BIOINOCULANTS OF *AZOTOBACTER VINELANDII* NBIMCC 1619 - PHYSIOLOGICAL, KINETIC, AND TECHNOLOGICAL CHARACTERISTICS

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Received 27 July 2026
Accepted 30 August 2026 *DOI: 10.59957/jctm.v61.i6.2026.2*

ABSTRACT

Considerable attention is now being paid to environmentally friendly agricultural practices that reduce reliance on chemical inputs while maintaining high crop productivity and preserving soil health. Among the most promising sustainable approaches is the use of plant growth-promoting rhizobacteria (PGPR), which are essential components of sustainable agriculture and integrated nutrition management practices. Among them, bacteria of the genus Azotobacter have attracted scientific and commercial interest because they combine efficient atmospheric N_2 fixation with numerous plant growth-promoting (PGP) properties.

The present study focuses on Azotobacter vinelandii NBIMCC 1619 PGP characteristics and the formulation of a bioinoculant preparation. A systematic fermentation and processing strategy was developed under controlled conditions to address the key challenges of producing effective N_2-fixing PGPR bioinoculants. The biochemical profile of the strain revealed simultaneous expression of N_2 fixation, phytohormone biosynthesis, siderophore production, phosphate solubilization, and extracellular enzyme production. An optimization process resulted in the development of a nutritionally balanced, agriculturally relevant N-free medium and a cultivation regime with DO_2 of 30 %, a temperature of 29°C, and a pH of 7.1. A fed-batch strategy of continuous substrate feeding over a 12 h period was implemented to result in high biomass production and N_2-fixing performance of the bioinoculant. Its stability at 4°C and in freeze-dried form with cryoprotection was proved. Thus, the physiological, kinetic, and technological characteristics of A. vinelandii NBIMCC 1619 demonstrate its potential for large-scale production under economically satisfactory conditions as a bioinoculant to increase crop productivity.

Keywords: Azotobacter vinelandii, plant growth-promoting rhizobacteria (PGPR), biological nitrogen fixation, fed-batch cultivation, bioinoculant formulation, cryoprotection, sustainable agriculture.

INTRODUCTION

The security of agricultural production, which underpins sustainable economic and societal development, is one of the principal objectives of the United Nations 2030 Agenda for Sustainable Development, particularly Sustainable Development Goal 2 (SDG 2, Zero Hunger), which aims to end hunger, achieve food security, improve nutrition, and promote sustainable agriculture [1]. The increasing demand for agricultural products, together with the depletion of natural resources and the impacts of climate change, has strengthened the need for sustainable crop production systems [2]. Over the past decades, remarkable increases in agricultural productivity have largely been achieved through the intensive use of synthetic fertilizers and chemical pesticides [3]. Although these agrochemicals have substantially

increased crop yields, their prolonged and excessive use has resulted in numerous environmental problems, including soil acidification, nutrient imbalances, groundwater contamination, greenhouse gas emissions, biodiversity loss, and deterioration of soil microbial communities, thereby compromising long-term soil fertility and ecosystem sustainability [3, 4]. Consequently, considerable attention has been directed toward environmentally friendly agricultural practices that reduce dependence on chemical inputs while maintaining high crop productivity and preserving soil health [5].

Among the most promising sustainable approaches is the use of plant growth-promoting rhizobacteria (PGPR), a diverse group of beneficial microorganisms that colonize the rhizosphere and enhance plant growth through direct and indirect mechanisms [6]. These microorganisms establish beneficial interactions with plant roots, improving nutrient availability, stimulating plant metabolism, increasing tolerance to abiotic stresses, and suppressing phytopathogenic microorganisms, thus improving soil and plant health while reducing environmental pollution [7]. As a result, they have become essential components of sustainable agriculture and integrated nutrition management practices [8].

Various types of PGPR are recognized, some of which are marketed as biofertilizers. These include the genera *Azotobacter, Azosprillum, Bacillus, Burkholderia, Enterobacter, Klebsiella, Pseudomonas*, and *Serratia* [5]. Among free-living diazotrophs, bacteria of the genus *Azotobacter* have attracted scientific and commercial interest because they combine efficient atmospheric N_2 fixation with numerous plant growth-promoting properties [9]. *Azotobacter* spp. is abundant in agricultural soils and the rhizosphere of cereals, vegetables, legumes, and industrial crops. There, they contribute to nutrient cycling and maintenance of soil fertility [10]. In addition to N_2-fixation, *Azotobacter* spp. produce phytohormones, siderophores, vitamins, extracellular polysaccharides, ammonia, and enzymes involved in mineral nutrient mobilization, making them multifunctional microbial inoculants [11].

A versatile approach to increasing crop production under economically satisfactory conditions while maintaining the necessary quality is the formulation and large-scale production of *Azotobacter* inoculants. Although *Azotobacter* species have been extensively investigated as PGPR, information regarding optimization of large-scale cultivation processes and formulation technologies for individual industrial strains remains relatively limited. Therefore, detailed investigations of growth kinetics and nutrient utilization, physiological characteristics, in particular N_2-fixing activity, and storage stability are required to facilitate the commercial production of effective microbial inoculants [12, 13].

The present study aimed to characterize the plant growth-promoting (PGP) properties of *Azotobacter vinelandii* NBIMCC 1619 and to optimize the technological parameters (medium composition, dissolved oxygen regulation, substrate feeding strategies, cultivation kinetics, and final product stability) governing biomass production and N_2 fixation under batch and fed-batch cultivation.

EXPERIMENTAL

Bacterial strain and culture media

Azotobacter vinelandii NBIMCC 1619 strain was used in this study. The strain was maintained on Azotobacter medium II (NBIMCC catalogue https://nbimcc.org/www_2020/en/m_catalog.php?id=59). Four nutrient media that differ in their N-source were used to select the optimal one for the cultivation of the model strain, as follows:

A: Nitrogen-free medium (g L^{-1}) (Sucrose - 20.0, K_2HPO_4 - 1.0, KH_2PO_4 - 0.5, $MgSO_4$ - 0.5, Na_2MoO_4 - 0.005, NaCl - 0.5, $FeSO_4$ - 0.1, and $CaCO_3$ - 1);

B: A + organic N-source (g L^{-1}) (Fish hydrolysate (Nofima, Norway) - 5);

C: A + inorganic N-source (g L^{-1}) ($(NH_4)_2SO_4$ - 5);

D: A + organic + inorganic N-source (g L^{-1}) (Peptone - 1; Yeast extract - 1; $(NH_4)_2SO_4$ - 3)

In addition, the nitrogen-free medium A was further modified using Response Surface Methodology [14] (RSM, a second-order regression model for response prediction) to result in 3^2 variants (V1-V9) that differ in the concentrations of the C, P, and K sources (Table 1).

Cultivation conditions

Batch cultivation

The experimental strain was cultivated batch-wise at 29 ± 0.5°C, pH 7.1 ± 0.1 for 72 h on a rotary shaker and/or in a bioreactor (Applikon ADI 1030/1035, 6 L vessel) with a working volume of 2 L. The effect of dissolved oxygen (DO_2) on strain growth was assessed using aeration

Table 1. Nutrient media used in batch cultivation of *Azotobacter vinelandii* NBIMCC 1619.

	Sucrose, g L^{-1}	K_2HPO_4, g L^{-1}	KH_2PO_4, g L^{-1}	$MgSO_4$, g L^{-1}	Na_2MoO_4, g L^{-1}	NaCl, g L^{-1}	$FeSO_4$, g L^{-1}	$CaCO_3$, g L^{-1}
V1	10	0.5	0.25	0.5	0.005	0.5	0.1	1
V2	20	0.5	0.25	0.5	0.005	0.5	0.1	1
V3	40	0.5	0.25	0.5	0.005	0.5	0.1	1
V4	10	1.0	0.5	0.5	0.005	0.5	0.1	1
V5	20	1.0	0.5	0.5	0.005	0.5	0.1	1
V6	40	1.0	0.5	0.5	0.005	0.5	0.1	1
V7	10	2.0	1.0	0.5	0.005	0.5	0.1	1
V8	20	2.0	1.0	0.5	0.005	0.5	0.1	1
V9	40	2.0	1.0	0.5	0.005	0.5	0.1	1

regimens of 20 %, 30 %, 40 %, and 60 % DO_2. Culture growth monitoring was performed by measuring the cellular density (OD_{600nm}) and extracellular protein concentration ($A_{260}/_{280}$). Both measurements were performed on UV/Vis spectrophotometer VWR UV-1600PC, and the protein concentration $C_{protein}$ (mg mL^{-1}) was calculated by the formula $C_{protein}$ (mg mL^{-1}) = $(1.55*A_{280}) - (0.76*A_{260})$, where A_{280} and A_{260} are the absorptions measured at 280 nm and 260 nm, respectively. Biomass growth kinetics were evaluated using conventional unstructured kinetic models commonly applied in aerobic microbial fermentations [15], and the kinetic parameters specific growth rate (μ), biomass yield coefficient ($Y_{x/s}$), and doubling time (t_d) were calculated as described below [16, 17].

Fed-batch cultivation

Fed-batch cultivation was also performed in a bioreactor Applikon ADI 1030/1035 6 L vessel with a working volume of 2 L, in two experimental variants. In the Single-pulse Fed-Batch variant, the feeding solution (200 mL) was introduced into the bioreactor once, as a single pulse, during the exponential growth phase (18 h). In the Continuous Fed-Batch variant, the feeding solution was supplied continuously at a constant flow rate of 0.0167 L h^{-1} for 12 h (between the 18 h and 30 h of cultivation).

In both variants, the total amount of feeding solution was 200 mL, and the C-source concentration was 400 g L^{-1}. Process parameters (OD_{600nm}, $C_{protein}$ (mg mL^{-1}), DO_2 dynamics, and cultivation kinetics) were monitored throughout the fermentation process by the methods described in sections Batch cultivation and Biochemical analyses.

Upon completion of the fermentation process, the cultural broth was used for the analyses specified in Biochemical analyses.

Biochemical analyses

Assimilation characteristics and enzymatic activities

Assimilation characteristics (carbohydrate assimilation profiles) and enzymatic activities/ physiological properties of *A. vinelandii* NBIMCC 1619 were evaluated using the commercial identification systems of non-fastidious, non-enteric Gram-negative rods API 50 CH and API 20 NE (bioMérieux, France), following the manufacturer's instructions [18]. Results were recorded after incubation under the recommended conditions and interpreted according to the standardized evaluation criteria described by Humble et al., [19] and Donnelly [20].

Sucrose measurement

Residual sucrose concentration in the fermentation broths was determined by the colorimetric phenol - sulfuric acid method of Dubois et al. [21]. Samples were treated with phenol and concentrated sulfuric acid. The absorbance of the resulting yellow - orange complex was measured at 490 nm against a sucrose standard curve. The concentration was expressed as g L^{-1} [21].

Measurement of N_2-fixing capacity

The nitrogen-fixing capacity of the model culture was estimated spectrophotometrically following

Cordova-Rodriguez et al. [22]. Cultures were grown in the nitrogen-free medium (29 ± 0.5 °C, pH 7.1 ± 0.1) with bromothymol blue as a pH indicator. The alkalinisation accompanying atmospheric N_2 fixation shifts the medium colour towards blue. After 72 h of incubation, the amount of biologically fixed nitrogen was quantified from the absorbance measured at 610 nm against a calibration curve prepared with a standard nitrogen solution. The results were expressed as μg mL^{-1} [22].

Siderophore production

Siderophore production was checked *in vitro* following the method of Alexander and Zuberer using Chrome Azurol S (CAS) agar medium [23]. In this assay, Fe^{3+} forms a blue complex with the CAS dye, and siderophores secreted by the culture sequester the iron and release the free dye, producing an orange - yellow halo. The strain was spot inoculated onto CAS plates and incubated at 29 ± 0.5°C. After the incubation period (7 days), the development of an orange halo against the blue background was recorded as a positive result, with uninoculated petri dishes used as controls (Fig. 1A) [23].

Phosphate solubilization assay

The phosphate-solubilizing ability of *A. vinelandii* NBIMCC 1619 was evaluated on the NBRIP medium containing insoluble tricalcium phosphate as the sole phosphorus source, following Nautiyal [24]. The strain was spot-inoculated and incubated at 29 ± 0.5°C for a period of 5 days. Solubilization of the mineral phosphate was detected as a translucent clearing zone around the colony (Fig. 1B). The solubilization index was calculated as the ratio of the total diameter (colony plus halo) to the colony diameter [24].

Determination of essential phytohormones

The strain biosynthetic potential for essential phytohormones production was evaluated by HPLC-MS (qualitative and quantitative) analyses performed using the following chromatographic and spectrophotometric analytical devices and conditions: Q Exactive® hybrid quadrupole - Orbitrap® mass spectrometer (ThermoScientific Co, USA), equipped with a HESI® module (heated electrospray ionisation), TurboFlow® Ultra High-Performance Liquid Chromatography (UHPLC) system (ThermoScientific Co, USA) and HTC PAL® autosampler (CTC Analytics, Switzerland).

Kinetic parameters calculation

The kinetic parameters growth yield coefficient ($Y_{x/s}$), doubling time (t_d, h), specific growth rate (μ, h^{-1}), maximum specific growth rate (μ^{max}, h^{-1}), and lag phase duration (T_L) were calculated in compliance with the principles of microbial growth kinetics [16].

Long-term stability and survival tests

Storage of the bioinoculant formulation

The bioinoculant formulation (a liquid 48 h broth culture of *A. vinelandii* NBIMCC 1619) was stored at 4°C for 3 months and then tested for viability and long-term stability by viable cell count analysis. The concentration of viable cells was determined by plating of serially diluted samples in PBS (HIMEDIA M1866) and counting colony-forming units per mL (CFU mL^{-1}) on NBIMCC Medium 529 agar plates (https://nbimcc.org/www_2020/en/m_catalog.php?id=59).

Freeze-drying

The bioinoculant formulation was subjected to vacuum-assisted freeze-drying under controlled conditions, as follows: freezing to a plate temperature of -60°C and the product to -40°C for 2 h, primary drying at a plate temperature of 40°C under vacuum for 24 h; secondary drying upon reaching a product temperature of 25°C and a moisture content below 2 %. In some experiments, a cryoprotective agent (soy milk) at a final concentration of 3 % was added to the initial bioinoculant suspension before freezing. After lyophilization, the dried preparations were stored at 4°C for 12 months and periodically evaluated for cell viability and physiological activity following rehydration using viable cell count analysis (see section Storage of the bioinoculant formulation above).

Statistical analyses

All assays were performed in triplicate and repeated three times. Data were processed using the MS Excel inbuilt functions, and results are presented as means ± standard deviations (n = 3).

RESULTS AND DISCUSSION

A bioinoculant preparation of *A. vinelandii* NBIMCC 1619 has been formulated through a fermentation and processing strategy implementation under controlled

conditions that meet the scientific and technological challenges for the selection of effective N_2-fixing PGPR.

A. vinelandii NBIMCC 1619 PGP metabolic characteristics

A. vinelandii NBIMCC 1619 demonstrated a broad spectrum of plant growth-promoting (PGP) metabolic characteristics, as summarized in Table 2. The strain tested positive for glucose, mannitol, and K-gluconate assimilation, as well as for several enzymatic activities including β-glucosidase, oxidase, esterase (C4), esterase lipase (C8), leucine arylamidase, α-galactosidase, α-glucuronidase, and acid phosphatase.

N_2-fixing activity, measured under N-free conditions (29°C, pH 7.1, DO_2 30 %), ranged from 16.1 to 20.8 μg mL^{-1}. LC-MS/MS analysis confirmed the biosynthesis of several key phytohormones: Indole-3-acetic acid/ Indole-3-pyruvic acid (IAA/IPA) (4.07 / 5.33 ng mL^{-1}), gibberellins GA_7 (0.73 ng mL^{-1}) and GA_9 (1.02 ng mL^{-1}), 1-aminocyclopropane-1-carboxylic acid (ACC) (1551.39 ng mL^{-1}), Jasmonic acid (JA) (0.23 ng mL^{-1}), and Abscisic acid (ABA) (9.25 ng mL^{-1}). The strain also produced siderophores (Fig. 1A) and demonstrated phosphate-solubilizing activity (Fig. 1B).

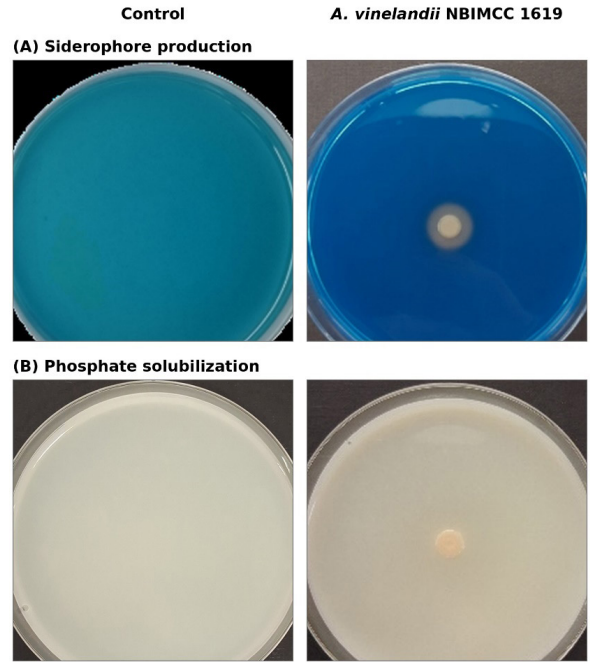


Fig. 1. *A. vinelandii* NBIMCC 1619 capacity to produce siderophores (A) and solubilize phosphates (B).

Table 2. *A. vinelandii* NBIMCC 1619 PGP metabolic characteristics.

No	Characteristics	Value
1	Glucose assimilation	Positive
2	Mannitol assimilation	Positive
3	K-gluconate assimilation	Positive
4	β-Glucosidase	Positive
5	Oxidase	Positive
6	Esterase (C4)	Positive
7	Esterase lipase (C8)	Positive
8	Leucine arylamidase	Positive
9	α-Galactosidase	Positive
10	α-Glucuronidase	Positive
11	Acid phosphatase	Positive
12	N_2-fix (N-free medium, T - 29°C, pH - 7.1, DO_2 - 30 %)	16.1 ± 0.21 to 20.8 ± 0.2, μg mL^{-1}
13	Gibberellin A7	0.73 ± 0.08, ng mL^{-1}
14	Gibberellin A9	1.02 ± 0.13, ng mL^{-1}
15	IAA/IPA	4.07 ± 0.71 / 5.33 ± 0.82, ng mL^{-1}
16	ACC	1551.39 ± 32.63, ng mL^{-1}
17	JA	0.23 ± 0.04, ng mL^{-1}
18	ABA	9.25 ± 1.02, ng mL^{-1}

The multifunctional PGP profile of *A. vinelandii* NBIMCC 1619 is consistent with what has been reported for agriculturally relevant *Azotobacter* strains [10, 25 - 27]. Effective biofertilizer candidates are generally expected to combine several complementary PGP traits, since plants are exposed to a variety of biotic and abiotic stresses throughout their growth cycle and no single mechanism is sufficient to consistently promote productivity across different field conditions [7, 8].

The ability to fix atmospheric N_2 is among the most agronomically valuable traits of the investigated strain. It converts atmospheric N_2 into plant-available ammonia via the nitrogenase complex, contributing to soil nitrogen cycling without requiring symbiotic associations with leguminous hosts [28, 29]. This makes it applicable across a broad range of crops and soil types [9].

The phytohormonal profile detected by LC-MS/MS reflects a diverse biosynthetic capacity. IAA is the predominant auxin produced by PGPR and regulates cell elongation, root initiation, and lateral root development, thereby increasing the absorptive surface area of the root system and improving water and nutrient uptake [30, 31]. The gibberellins GA_7 and GA_9, although detected at relatively low concentrations, are biologically active at nanomolar levels and may contribute to gibberellin-mediated stimulation of seed germination, stem elongation, and root biomass [32, 33]. The presence of ACC indicates bacterial involvement in ethylene-related signalling pathways; it is the direct precursor of ethylene, and its detection suggests active participation in plant hormone metabolism [34, 35]. ABA and JA, detected at lower concentrations, broaden the strain's growth-promoting potential by contributing to plant adaptation to drought and osmotic stress [36] and induced systemic resistance against pathogens [37], respectively.

Siderophore production provides an important indirect PGP mechanism. Iron is essential for respiration, DNA synthesis, and N_2 fixation, yet its bioavailability in aerobic soils is severely limited due to the low solubility of Fe^{3+} hydroxides [38]. Siderophores synthesized by *A. vinelandii* - including azotochelin, aminochelin, protochelin, and azotobactin - chelate Fe^{3+} with high affinity and make iron available to both the bacterium and surrounding plants [39 - 41]. Additionally, by competing with pathogens for available iron, siderophore-producing PGPR can limit the development of soil-borne phytopathogens [8].

Phosphate solubilization is another key trait that enhances the agricultural value of the strain. Phosphorus is the second most limiting macronutrient for plant growth after nitrogen, yet a large proportion of soil phosphorus exists in insoluble mineral forms unavailable for root uptake [42]. Phosphate-solubilizing bacteria release organic acids and phosphatases that convert insoluble phosphates into plant-available forms [43]. The combination of biological N_2 fixation and phosphate solubilization in a single strain is particularly valuable since these two nutrients most frequently co-limit crop productivity [5].

The extracellular enzyme activities detected (proteases, esterases, glucosidases, and acid phosphatase) further support the strain's suitability for rhizosphere colonization. These enzymes facilitate the hydrolytic breakdown of complex organic substrates, increase nutrient mineralization, and may suppress phytopathogenic fungi by degrading their cell-wall polymers [6, 8].

Taken together, the simultaneous expression of N_2 fixation, phytohormone biosynthesis, siderophore production, phosphate solubilization, and extracellular enzymes production places *A. vinelandii* NBIMCC 1619 among the multifunctional PGPR strains considered most promising for biofertilizer development [13, 44]. These characteristics provide a solid biological foundation for the subsequent optimization of cultivation conditions and formulation of a commercially viable bioinoculant.

Optimization of *A. vinelandii* NBIMCC 1619 fermentation process

The successful commercialisation of microbial biofertilizers is guaranteed by both the microorganism's beneficial properties and the efficient cultivation technologies that produce high concentrations of metabolically active cells. Fermentation parameters such as nutrient composition, dissolved oxygen concentration, pH, temperature, agitation, and feeding strategy substantially influence microbial growth kinetics, biomass productivity, N_2 fixation, and metabolite biosynthesis [15].

Nutrient media optimization

A two-step procedure was applied to optimize nutrient media formulations that support high growth and

enhanced culture productivity. To choose the essential composition of a nutrient medium that leverages the N_2-fixing capacity of the model strain, four variants (A to D, as described in the Materials and Methods section) were tested for growth promotion. As indicated by the culture growth curves (Fig. 2) and growth parameters (Table 3), the N-free nutrient medium (A) best fits the criteria t_d, biomass accumulation, μ, and μ^{max}. Furthermore, the culture N_2-fixing capacity was the highest.

To further optimize the cultivation medium, the Response Surface Methodology (RSM) was applied to the N-free medium. The results obtained indicated that sucrose of 20 g L^{-1} and K_2HPO_4:KH_2PO_4 g L-1 of 0.5:0.25 (V2) and 1.0:0.5 (V5) as well as V3 (sucrose of 40 g L^{-1} and K_2HPO_4:KH_2PO_4 g L^{-1} of 0.5:0.25) are the one that resulted in best culture performance in terms of biomass accumulation and extracellular protein production (Fig. 3). Although these variants exhibited similar growth performance, V5 was selected as the optimal one. The V3 was eliminated as an option because the higher sucrose concentration (40 g L^{-1}) did not improve growth. Between V2 and V5, V5 was preferred because of its higher potassium and phosphate content. Potassium is an essential soil macronutrient. In plant cells, it plays a pivotal role in osmotic regulation, carbohydrate metabolism, and enzymes activation. In

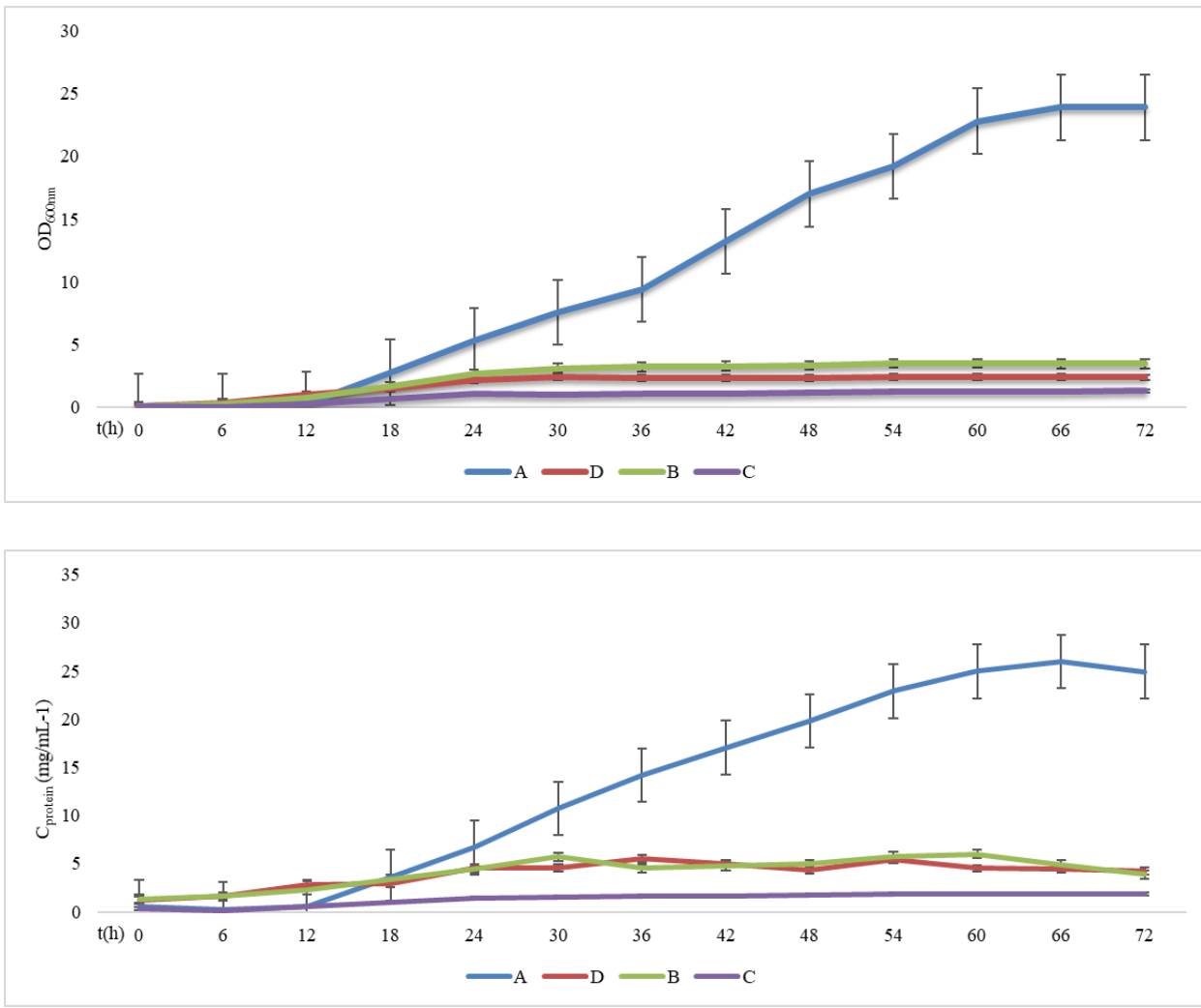


Fig. 2. Growth dynamics - (A) and extracellular protein accumulation (B) of *A. vinelandii* NBIMCC 1619 cultivated for 72 h on different nutrient media at T = 29°C and pH = 7.1.

Table 3. Effect of the nutrient medium composition on *A. vinelandii* NBIMCC 1619 growth and N_2-fix capacity.

Parameter / Nutrient media	t_d, h	$Y_{x/s}$	μ, h^{-1}	μ^{max}, h^{-1}	N_2-fix, μg mL^{-1}
A	5.50 ± 0.05	0.522	0.086	0.126	16.1 ± 0.21
B	5.63 ± 0.02	0.079	0.070	0.106	n.a.
C	6.54 ± 0.02	0.029	0.041	0.123	n.a.
D	6.08 ± 0.05	0.055	0.063	0.114	n.a.

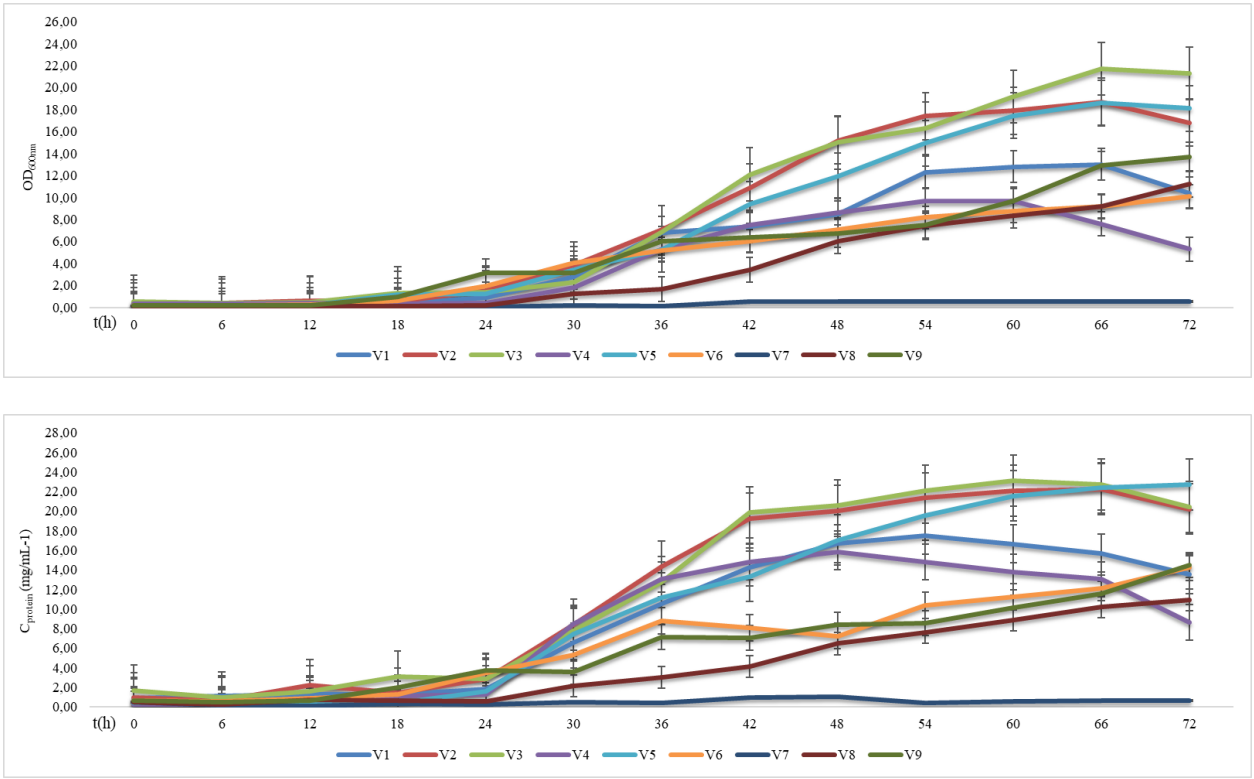


Fig. 3. Growth dynamics (A) and extracellular protein accumulation (B) of *A. vinelandii* NBIMCC 1619 cultivated for 72 h on different variants (V1-V9) of the basic N-free nutrient medium at T = 29°C and pH = 7.1.

agricultural systems, sufficient potassium availability improves biomass production, nutrient uptake efficiency, and stress tolerance in plants. Therefore, V5 represents a more nutritionally balanced and agriculturally relevant medium, offering comparable microbial growth to V2 while providing a higher potassium supply. Thus, the batch/fed-batch cultivation experiments were done with V5 medium.

Optimization of culture aeration

In addition to the composition of the nutrient medium, bacterial growth is strongly influenced by physical/chemical parameters that affect metabolic activity, oxygen transfer, and culture productivity. In the present study, the effect of dissolved oxygen (DO_2) on biomass accumulation and N_2-fixation capacity was evaluated in 48 h batch cultures grown at the optimal temperature. Four DO_2 concentrations were tested, namely 20 %, 30 %, 40 %, and 60 %, and the results are presented in Fig. 4 and Table 4.

The data showed that DO_2 values of 30 - 40 % were optimal for culture growth, biomass accumulation, and extracellular protein production, with the highest N_2-fixation capacity recorded at 30 % DO_2. There was comparable growth performance at DO_2 of 30 % and 40 %. However, 30 % was selected due to technological

Table 4. Effect of DO_2 concentration on *A. vinelandii* NBIMCC 1619 growth and N_2-fix capacity.

Parameter DO_2 (%) / rpm	t_d, h	$Y_{x/s}$	μ, h^{-1}	μ^{max}, h^{-1}	N_2-fix, μg mL^{-1}
20/100 (±20)	9.36 ± 0.05	0.502	0.046	0.074	11.3 ± 0.09
30/150 (±20)	6.60 ± 0.05	0.525	0.051	0.105	20.82 ± 0.23
40/200 (±20)	5.73 ± 0.05	0.498	0.086	0.121	16.5 ± 0.11
60/300 (±20)	10.34 ± 0.05	0.385	0.028	0.067	11.4 ± 0.09

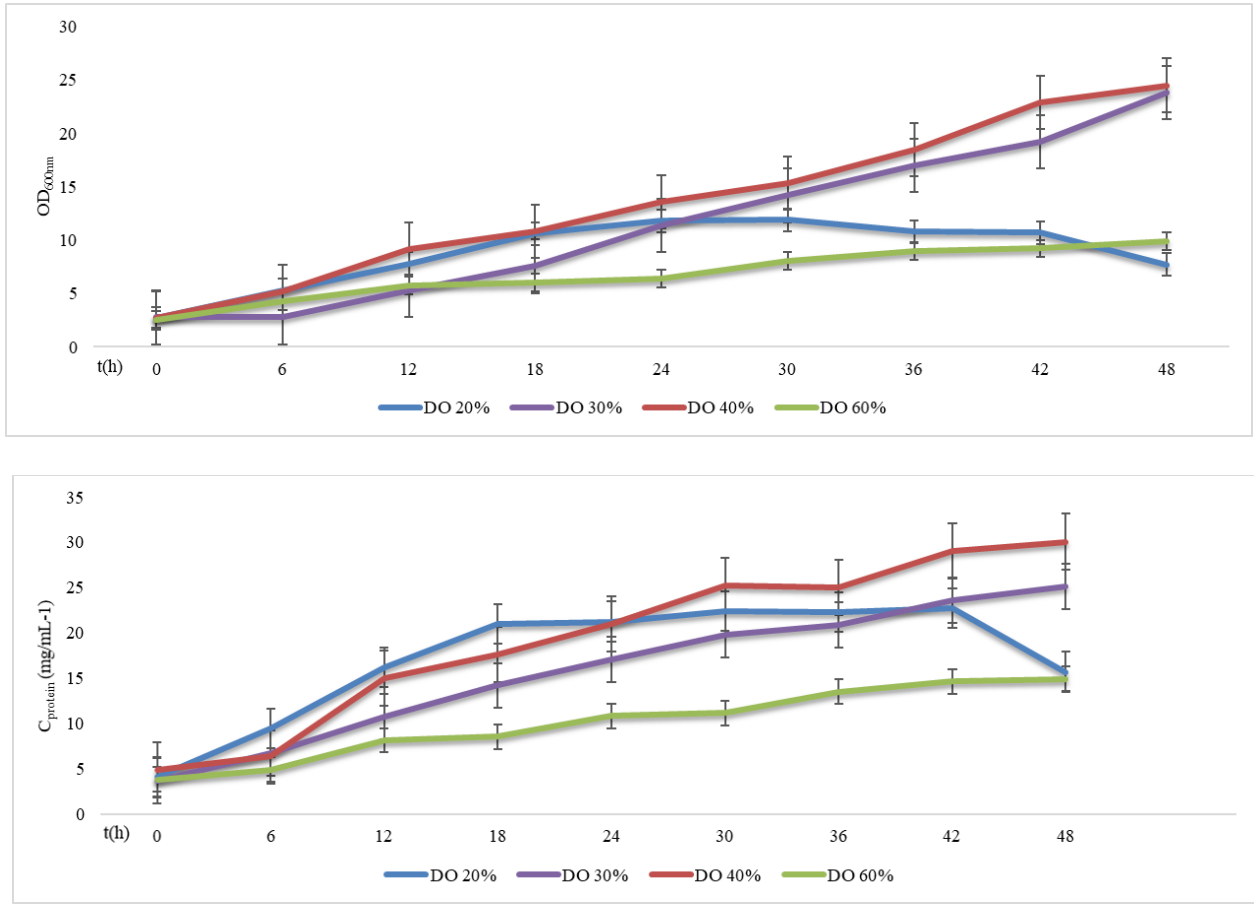


Fig. 4. Growth dynamics and extracellular protein accumulation of *A. vinelandii* NBIMCC 1619 cultivated for 48 h on N-free medium (V5) at T = 29°C, pH = 7.1, and various DO_2.

and economic advantages. In contrast to most N_2-fixing microorganisms, *A. vinelandii* fixes atmospheric N_2 under fully aerobic conditions. Respiratory protection mechanisms are operating intracellularly to maintain low oxygen concentrations in the vicinity of the oxygen-sensitive nitrogenase complex [45]. The organism achieves this through remarkably high respiratory activity and conformational protection of nitrogenase proteins, thus enabling synchronised aerobic growth and biological N_2 fixation [46]. Thus, the results for the effect of DO_2 concentration on *A. vinelandii* NBIMCC 1619 growth and N_2-fix capacity are consistent with the physiological regulation of this species.

The cultivation regime with the following parameters: N-free V5 medium, DO_2 at 30 %, a temperature of 29 ± 0.5°C, and a pH of 7.1 ± 0.1 was chosen because of the optimization process. *A. vinelandii* NBIMCC 1619 showed the growth pattern typical for

aerobic diazotrophs cultivated under these conditions (Fig. 4) with a T_L of 1.83 t_d and an exponential phase lasting approximately 12 h (16 - 28 h), during which the maximum extracellular protein accumulation was observed. The kinetic data indicated μ and μ^{max} values of 0.051 and 0.105 h^{-1}, and $Y_{x/s}$ of 0.525 (Table 5). These results support data reported by other authors of efficient growth of *Azotobacter* spp. under oxygen supply balanced with the culture's respiratory demand [10, 17].

During cultivation, active substrate consumption is registered for both biomass synthesis and the energy-demanding process of N_2 fixation. [28, 45]. As the culture approached the stationary phase, growth rate declined, most likely due to substrate depletion and increased maintenance requirements. Similar growth behaviour has been reported for other diazotrophic fermentations, where depletion of readily metabolizable carbon sources limits further biomass accumulation and reduces nitrogenase activity [15, 46].

The batch cultivation data indicated that this fermentation approach imposed nutritional limitations that hindered maximum biomass productivity. Thus, fed-batch cultivation, as a strategy to prolong the exponential phase, improve substrate utilization, and increase final biomass concentration, is justified.

Formulation of *A. vinelandii* NBIMCC 1619 bioinoculants through fed-batch cultivation

The production of metabolically active biomass of *Azotobacter vinelandii* NBIMCC 1619 as a PGP bioinoculant was achieved through a fed-batch cultivation strategy. Fed-batch cultivation is widely recognized as one of the most efficient industrial fermentation strategies because it enables precise control of substrate availability, prevents carbon excess or depletion, prolongs the exponential growth phase, and improves biomass productivity compared with batch cultivation [15 - 17]. Since the overall performance of a fed-batch process depends primarily on the composition of the feeding medium and the feeding strategy employed, two substrate addition regimes were evaluated in the present study: a single-pulse feeding and continuous feeding over a 12 h period, as described in the Experimental section. The effects of both feeding strategies on bacterial growth, biomass accumulation, and extracellular protein production are summarized in Fig. 6 and Table 5.

The experimental data indicate that during the batch process, less biomass is produced than in both fed-batch cultivations. The extracellular protein accumulation and N_2-fixing capacity also increased significantly. It is well known that fed-batch cultivation supports cellular metabolism during aerobic microbial fermentations. Findings have been reported that controlled nutrient feeding prevents inhibitory substrate concentrations and excessive overflow metabolism while minimizing substrate limitation [16, 47]. The superior performance of the fed-batch process can be attributed to the maintenance of an adequate carbon supply during the later stages of cultivation, resulting in

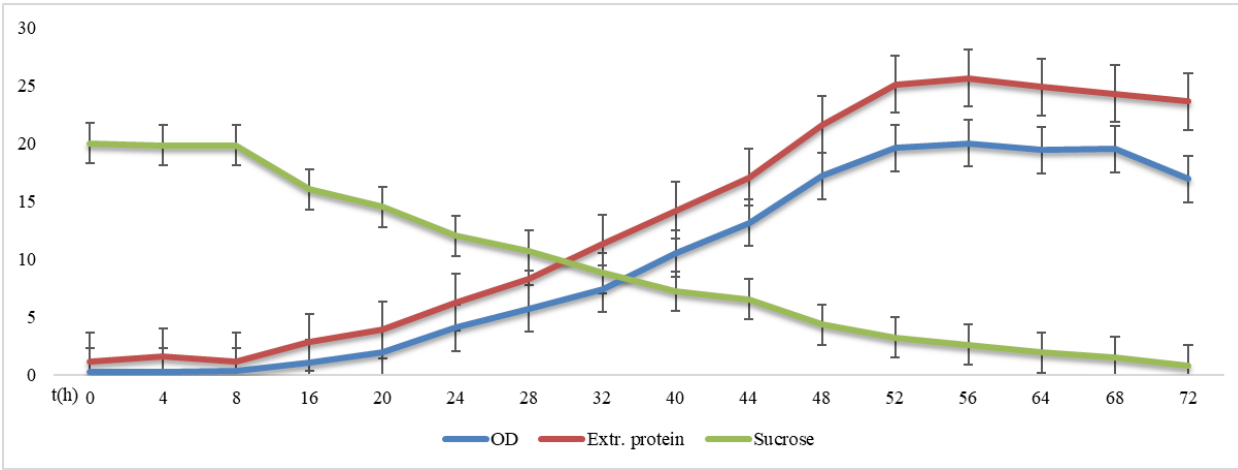


Fig. 5. Growth dynamics, extracellular protein accumulation, and sucrose consumption of *A. vinelandii* NBIMCC 1619 cultivated for 48 h on N-free medium (V5) at T = 29°C, pH = 7.1, and DO_2 = 30 %.

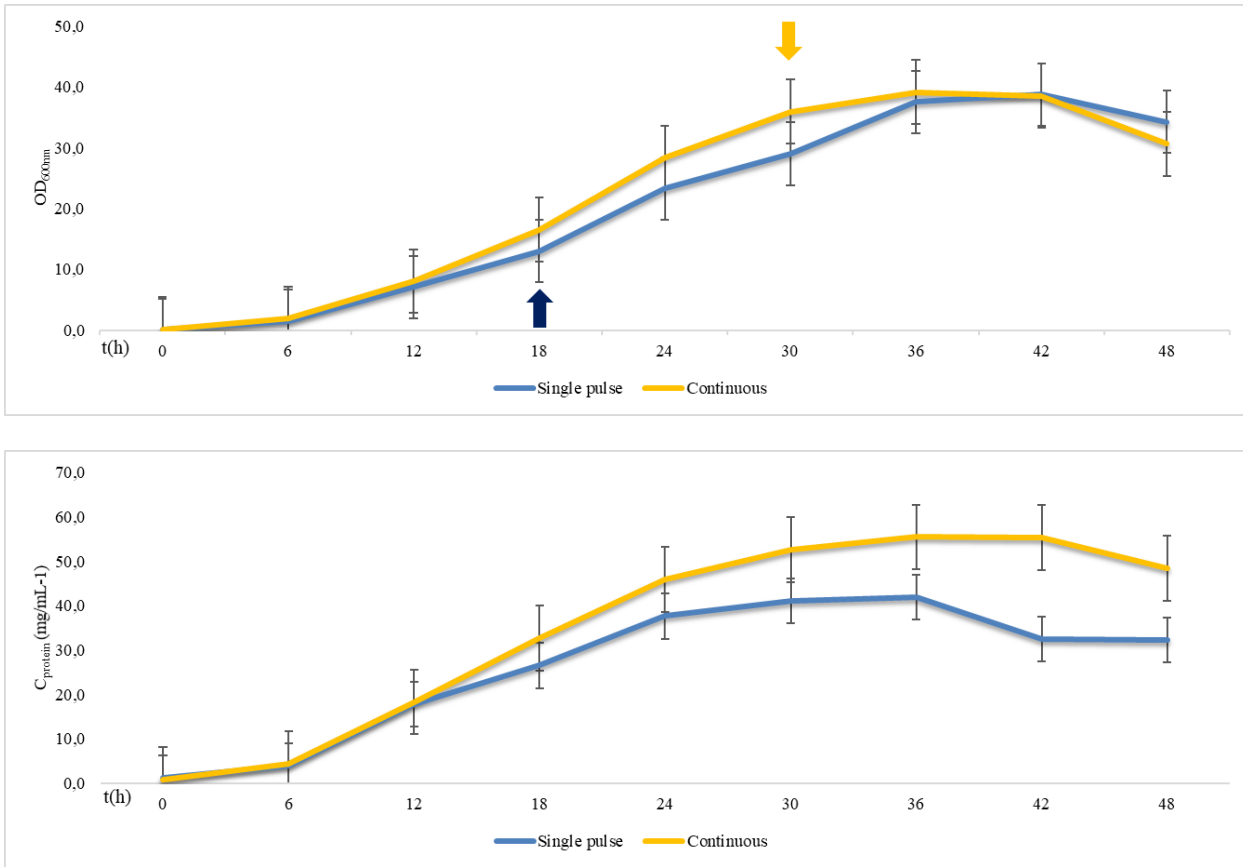


Fig. 6. Growth dynamics and extracellular protein accumulation of *A. vinelandii* NBIMCC 1619 cultivated in a fed-batch mode for 48 h on N-free medium (V5) at T = 29°C, pH = 7.1, and 30 % DO_2. Feed supply was initiated at 18 h for both regimes (blue arrow), and the continuous feed supply was stopped at 30 h (yellow arrow).

Table 5. *A. vinelandii* NBIMCC 1619 kinetic characteristics during batch and fed-batch cultivation.

Parameter / Nutrient media	t_d, h	$Y_{x/s}$	μ, h^{-1}	μ^{max}, h^{-1}
Batch culture	2.30 ± 0.02	0.520	0.210	0.277
Single-pulse feeding	2.12 ± 0.02	0.568	0.327	0.347
Continuous feeding	1.95 ± 0.02	0.582	0.332	0.355

prolonged exponential growth phase, sustained biomass synthesis, continuous ATP generation, and more efficient conversion of substrate into cellular material [15]. This is especially important for *A. vinelandii* since its high respiratory activity is essential both for growth and shielding the oxygen-sensitive nitrogenase complex through respiratory protection mechanisms [45, 46].

The process kinetics data additionally support the technological advantages of fed-batch cultivation (Table 5). The μ and t_d values of both feeding strategies were comparable. However, continuous substrate feeding resulted in higher $Y_{x/s}$, exceeding that obtained with single-pulse feeding and approaching the $Y_{x/s}^{max}$ of sucrose (0.603). The higher $Y_{x/s}$ under continuous feeding conditions indicates more efficient substrate utilization and greater conversion of carbon into biomass. Similar observations have been reported for industrial fed-batch fermentations, where steady

nutrient addition improves carbon utilization efficiency, reduces metabolic fluctuations, and enhances biomass productivity [16, 48]. Continuous feeding also provided a more stable physiological environment by avoiding the abrupt nutrient fluctuations associated with single-pulse feeding, conditions especially advantageous for aerobic diazotrophs [17].

Overall, the fed-batch strategy of continuous substrate feeding over a 12 h period resulted in substantially improved biomass production and N_2-fixing performance of *A. vinelandii* NBIMCC 1619 compared with batch cultivation. These findings establish a solid technological basis for the large-scale production of *A. vinelandii* NBIMCC 1619 as a microbial biofertilizer and confirm the suitability of fed-batch cultivation with a continuous substrate addition regime for industrial biomass production.

A. vinelandii NBIMCC 1619 bioinoculant long-term stability and survival

The long-term stability and survival of the biological component are critical parameters in bioinoculant manufacturing. They directly determine the shelf life, viability, and commercial effectiveness of each production lot. To exert their PGP effects under field conditions, bioinoculants must be stored in a way that ensures they retain enough metabolically active cells. According to current regulatory and quality standards, effective microbial inoculants should contain a minimum of 10^7 - 10^8 CFU mL^{-1} or gram at the time of application [12, 13]. In the present study, the stability of *A. vinelandii* NBIMCC 1619 was evaluated under two formulation approaches: liquid storage at 4°C and freeze-drying with or without cryoprotection.

A. vinelandii NBIMCC 1619 liquid formulation

Monitoring the survival of the bioinoculant in liquid form during storage at 4°C is an important indicator of the effectiveness of post-fermentation processing of the cultural broth. The bioinoculant behaviour during a 3-month storage at 4°C was assessed by determining viable cell counts. As shown in Fig. 7, the culture retained 3.4×10^8 CFU mL^{-1} compared to 7×10^9 CFU mL^{-1} in the initial fresh culture (4.9 % viable cell retention). This decline of approximately one order of magnitude is consistent with the gradual loss of culturability reported for other aerobic diazotrophs stored in liquid form at refrigeration temperatures over comparable periods [13]. Nevertheless, the final cell concentration remains above the minimum threshold recommended for effective bioinoculant application, indicating that the liquid formulation retains agronomically relevant viability during 3 months of cold storage. This stability may be attributed to the resistant cysts, which are metabolically dormant structures capable of surviving adverse environmental conditions, including desiccation, oxidative stress, and low-temperature storage [49]. The exopolysaccharide matrix, primarily alginate, produced by *A. vinelandii* may additionally contribute to cell protection during storage by reducing oxidative damage and maintaining membrane integrity [46]. All these physiological characteristics support the relative stability observed in the liquid formulation and reinforce the suitability of *A. vinelandii* NBIMCC 1619 for refrigerated liquid bioinoculant products.

Freeze-dried A. vinelandii NBIMCC 1619

Among the methods for long-term preservation of microbial inoculants, freeze-drying (lyophilization) is regarded as one of the most effective ones. It minimizes metabolic activity and cellular deterioration over extended periods, maintaining low moisture content at low storage temperatures [13]. However, the freeze-drying process exposes cells to multiple physiological stresses, including rapid freezing, ice crystal formation, vacuum-induced dehydration, and oxidative stress upon rehydration. These stresses can cause irreversible damage to cellular membranes, proteins, and DNA, resulting in substantial losses of cell viability [12]. To evaluate the effect of cryoprotection on survival during lyophilization, the *A. vinelandii* NBIMCC 1619 bioinoculant was freeze-dried with/without a cryoprotective agent (3% soy milk, added before freezing). The results presented in Fig. 7 demonstrate a definite difference in viable cells' recovery between the two treatments. Without a cryoprotectant, cell viability after lyophilization was reduced to 1.0×10^6 CFU mL^{-1}, representing less than 0.02 % of the initial viable count of 7×10^9 CFU mL^{-1} - a reduction of more than three orders of magnitude. In contrast, the addition of 3 % soy milk as a cryoprotectant substantially improved cells' survival, yielding 5.0×10^7 CFU mL^{-1} after lyophilization. Although this value is significantly lower than that of the fresh culture, it is close to the lower limit

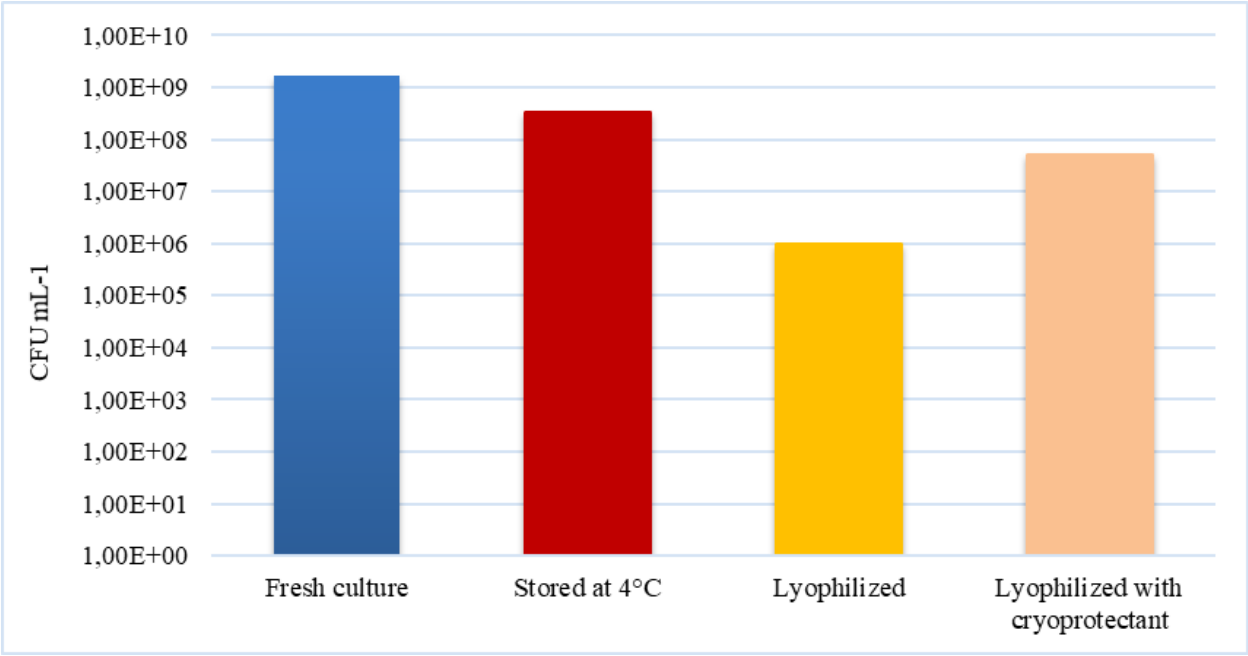


Fig. 7. Viability of *A. vinelandii* NBIMCC 1619 under various storage conditions.

of the recommended minimum viable cell concentration for effective bioinoculants. Cryoprotectants such as skimmed milk, soy milk, sucrose, trehalose, and glycerol are commonly used in the preservation of microbial inoculants because they stabilize cellular membranes and proteins during dehydration by replacing water molecules in the hydration shell and preventing ice crystal-induced mechanical damage [12, 13]. The protective effect of soy milk observed in the present study is consistent with previous reports demonstrating the efficacy of plant-derived protein hydrolysates as cryoprotectants for *Azotobacter* and other PGPR [13]. These compounds likely act through a combination of membrane stabilization, protein protection, and reduction of oxidative damage during both freezing and vacuum dehydration phases of the lyophilization cycle.

The freeze-dried preparations were stored at 4°C for 12 months and periodically evaluated for cell viability after rehydration. They retained about 85 - 80 % viability (data not shown).

The long-term stability and survival data confirm that *A. vinelandii* NBIMCC 1619 can be formulated as both a liquid and a freeze-dried bioinoculant. It retains viability during prolonged storage periods, especially when freeze-dried with a cryoprotective agent.

CONCLUSIONS

In this study, the PGP properties of *Azotobacter vinelandii* NBIMCC 1619 were characterized, and the technological parameters (medium composition, dissolved oxygen regulation, substrate feeding strategies, cultivation kinetics, and final product stability) were optimized to govern biomass production and N_2 fixation under batch and fed-batch cultivation. *A. vinelandii* NBIMCC 1619 exhibited a broad and agronomically relevant spectrum of PGP traits, including efficient atmospheric N_2 fixation, phytohormone biosynthesis (IAA, GAs, ACC, JA, ABA), siderophore production, phosphate solubilization, and extracellular enzyme activities, confirming its potential as a multifunctional biofertilizer. A nutritionally balanced N-free medium and a relevant cultivation regimen were identified as optimal for biomass production and N_2-fixing activity. A continuous fed-batch feeding strategy over a 12 h period was implemented to significantly improve biomass yield and substrate utilization compared with conventional batch cultivation, establishing a technically viable and scalable production platform. The bioinoculant formulation retained agronomically relevant viability (80 - 85 %) during both refrigerated liquid storage and

freeze-drying supplemented with a cryoprotective agent.

Together, the biological, physiological, and technological characteristics of *A. vinelandii* NBIMCC 1619 meet the requirements for the industrial production of high-quality microbial inoculants and support its commercial development as a sustainable biofertilizer that reduces dependence on synthetic nitrogen inputs. Future studies should evaluate the field performance of this formulation under diverse agronomic conditions, including greenhouse and field trials, and assess its effects on crop yield, nutrient-use efficiency, and soil microbiome composition.

Funding

The present work was funded by project No. 80-10-59/ 06.04.2026, Sofia University "St. Kliment Ohridski".

Authors' contributions

T.N., P.H., I.R.: conceptualization; T.N., P.H., and I.R.: methodology; T.N., P.H., I.R., V.P., B.R.: validation; T.N. and V.P.: formal analysis; T.N., P.H., I.R., V.P.,B.R.: investigation; T.N., P.H., I.R.: resources; T.N., P.H., I.R.: data curation; V.P., B.R., T.N.: writing - original draft preparation; T.N., P.H., V.P.: writing - review & editing; T.N., P.H., I.R.: supervision. All authors have read and agreed to the published version of the manuscript.

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