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Biofertilizer based on *Agrobacterium* as a key to food security

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ABSTRACT

Background: The concept of food security is contingent upon the fulfillment of two fundamental requirements: physiological and socioeconomic. The former comprises providing sustenance that is both safeguarded and enhanced in line with people's nutritional needs and priorities, whereas the latter includes the mechanisms by which these needs are supplied. The objective is to ensure a state of robust and healthy well-being. As a sustainable solution, biofertilizers play a pivotal role in addressing the challenges of food security. Biofertilizers represent a cost-effective and environmentally friendly solution, with the potential to enhance soil productivity through nitrogen fixation. They also enhance crop yield by increasing the concentration of nutrients. Access to functional foods derived from biofertilizer-enhanced yields may contribute to improved dietary diversification and overall well-being.

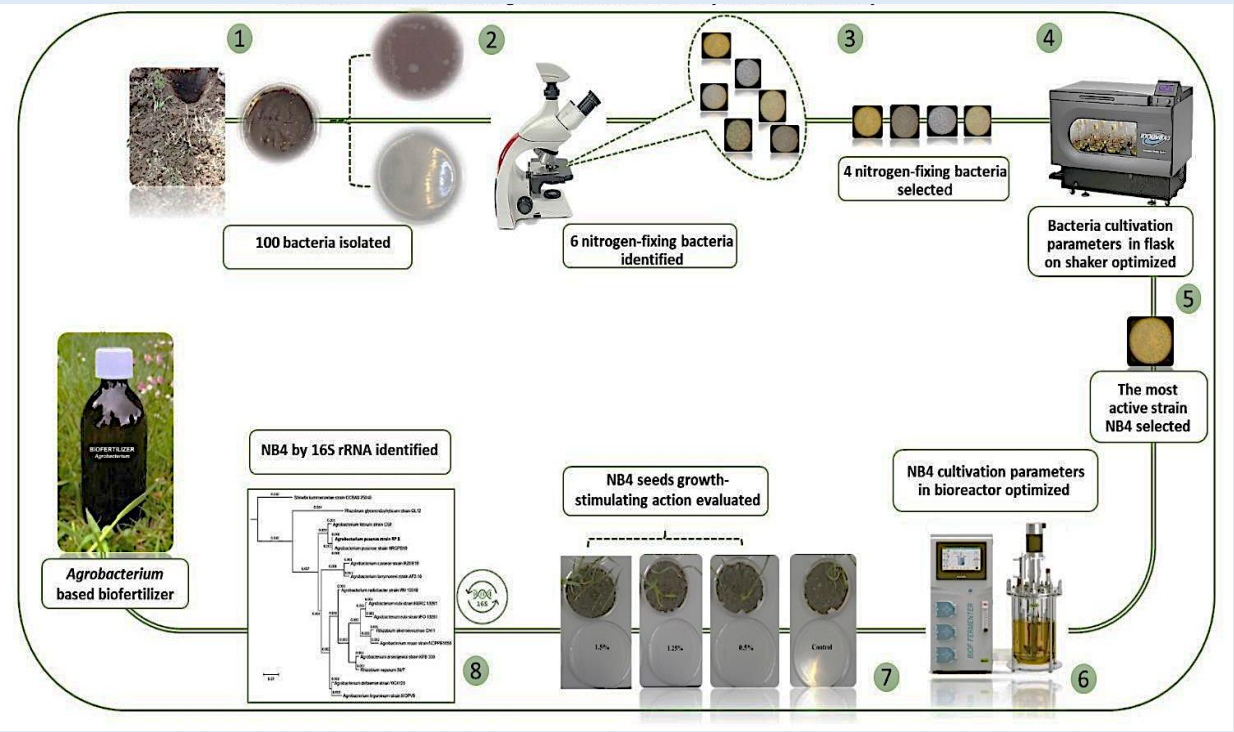
Objective: The objective is to isolate and screen the most active nitrogen-fixing bacterium as a basis to a new biofertilizer.

Methods: The isolation of nitrogen-fixing bacteria was conducted using the soil sowing technique. The selection of nitrogen-fixing bacteria was based on their morphophysiological characteristics. Bacterial growth was evaluated by viable cell count. Cultivation of the selected bacterium occurred in both flasks (on a shaker) and a bioreactor to compare growth conditions. The stimulatory effect of the selected bacterium on seed germination was assessed based on the final germination rate. The selected bacterium identified through molecular taxonomy.

Results: A total of 100 pure cultures were isolated, from which nitrogen-fixing bacteria were selected. The cultivation conditions for these bacteria were optimized regarding pH, temperature and process duration in a flask on a shaker. Strain NB4 exhibited the most favorable development under optimal settings. Additionally, cultivation parameters for this strain were optimized in a laboratory bioreactor. Notably, a bacterial liquid culture water solution comprising 1.5% strain NB4 facilitated complete germination of wheat (*Triticum aestivum* L.), seeds and produced high-quality sprouts. The 16S rRNA gene sequence of strain NB4 was submitted to the GenBank database under accession number MT670424.1, identifying it as *Agrobacterium Pusense* RP 1.

Conclusion: This research aimed to develop an effective biofertilizer to tackle the pressing issue of food security. The project's cornerstone was *Agrobacterium pusense* RP 1, nitrogen-fixing strain isolated and identified through rigorous research.

Keywords: Isolation and identification of nitrogen-fixing bacteria, cultivation in bioreactor, germination of wheat seeds, *Agrobacterium pusense.*



Graphical Abstract: Biofertilizer based on Agrobacterium as a key to food security

INTRODUCTION

Currently, there is a global consensus on the importance of food purity due to its ecological impact. The global market demand for organic food is increasing, along with requirements for food origins and safety [1].

Food security is defined as accessing organic food that meets nutritional requirements for food origins and safety. Nutrition insecurity arises when individuals lack the resources and knowledge to access safe, nutritious food, hindering their ability to maintain well-being. This

can result from nutrient deficiencies or inefficient utilization of reserves at the individual level. Continuous cultivation and chemical fertilizer application for uninterrupted crops may contribute to food security crises. This has led to decreased soil fertility, rendering most produce unsafe for human consumption due to chemical residues [2-6].

The widespread use of chemical fertilizers has contaminated soil, atmosphere, water sources, and even food products. The contamination leads to the accumulation of nitrates in plants, forming carcinogenic nitrosamines. Consequently, chemical fertilizer use poses significant environmental concerns. Biotechnological methods offer a promising solution through the development and production of biofertilizers. In agriculture, demand for biofertilizers-biologically based alternatives to chemical and synthetic fertilizers-is growing [2]. As a crucial component of organic farming, biofertilizers maintain soil fertility, mitigating adverse environmental and life-supporting impacts on the planet [4-6].

The utilization of biofertilizers represents an environmentally conscious approach to sustainable agricultural practices, as evidenced by the literature [7, 8]. As the global population continues to grow, demand for higher food production increases. Biofertilizers have the potential to enhance food production rates while ensuring farm product safety for consumers. Consequently, biofertilizers are an optimal alternative for producing safer crops and advancing global food security [1, 6-13].

Biofertilizers are frequently used to augment bacterial activity, increasing nutrient availability for plant absorption. Their utilization enhances soil fertility by facilitating atmospheric nitrogen fixation and insoluble phosphate solubilization, stimulating plant growth. By exploiting natural nutrient mobilization processes, biofertilizers considerably improve soil fertility and crop yield [6]. The biofertilizer market is expected to experience a 14.0% compound annual growth rate (2015-2020), reaching USD 1.88 billion by 2025. Due to stringent regulations governing chemical fertilizer use, biofertilizers have become the preferred choice in Europe and Latin America.

Biofertilizers can substantially impact sustainable agriculture's economic growth, environmental sustainability, and human well-being. Advancing organic farming is crucial in addressing interconnected societal health, food security, and environmental challenges. By prioritizing food security, organic farming development can play a pivotal role in understanding and addressing these complex issues. This study aims to isolate and select the most active nitrogen-fixing bacterium as the foundation for a novel biofertilizer, ensuring global food security.

MATERIALS AND METHOD

Media: (1) Nutrient agar (g/l): Beef extract – 11.0, peptone – 10.0, NaCl – 5.0, agar – 15.0, distilled water – 1 l, pH – 7.0-7.2. (2) Ashby's agar (g/l): Sucrose – 20.0, K_2HPO_4 – 0.2, $MgSO_4 \times 7H_2O$ – 0.2, NaCl – 0.2, K_2SO_4 – 0.1, agar – 15.0, distilled water – 1 l, pH – 7.0. (3) Bean agar (g/l): Sucrose – 2.0, K_2HPO_4 – 1.0, $MgSO_4 \times 7H_2O$ – 0.3, agar – 15.0, bean broth – 1 l, pH – 7.0.(4) Bean liquid medium (g/l): Sucrose – 2.0, K_2HPO_4 – 1.0, $MgSO_4 \times 7H_2O$ – 0.3, bean broth – 1 l, pH – 5.0-8.0.

Isolation, screening, and identification: Soil sampling was conducted in Armenia at various soil horizons, to a depth of 10–15 cm. Sampling was done using aseptic techniques with sterile flasks and test tubes. The samples were then purified to remove broken stones, small stones, mechanical particles, and root pieces. Nitrogen-fixing bacteria were isolated using soil grain sowing and

soil dilution techniques, as described by [2]. For identification, cultures were incubated on selective media (2 and 3). The screening was conducted according to "Bergey's Manual of Determinative Bacteriology" criteria.

Microscopic assays: Morphological observation of pure cell cultures was made following Avetisova et al.'s methodology [14], using a Leica DM500 trinocular microscope (×1000) with a digital camera (Leica EC3 microsystem, ×10) for visualization.

Viable cell determination: Nitrogen-fixing bacteria viability was assessed by colony-forming unit (CFU) counting. Serial dilutions were plated on medium (3) and incubated at 30°C for 48h. Colonies were counted using a Colony Star counter, and CFU/mL were calculated using: The colonies were counted using colony counter ColonyStar (Funke Gerber). The CFU in 1 ml sample was calculated by the formula:

$$CFU = \frac{N * 10^{D}}{V}$$

Where N is the number of bacterial colonies, D is the dilution, and V is the volume, of plated sample.

Cultivation of nitrogen-fixing bacteria in flasks: The submerged cultivation of selected nitrogen-fixing bacteria was carried out in Erlenmeyer flasks containing medium (4) on an Innova 43 Shaker (New Brunswick Scientific) at200-250 rpm and 26-32°C for 24-72 hours.

Cultivation of strain NB4 in a bioreactor: Strain NB4 was cultivated in a LabFreez bioreactor with automated monitoring of temperature, dissolved oxygen, pH, pCO2, pO2, air consumption, and other parameters. Physiological activity was assessed by calculating the average specific growth rate (h-1).

$$\mu = \frac{ln\,(x/x_o)}{t}$$

Where x is the quantity of biomass after the culture, x_o is the amount of biomass at the start of cultivation, and t is the duration of cultivation.

Seeds germination: To investigate the germination and growth-stimulating properties of strain NB4, its impact on wheat seeds (*Triticum aestivum* L.) was studied in a laboratory setting. The study was conducted in soil-filled Petri dishes, with eight seeds planted in 50 g of soil per dish. The Petri dishes were treated with aqueous solutions of bacterial liquid culture (BLC) at concentrations of 0.5, 1.0, 1.25, and 1.5% (w/v). The soil treated with tap water served as a control. The final germination percentage (FGP) was estimated using the formula:

$$FGP = \left(\frac{T_s}{I_s}\right) * 100\%$$

Where Ts is the total number of seeds germinated at the end of the trial and is the number of initial seeds.

Molecular taxonomic identification of strain NB4: Strain NB4's taxonomic identification was performed through DNA extraction, 16S rRNA gene PCR amplification, and = sequencing by Keleshyan et al. [15]. The PCR product sequencing was outsourced to Macrogen, South Korea. A phylogenetic tree was constructed using MEGA 7.0 software, employing the neighbor-joining (NJ) tree method.

RESULTS AND DISCUSSION

Isolation, and screening of nitrogen-fixing bacteria: The isolation scheme for nitrogen-fixing bacteria is illustrated in Figure 1.

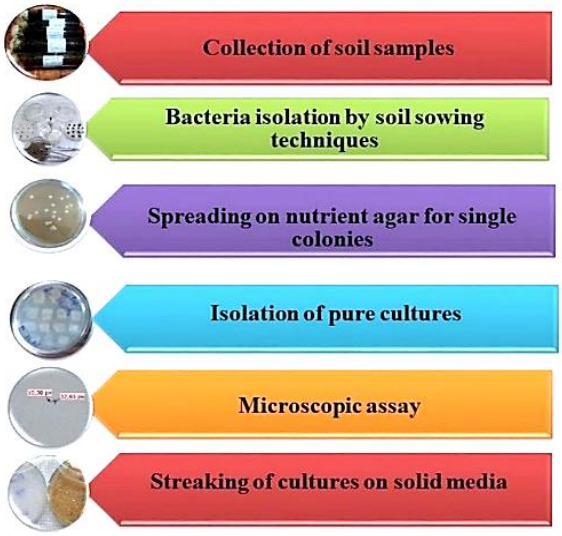


Figure 1. Isolation steps of nitrogen-fixing bacteria

Stored cultures from five soil samples were obtained to isolate and screen nitrogen-fixing bacteria. A total of 100 colonies were selected for incubation and pure culture isolation. *Azotobacter* was identified by selecting colonies with distinct characteristics: white, opaque, mucous, creasy texture, and color changes to yellow-greenish, pink, brown, or black. Symbiotic bacteria were identified by selecting white jelly-like colonies.

Six selected cultures exhibited morphophysiological characteristics like free-living and symbiotic nitrogen-fixing bacteria. Microscopic analysis of the selected cultures revealed the presence of relatively large cells, 1-2 µm in diameter, with oval, rod-shaped, or spherical morphology.

Microscopic examination revealed diverse cellular arrangements, including single pairs, irregular clusters, and occasional chains of varying lengths. Additionally, resting forms (cysts) were observed in some cases, whereas spore forms were absent. Both motile and non-motile cells were present. These findings suggest that some isolated cultures exhibit morphophysiological characteristics consistent with nitrogen-fixing bacteria.

The generic specificity of the selected strains was determined by observing their growth on selective media designed for nitrogen-fixing bacteria. Growth on agar media revealed that two of the six chosen strains grew poorly on the selective medium, whereas the remaining four cultures thrived, particularly on bean agar. Colonies formed on the bean agar were colorless and semi-transparent, with mucous-like. In bean broth, numerous white, mucous streaks were observed. By the third day, white, spherical, conspicuous, mucous colonies measuring 1-2mm in diameter and formed.

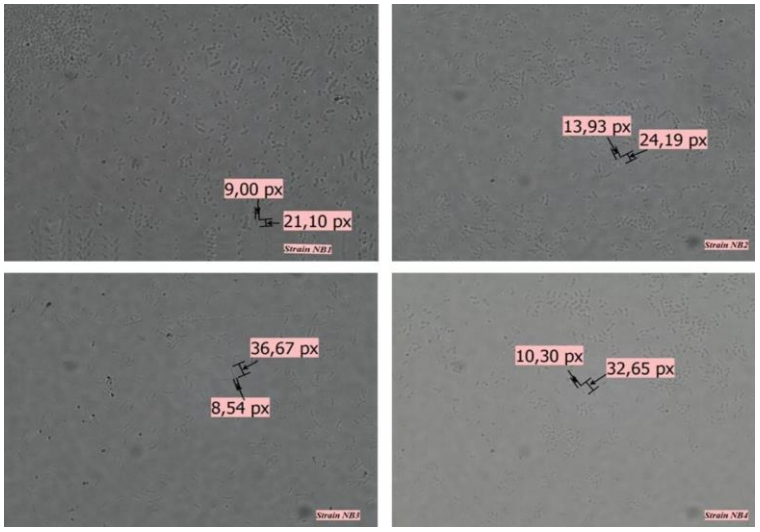


Figure 2. Micrographs of selected nitrogen-fixing bacteria (× 10 000; 1 px = 263.6 µm).

Microscopic analysis showed that the cultured cells exhibited rod-like morphology with rounded ends, developing into banded rods with straight and branched forms as they matured. The cells grew optimally on bean agar, a selective medium for nitrogen-fixing bacteria, indicating their ability to fix nitrogen as indicated in Figure 2.

Nitrogen-fixing bacteria cultivation in flasks on shaker: The effects of pH, temperature, and cultivation duration on nitrogen-fixing bacteria growth were assessed in three separate experiments. Each factor varied while keeping others constant (pH: 30°C, 220 rpm, 48h; temperature: pH 7.0, 220 rpm, 48h; duration: 30°C, 220 rpm, pH 7.0), with results presented in Table 1.

Table 1. Comparative data on nitrogen-fixing bacteria cultivation.

Strain	pH	Viable cells (CFU/ml)	Temperature (°C)	Viable cells (CFU/ml)	Cultivation duration (h)	Viable cells (CFU/ml)
NB1	5.0	1.4×10^7	26	2.9×10^7	18	1.8×10^7
	6.0	2.8×10^8	28	1.8×10^8	24	4.3×10^8
	7.0	5.7×10^8	30	5.9×10^8	48	5.9×10^8
	8.0	1.7×10^7	32	3.5×10^8	72	3.9×10^8
NB2	5.0	1.1×10^7	26	2.1×10^7	18	1.1×10^7
	6.0	1.8×10^8	28	3.0×10^8	24	4.7×10^8
	7.0	4.8×10^8	30	6.7×10^8	48	5.7×10^8
	8.0	2.0×10^7	32	5.5×10^8	72	4.0×10^8
NB3	5.0	1.3×10^7	26	2.1×10^7	18	1.9×10^7
	6.0	2.1×10^8	28	2.0×10^8	24	3.9×10^8
	7.0	6.9×10^8	30	6.8×10^8	48	4.8×10^8
	8.0	2.1×10^7	32	5.5×10^8	72	7.7×10^8
NB4	5.0	1.6×10^7	26	3.2×10^7	18	5.5×10^7
	6.0	2.5×10^8	28	5.6×10^8	24	7.8×10^8
	7.0	7.8×10^8	30	7.8×10^8	48	9.9×10^8
	8.0	3.5×10^7	32	7.0×10^8	72	8.0×10^8

The selected nitrogen-fixing strains exhibited optimal growth at pH 7.0. Although certain nitrogen-fixing bacteria can grow across a wide pH range, their growth is more pronounced at neutral pH [13]. The optimal temperature for strain growth was found to be 30°C; although, some nitrogen-fixers demonstrated growth capabilities to grow at 34°C. Notably, the ideal cultivation conditions for the selected strains were determined to be pH 7.0, 30°C, and a 48-hour duration. These optimal growth conditions are consistent with those reported for other nitrogen-fixing bacteria [16].

As shown in Table 1, strain NB4 exhibited the highest under the selected cultivation conditions. Notably, NB4 demonstrated the most significant improvement from the previous optimization stage in terms of aeration impact. Consequently, this strain was selected for further investigation. Cultivation was conducted under optimized conditions (pH 7.0, 30°C, 220 rpm), and on a shaker with increased revolutions per minute (230 and 250 rpm). The impact of aeration on microbial growth was assessed by quantifying viable cells.

To explore potential reductions in incubation time at 30°C and elevated aeration levels, CFU/ml were measured at both 24 and 48 hours. Figure 3 graphically illustrates that an increase in aeration levels significantly accelerates growth and decreases the time required to achieve the highest viable cell count in specific conditions.

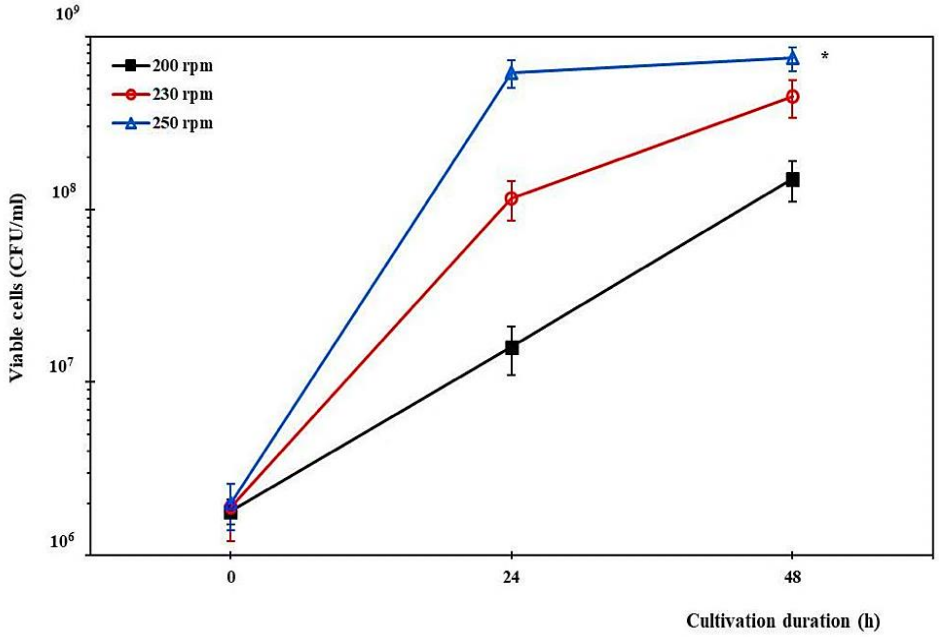


Figure 3. NB4 growth in different aeration conditions. Data are shown as mean ± SD (n=2). Figure 3 demonstrates that the highest aeration level intensified the growth of NB4, leading to a more cost-effective outcome.

Bacterium NB4 cultivation in bioreactor BIOF-10L: Following optimization of aeration, the technological parameters for strain NB4 cultivation in a bioreactor were further refined. Medium pH significantly influences bacterial cultivation. To investigate this, the impact of pH on strain NB4 growth was examined. The optimal pH value was determined by culturing the strain in media with initial pH values ranging from 5.0 to 8.0, in 0.5 increments. pH levels remained stable during the development phase, with 35% hydrochloric acid used as the titrant. Results are presented in Table.

Table 2. Cultivation data on culture NB4 in a bioreactor.

pH	Cultivation duration (h)	Viable cells (CFU/ml)	Temperature (^{0}C)	Average specific growth rate (h^{-1})	Time of achieving average specific growth rate(h)	Cultivation duration (h)	Viable cells (CFU/ml)
5.0	24	1.2×10^4	24	0.46	36	46	2.2×10^7
5.5	40	2.0×10^6	26	0.81	16	20	5.2×10^8
6.0	**20**	**2.2×10^9**	**28**	**0.70**	**18**	**22**	**4.0×10^9**
6.5	**18**	**3.8×10^9**	**30**	**0.66**	**24**	**28**	**4.5×10^9**
7.0	28	7.2×10^8	32	0.58	26	30	1.2×10^8
7.5	38	1.1×10^8	34	0.50	34	40	6.3×10^7
8.0	24	1.8×10^6	36	0.44	38	52	7.4×10^6

As shown in the table, the optimal method for cultivating the NB4 culture in a bioreactor involves maintaining an initial pH of 6.0-6.5, which yields the highest CFU/ml. Deviating from this pH range reduces viable cell counts and prolongs the procedure.

Bacterium effects of temperature on strain NB4 growth and activity: To determine the optimal temperature of cultivating strain NB4, experiments were conducted in the same bioreactor, with temperatures ranging from 24 to 36 °C in 2 °C increments (Table 2). The data indicates that the highest specific growth rate was achieved between 18-24 hours of cultivation at 28-30°C. Temperature alterations, whether they increased or decreased, adversely affect cultivation indices, leading to growth inhibition and reduced culture activity.

The degree of medium oxygen saturation significantly influences the growth and development of aerobic bacteria. Moreover, oxygen levels impact biological nitrogen fixation and symbiotic partnership with plants in nitrogen-fixing bacteria [17].

Given that the selected nitrogen-fixing strain belongs to this group, we investigated the effect of oxygen solubility and mass transfer coefficient on the cultivation process. Experiments were conducted throughout the development phase using our technology, maintaining temperatures between 28-30 °C and pH levels of 6.0-6.5. Oxygen dissolution rates varied from 1.0-5.0 $g0_2$/ l per hour. We determined the time required to achieve average culture-specific growth rates and the duration for achieving the highest viable cell counts under specific conditions. Results are presented in Table 3. As evident from Table 3, optimal results were achieved at an oxygen dissolution rate of 2.0 $g0_2$ /l per hour. Deviations from this parameter, either higher or lower, adversely affected the process.

Table 3. Dependence of culture NB4 growth parameters on the rate of oxygen dissolution.

Rate of oxygen dissolution ($g0_2$ /l per h)	Average specific growth rate (h^{-1})	Time of achieving average specific growth rate (h)	Cultivation duration (h)	Viable cells (CFU/ml)
1.0	0.32	38	45	2.5×10^7
2.0	**0.78**	**20**	**24**	**4.4×10^9**
3.0	0.69	30	37	8.1×10^8
4.0	0.55	45	50	1.0×10^7
5.0	0.1	39	57	5.7×10^6

To analyze aeration effects, oxygen consumption was investigated throughout the procedure. Specifically, oxygen consumption rates were studied at various stages of the culture growth cycle to determine the mass transfer (Kla) required to meet the culture's oxygen demands. Preliminary analysis of the device's mass transfer characteristics employed static degassing with nitrogen under various aeration and stirring modes. Results showed that oxygen requirements during the stationary phase exceeded those during the logarithmic phase. Experimental studies revealed that maintaining a Kla value of 100-120 h-1 for the initial 12 hours followed by a reduction to 70-80 h-1, yielding optimal results. The aeration mode resulted in higher CFU/mL compared to maintaining a constant Kla value of 100-120 h-1 throughout its entirety.

Cultivating strain NB4 in a laboratory bioreactor optimized technological parameters (pH, T°C, KLa) and pH starting points along with stepwise adjustments to the mass transfer coefficient, reduces process duration and enhances overall efficiency. This, in turn, decreases energy consumption.

Seeds germination stimulating activity: The germination stimulating activity of nitrogen-fixing strain NB4 was investigated in the laboratory by examining the effects of various concentrations of bacterial liquid culture (BLC) on wheat seed germination. Wheat seeds were treated with 0.5, 1.0, 1.25, and 1.5% BLC water solutions, and their germination was observed over several days under controlled irrigation conditions. Results are presented in Figure 4.

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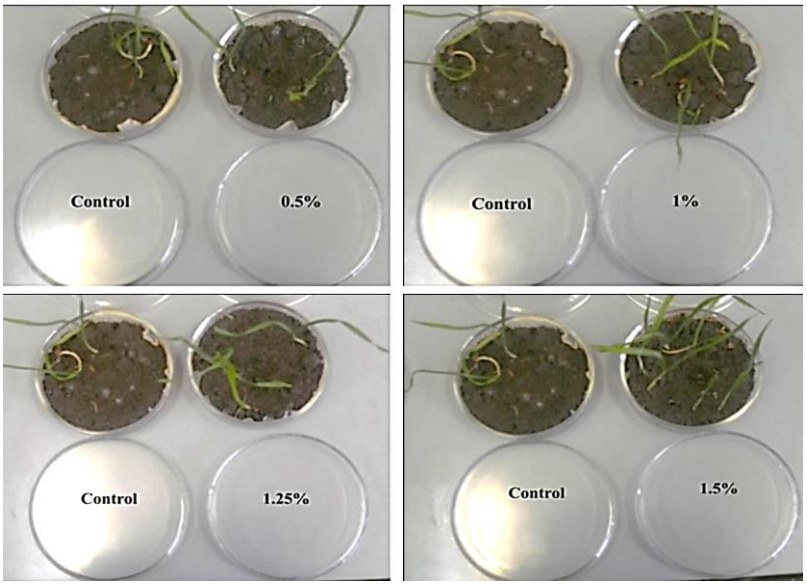

Figure 4. The effect of strain NB4 on wheat seed germination.

The emergence of seedlings occurred on the sixth day in the control and 0.5, 1.0, and 1.25% BLC treatments, whereas the 1.5% BLS treatment showed emergence on the fourth day. Notably, the 1.5% BLC solution resulted in 100% final germination percentage (FGP), compared to 25% in control. Moreover, wheat sprouts exhibited enhanced growth in terms of length and color saturation. Consistent with previous findings [18,19], plant growth-promoting rhizobacteria (PGPR) inoculation significantly enhanced wheat germination rates and vigor indices. Specifically, combined PGPR inoculation increased wheat plant height, tiller count, fresh weight, and dry weight. The results demonstrate that a 1.5% BLC solution of NB4 in laboratory conditions yields the highest germination rates, sprout length, and quality. However, further research is necessary to elucidate the specific mechanisms by which nitrogen-fixing bacteria NB4 influences plant development. Further research is necessary to determine the specific mechanisms by which nitrogen-fixing bacteria NB4 influences plant development.

Molecular identification of strain NB4: The 16S rRNA gene sequence is a widely used method for bacterial identification, allowing for the determination of conserved traits transmitted across generations. This gene region facilitates the identification of novel bacterial species and the analysis of phylogenetic relationships with other taxa. Bacterial classification is determined by similarity indices derived from 16S rRNA gene sequencing. Specifically, a bacterial strain is assigned to a genus if its similarity index falls within the 89-99% range. A strain is considered a distinct species if its similarity index exceeds 97%. Ultimately, a strain is defined as a unique entity when its similarity index reaches 100% [20].

Phylogenetic analysis revealed that strain NB4 shares a 100% similarity with Agrobacterium *pusense*

NRCPB10. A partial sequence 16S rRNA gene sequence of strain NB4 was deposited in the GenBank database as Agrobacterium pusense RP 1 (accession number MT670424.1). To elucidate phylogenetic relationships, a neighbor-joining (NJ) tree was constructed using GenBank and EzTaxon databases (Figure 5). This analysis confirmed that strain NB4 belongs to the same species as *Agrobacterium pusense.*

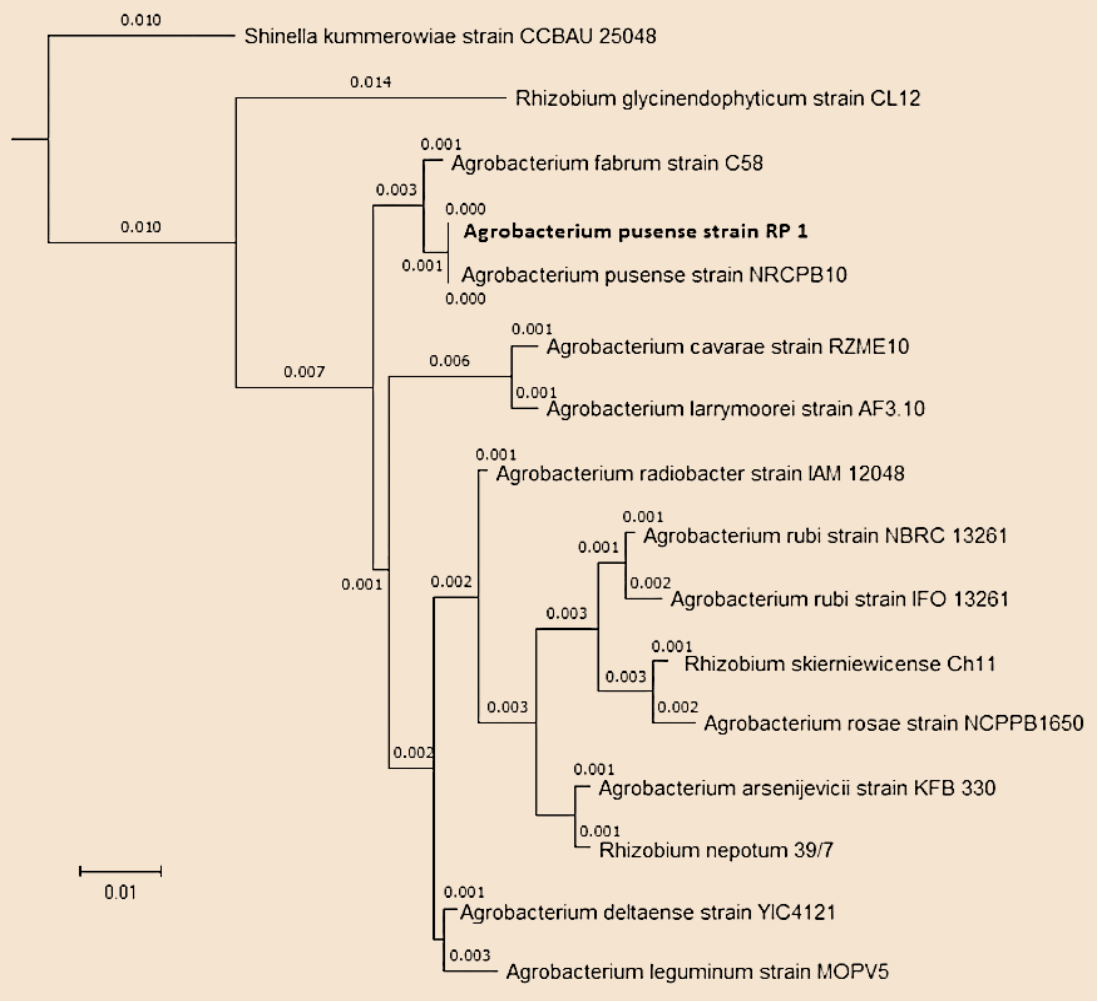

Figure 5. The phylogenetic topology of *A. pusense* RP 1 with the genus *Agrobacterium* used NJ.

CONCLUSION

Utilizing biofertilizers as an alternative to chemical fertilizers is crucial for ensuring food safety and security. Adopting these eco-friendly techniques will have a profound impact on the sustainability of agricultural practices, contributing to enhanced human well-being. This research lays the groundwork for developing an innovative biofertilizer, offering an economically viable and environmentally sustainable solution for advancing sustainable agriculture.

List of Abbreviation: CFU: colony-forming unit, BLC: bacterial liquid culture, FGP: final germination percentage, MEGA: molecular evolutionary genetic analysis, NJ: neighbor-joining, PGPR: plant growth-promoting *rhizobacteria.*

Competing interests: The authors confirmed that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Authors' contributions: GA: Supervision, investigation, methodology, writing – original draft preparation, writing – review and editing. LM: Investigation, methodology, writing – original draft preparation, writing – review and editing. VGh, GTs, ZhK, and SK: Investigation, methodology. AT: Investigation, writing – review and editing.

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