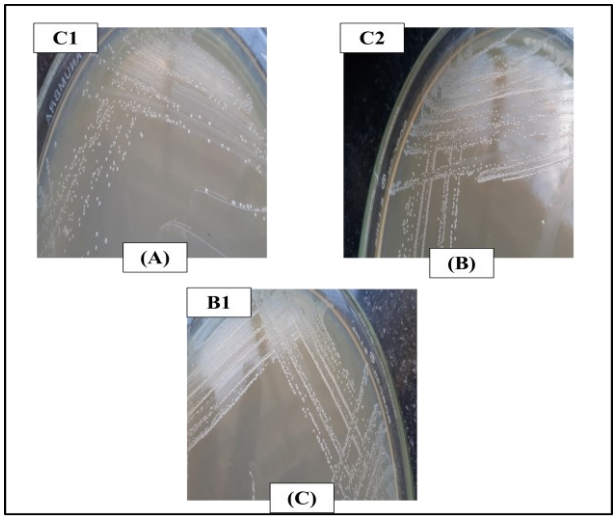

Fig. No. 1: Isolation of bacteria from curd, buttermilk and soil. **(A)** Isolate from curd sample(C1), **(B)** Isolate from curd sample (C2), **(C)** Isolate from buttermilk sample

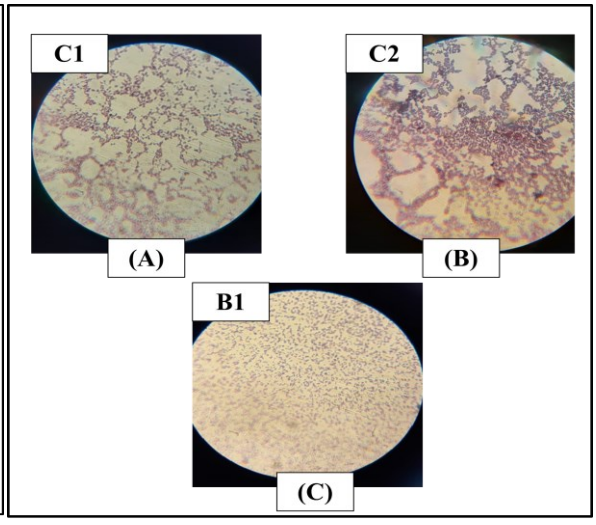

Fig. No. 2: Microscopic field of gram staining of isolates **(A)** Gram positive cocci isolated from curd sample(C1) **(B)** Gram positive cocci isolated from curd sample(C2) **(C)** Gram positive

16S rRNA sequencing and phylogenetic analysis

LAB isolates C1, C2 and B1 were subjected for phylogenetic analysis of 16S rRNA sequencing. A phylogenetic tree was constructed for the evolutionary analysis of organisms by using the isolates and related reference strains, as shown in (Fig. no. 3, 4, 5). All sequences of isolates C1,C2 and C3 obtained in the current study were deposited in GenBank under accession numbers:LC801477, LC801478 and LC801479, respectively. Analysis of 16S rRNA sequence of C1 isolate identified as*Lactococcus cremoris subsp. Tructae*, while isolate C2 belonged to *Streptococcus lactaricus* and isolate B1 belonged to *Lacticaseibacillus sharpeae* (Table No.4).

Table No.4: Identified organism along with accession and strain no. for the isolates

LAB isolates	Strain name	Identified organism	Gene bank Accession no.	Similarity %
C1	MSS24	*Lactococcus cremoris subsp. tructae*	LC801477	100%
C2	SPS26	*Streptococcus lactaricus*	LC801478	100%
B1	APS18	*Lacticaseibacillus sharpeae*	LC801479	100%

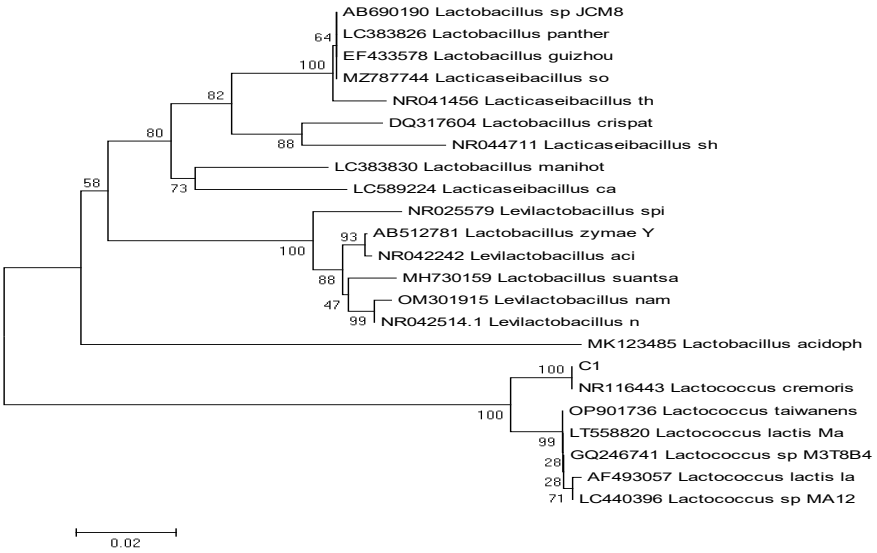

Fig. No. 3: Evolutionary analysis of isolate C1 Organism identified as *Lactococcus cremoris sub species tructae*

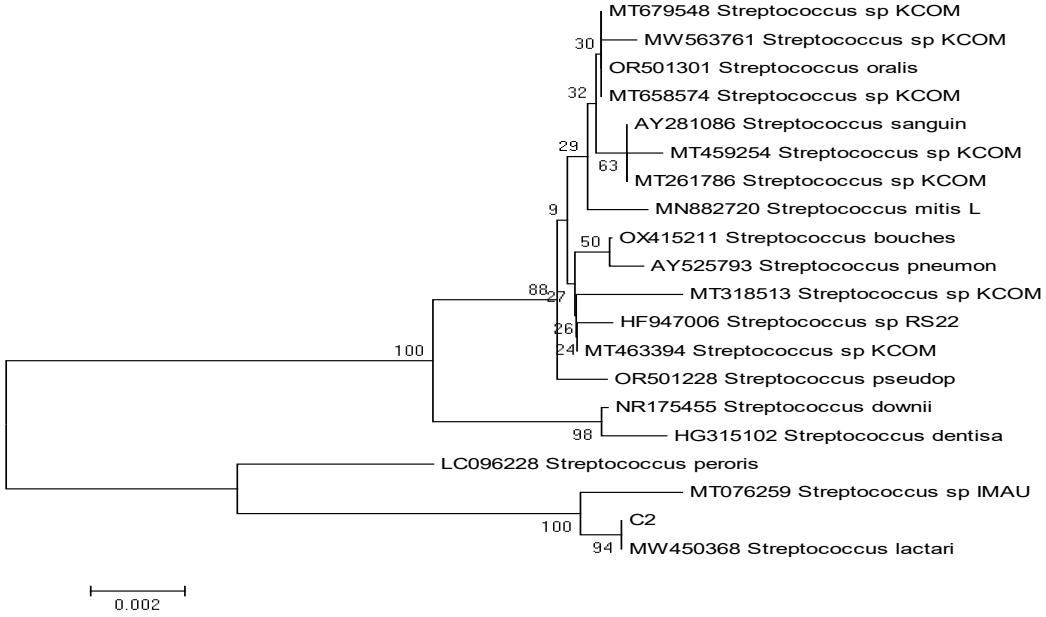

Fig. No. 4: Evolutionary analysis of isolate C2 organism identified as

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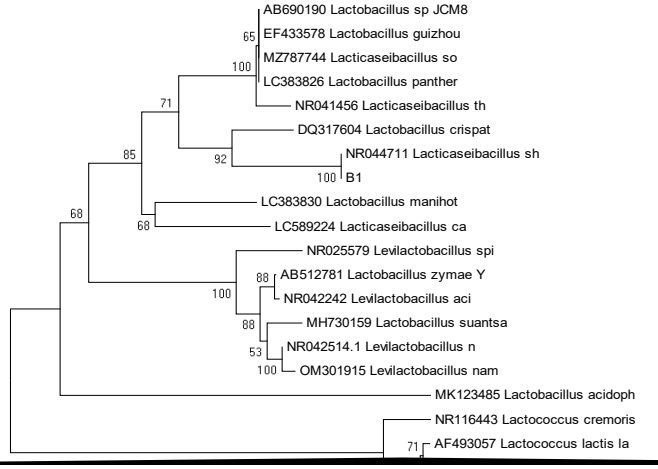

Fig. No. 5: Evolutionary analysis of isolate B1 organism identified as *Lacticaseibacillus sharpea*

GQ246741 Lactococcus sp M3T8B4

0.02

Phosphate solubilization

The isolates were studied to determine their ability to solubilize inorganic phosphate by qualitative test. The growth of the isolates was observed on Pikovasky's agar medium that contain inorganic phosphate for the development of a clear zone around the colony.[9]. Isolate B1was found to be phosphate solubilizer as clear zone was observed around the colony (Fig. No. 6 C) while growth of isolates C1 and C2 showed no clear zone around their colonies and thus were unable to solubilize inorganic phosphate (Fig. No. 6 A and B).

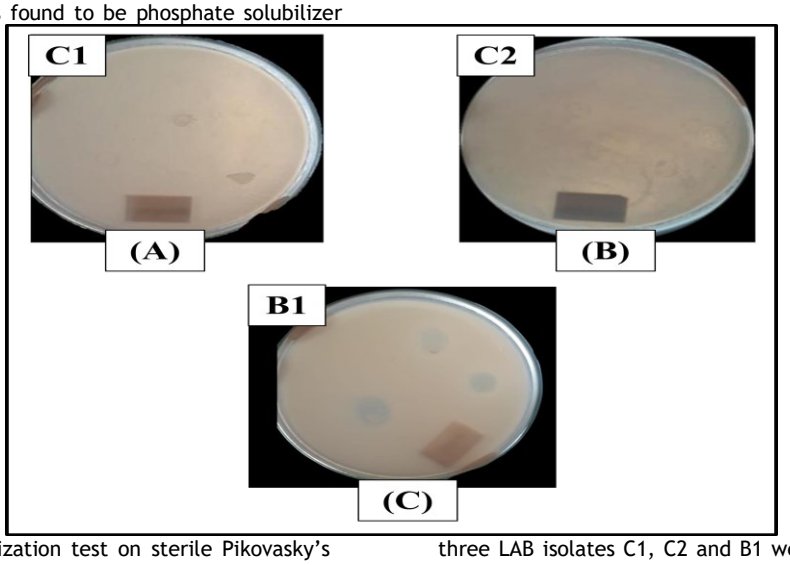

Fig. No.6: Phosphate solubilization test on sterile Pikovasky's agar medium **(A)** Phosphate solubilization on isolate C1(No zone observed) **(B)** Phosphate solubilization on isolate C2 (No zone observed) **(C)** Phosphate solubilization on isolate B1(Transparent zone observed around growth).

Nitrogen fixation

The growth of isolates on a sterile Ashby's N2 free Mannitol agar medium was examined in order to evaluate their ability to fix atmospheric nitrogen non-symbiotically (Fig no. 7 A and B). All three LAB isolates C1, C2 and B1 were able to grow excellently on N2 free medium demonstrating their nitrogen-fixing ability.

The Kjeldahl method was used to quantify the amount of nitrogen fixed by these isolates. Nitrogen content fixed by all three isolates C1, C2 and B1 were estimated as 3.38%, 3.09%, and 3.18 % respectively. By comparing with control sets (estimated as 0.61%, 0.51% and 0.50% respectively), all three isolates showed maximum nitrogen uptake capacity, highlighting their potential for non-symbiotic nitrogen fixation (Table No.5).

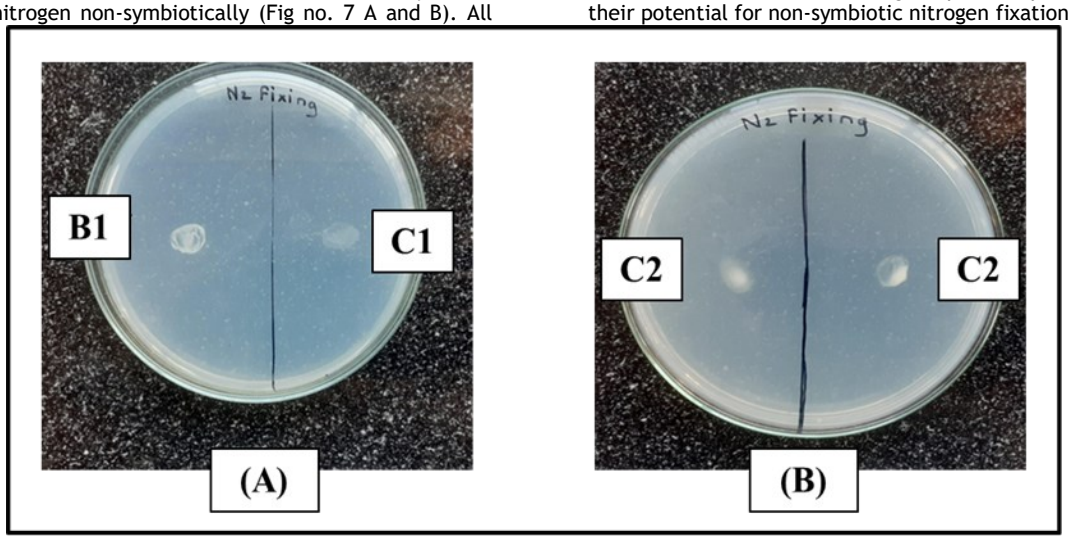

Fig. No.7: N_2 fixation test on sterile N_2 free mannitol agar medium **(A)** Growth of isolate B1 and C1 on N_2 free mannitol agar medium **(B)** Growth of isolate B1 and C1 on N_2 free mannitol agar medium

Table No.5: Estimation of nitrogen by Kjedahl's method

Isolate name	C1	C2	B1
Control N2 %	0.61%	0.51%	0.50%
Test N2 %	3.38%	3.09%	3.18%

Statistical analysis of estimation of nitrogen by Kjedahl's method

The data were statistically analyzed in Python software using Mann Whitney U Test (Non-Parametric Test) for checking the difference between control and isolates as shown in (Table No. 6). Here, the output U-statistic indicates the difference between the groups (Smaller value of U-statistic indicate a larger difference between the groups) and p-value indicates the probability of observed difference is due to chance. The p-values for C1, and Control for C1, C2, and Control for C2, B1, and Control for B1 are larger than the significance level (>0.05). This statistical analysis supports our findings and based

on the mean difference we conclude that C2, and Control for C2 isolate have the largest difference than others concerning the control group.

Table No.6: Mann-Whitney U test for estimation of Nitrogen by Kjedahl's method

Mann-Whitney U test between	U-Statistics	p-value	Mean difference	Significance (p<0.05)
C1 vs Control_C1	0.0	0.0636	2.76	No
C2 vs Control_C2	0.0	0.0636	3.39	No
B1 vs Control_B1	0.0	0.0636	2.68	No

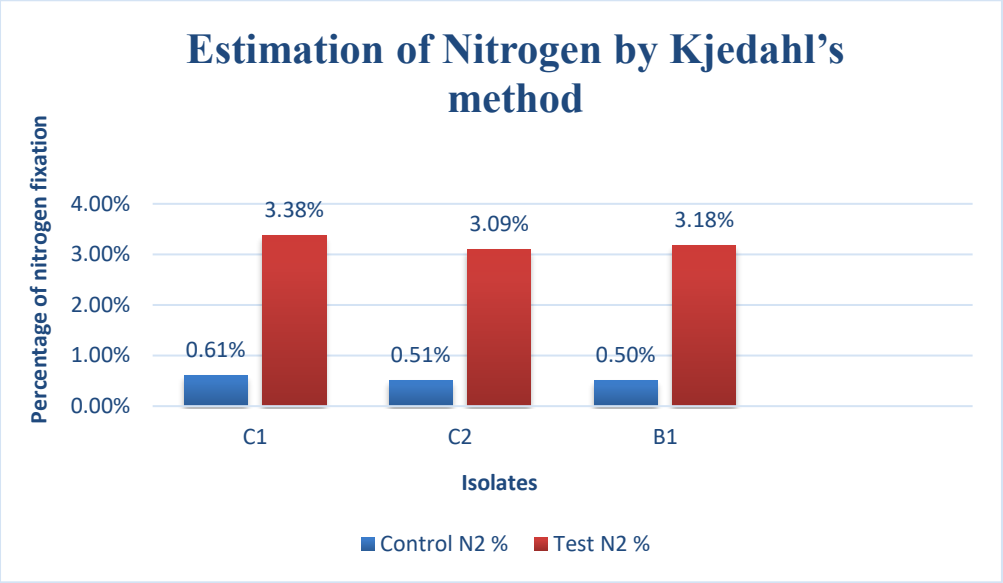

Fig. No. 8: Estimation of nitrogen fixed by isolates C1, C2 and B1 by using Kjedahl's method

Effect of LAB isolates on wheat (*Triticum aestivum L.*) growth

The effect of lactic acid bacteria based bioinoculant on growth of wheat plants (*Triticum aestivum L.*) was studied. Four pot sets of wheat plants were inoculated with isolates C1, C2, B1(TVC/ml $17.24*10^3$,$49.08*10^7$ and $16.30*10^7$ respectively) and with consortium of these three isolates along with the control set. The effect of lactic acid bacteria was evaluated by comparing the growth parameters of wheat plants with the control (soil without inoculums) as shown in (Fig No. 9). Various growth parameters including plant height, spike height, number of spikes, and number of grains (yield) of wheat was evaluated to determine the on-field efficiency of the lactic acid bacteria as plant growth promoter.

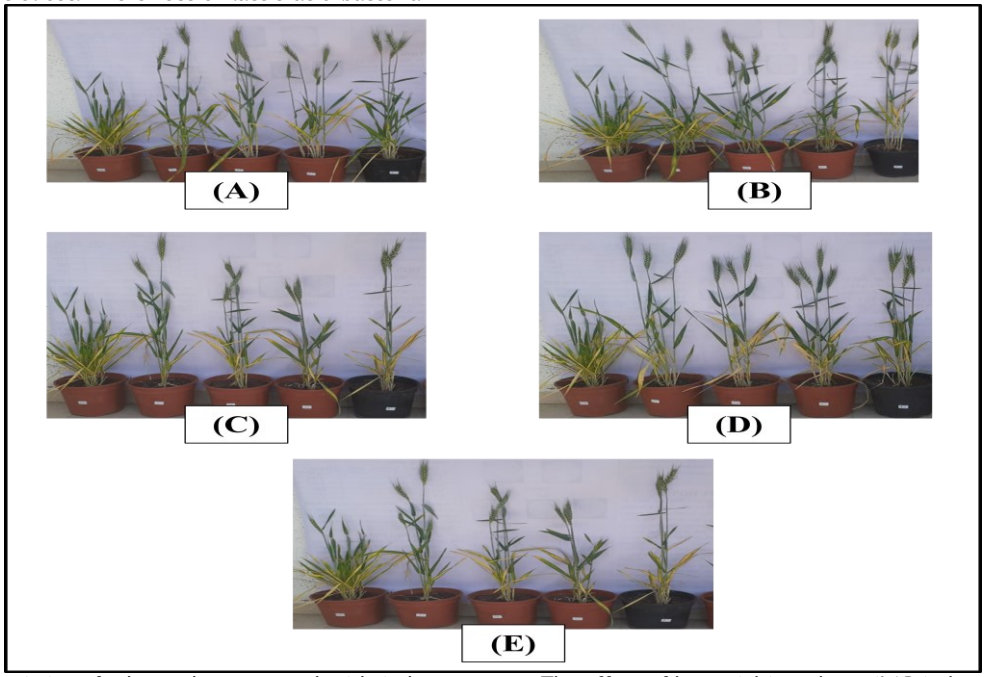

Fig. No.9: Characteristics of wheat plants treated with isolate C1, C2, B1 and Mix culture in pot experiment **(A)** Plant growth of set-1 with control **(B)** Plant growth of set-2 with control **(C)** Plant growth of set-3 with control **(D)** Plant growth of set-4 with control **(E)** Plant growth of set-5 with control.

Plant Height of Wheat Plants

The effect of bacterial inoculants (LAB isolates C1, C2, B1, and a mixed inoculum) on wheat plant height was assessed and compared to a non-inoculated control set of wheat. Wheat plants treated with isolate C1 showed the highest average height (61 cm), followed by the mixed inoculum group (60.4 cm). C2 and B1-treated plants also exhibited increased height as 57 cm

and 57.8 cm respectively. The graphical presentation of results showed that bacterial inoculation significantly enhanced plant growth when compared to the control group with average height is 35cm (Fig.No.10). Statistical analysis using the Mann-Whitney U test indicated that the differences between inoculated and control plants were notable but not statistically significant at $p < 0.05$. These findings suggest that inoculation with beneficial bacterial isolates, particularly C1, and the mixed culture enhance wheat plant height when compares with control set (Table No.7).

Table No.7: Average plant height in (cm) with statistical analysis (Mann-Whitney U test)

Treatment group	Average plant height in (cm)	U-Statistics	p-Value	Significance ($p < 0.05$)
Control	35.0	-	-	-
C1	61.0	0.0	0.0786	No
C2	57.0	0.0	0.0759	No
B1	57.8	0.0	0.0786	No
Mix	60.4	0.0	0.0952	No

Spike Height of Wheat Plants

The measurements of spike height showed considerable differences between treated set and the control set. The set treated with mixture of LAB isolates recorded the maximum spike height (9.6 cm), followed by C2 that recorded a spike height of 7.2 cm and B1 recorded 8.6 cm spike height all showed a significant increase in spike heights while isolate C1 recorded 6.4 cm height of spike with no significant increase (Fig No.10). Statistical analysis by the Mann-Whitney U test revealed that C2, B1, and Mix treatments had a significant spike height increase ($p < 0.05$), with the highest improvement in the Mix group over the control as shown in (Table No.8).

Table No.8: Average spike height in (cm) with statistical analysis (Mann-Whitney U test)

Treatment group of LAB isolates	Average spike height in (cm)	U-Statistics	p-Value	Significance ($p < 0.05$)
Control	6.0	-	-	-
C1	6.4	1.5	0.0214	No
C2	7.2	0.0	0.0088	Yes
B1	8.6	0.0	0.0099	Yes
Mix	9.6	0.0	0.0099	Yes

Number of Spikes of Wheat Plants

The control set had the least spikes, averaging 1 spike per plant, while set treated with C2 isolate produced the highest, averaging 4 spikes per plant, followed by isolate B1 with an average of 3.8 spikes, while set treated with C1 and the mixed inoculum showed average 3.4 spikes per plant (in Fig.No.10.) The Mann-Whitney U test statistical analysis showed increase in no. of spikes in all treated groups ($p < 0.05$) with the significant difference between control set and C2treated set as shown in (Table No.9).

Table No.9: No. of spikes per plant with statistical analysis (Mann-Whitney U test)

Treatment group	No. of spikes per plants	U-Statistics	p-Value	Significance ($p < 0.05$)
Control	1.0	-	-	-
C1	3.4	0.0	0.0097	Yes
C2	4.0	0.0	0.0097	Yes
B1	3.8	0.0	0.0097	Yes
Mix	3.4	0.0	0.0097	Yes

Yield of Wheat Plants

The study evaluated the effect of bacterial inoculation on the yield of wheat plant in term of number of grains produced per plant across different treatment groups. The control plants had the lowest average grain count (46 grains per plant), while the treated set with C1 isolate exhibited the highest grain production (52 grains per plant). The mixed inoculum followed closely with a total average of 51.4 grains per plant, while C2 and B1 isolates resulted in 49.8 and 48.8 grains per plant respectively (Fig no.10). Statistical analysis using the Mann-Whitney U test confirmed a significant increase in grain production across all treated groups ($p < 0.05$), with C1 showing the largest difference from the control as shown in (Table no. 10).

Table No. 10: No. of grains per plant with statistical analysis (Mann-Whitney U test)

Treatment group	No. of grains per plants	U-Statistics	p-Value	Significance ($p < 0.05$)
Control	46.0	-	-	-
C1	52.0	0.0	0.0106	Yes
C2	49.8	0.0	0.0101	Yes
B1	48.8	0.0	0.0109	Yes
Mix	51.4	0.0	0.0109	Yes

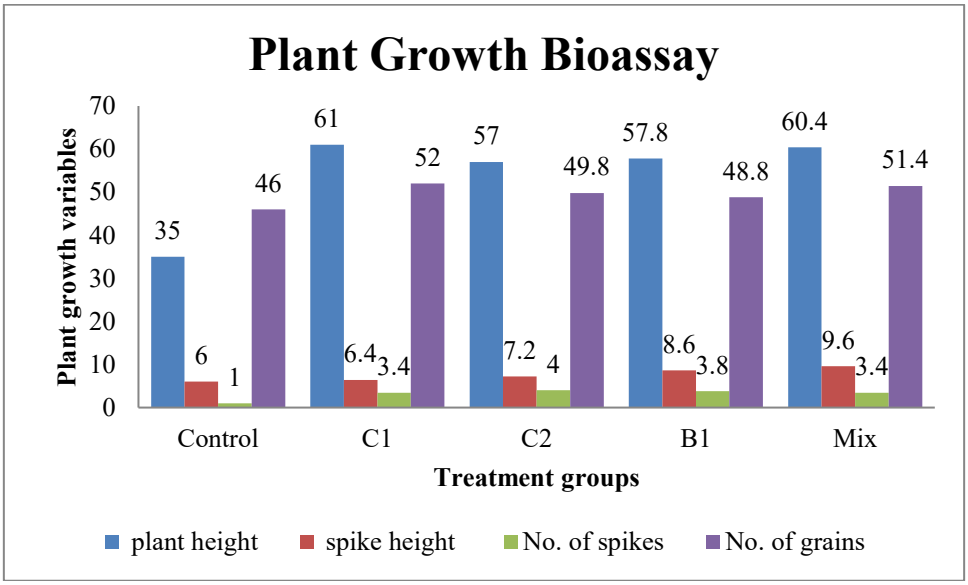

Fig. No.10: Evaluation of growth parameters of wheat plants treated with LAB isolates C1, C2, and B1 in comparison with control set (without treatment)

DISCUSSION

Sustainable agricultural development is promoted by the symbiotic link between plants and microorganisms. Gupta R. et al.,2013 reported use of plant growth-promoting microbes (PGPM) as bioinoculants to replace chemical fertilizers for agricultural sustainability [16]. In current study, the preparation of an efficient bioinoculant comprising indigenous nitrogen-fixing and phosphate-solubilizing probiotic organisms that provides a promising approach to enhance soil fertility and promote the growth of wheat (*Triticum aestivum L.*) was carried out. Through a systematic approach, three bacterial strains—*Lactococcus cremoris subsp. tructae* (C1), *Streptococcus lactarius* (C2), and *Lacticaseibacillus sharpeae* (B1) were isolated from curd and buttermilk samples under microaerophilic condition and thoroughly characterized for their plant growth-promoting properties. There are some previous reports that support the findings of present studies. According to Dadook, M. et al., (2014), plant growth promoting organisms and their role in sustainability were strongly identified and studied by their morphological, biochemical, and molecular characterizations [8]. In particular, these strains demonstrated notable activity related to nitrogen-fixing and phosphate-solubilizing, which are essential for improving soil nutrient availability. These microbial activities directly support plants capacity to absorb nutrients, which promotes better growth and development.

In earlier research by Li, H et al. (2023), the ability of lactic acid bacteria (LAB) strain *Limosilactobacillus spp.* LF-17 to solubilize phosphate was utilized for the preparation of bacterial agent and applied for promoting the growth of maize seedlings [28]. The phosphate-solubilizing capabilities of the isolated strains were evaluated using Pikovasky's agar medium. The present study reported *Lacticaseibacillus sharpeae* that exhibited the ability to solubilize phosphate.

In previous research by Higdon et al. (2020), total 23 *Lactococci spp.* associated with the plants mucilaginous aerial root were identified and characterized for their capability of biological nitrogen fixation (BNF) [17]. It has previously been documented (N. Nohwar et al., 2019) that nitrogen-fixing bacteria can be isolated using yeast extract mannitol agar [31]. In present study, all three isolates (*Lactococcus cremoris subsp. tructae* (C1), *Streptococcus lactarius* (C2), and *Lacticaseibacillus sharpeae* (B1) were found to be able to grow on nitrogen-free medium demonstrating their Nitrogen fixation ability.

The Kjeldahl technique, as outlined by R.K. Trivedy et al. (1984) was used to screen isolates in vitro, based on their fixing nitrogen efficiency [36]. The current study estimated the amount of nitrogen fixed by lactic acid bacteria by using Kjeldahl method and evidenced that all three LAB isolates were able to fix significant amount of nitrogen. These findings were supported by study on the nitrogen fixation capabilities of the isolates that ranges from 6.58 to 14.86 mg N/ml, demonstrating significant variance across them [12].

The plant-based bioassay, demonstrated by (Mingma Thundu Sherpa et al.,2021), showcased significant enhancements in various growth parameters of plants [41]. The present study reported the on-field effectiveness of bioinoculant on wheat plants. The efficiency of these isolates was assessed to promote wheat plant growth in pot assay experiment. The considerable enhancement in plant growth standards like plant height, spike height, number of spikes, and number of grains that resulted after treatment with LAB isolates demonstrated the efficiency of isolates as growth promoter.

The statistical analysis by Mann-Whitney U test emphasized the significant differences between each of treated group with LAB isolates and their respective control groups across multiple standards including plant height, spike height, number of spikes, and number of grains, suggesting that treatment with isolates had the most substantial impact on the measured parameters compared to the control. In previous investigations by Da Costa et al. (2012) performed the Mann-Whitney U test to compare Plant growth promoting traits under the same and different fertilization conditions [10].

CONCLUSION

The present study reported three LAB species; *Lactococcus cremorissubsp. tructae* (C1), *Streptococcus lactarius*(C2), *Lacticaseibacillus sharpeae*(B1) isolated from curd and buttermilk samples respectively. All three LAB isolates have potential to fix atmospheric nitrogen while *Lacticaseibacillus sharpeae* was found to solubilize phosphate. These abilities are crucial for improving soil fertility and are responsible for enhancement of crop yield. The application of these isolates as a bioinoculant had positive impacts on different growth parameters of wheat. All these findings lay the groundwork for the development of efficient bioinoculant mixtures that can significantly raise crop yields in an environment-friendly way. Such bioinoculants can help preserve soil health and reduce reliance on synthetic fertilizers. The study highlights the plant growth promoting potential of bioinoculant formulated from probiotic microbes isolated from curd and buttermilk samples to improve soil fertility and encourage environmentally friendly farming methods by replacing chemical fertilizers and pesticide and maintaining food security and agricultural productivity.

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