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RESEARCH ARTICLE
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Isolation, Selection, and Biological Evaluation for Bacteria that Fix Nitrogen and Produce Indole-3-Acetic Acid from Paddy Soils in Vietnam

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Abstract:

Introduction/Background: Acidic soils are limiting the production of crops and indirectly harming the environment due to the use of nitrogen (N) chemical fertilizer. Therefore, the current study aims to isolate a promising N_2-fixing candidate to solve this issue in some communes of Phu Luong District, Thai Nguyen Province, Vietnam.

Materials and Methods: The bacteria were isolated using the Ashby medium. The IAA generation and nitrogen fixation were assessed using spectroscopy. The selected bacteria were tested at temperatures ranging from 28°C to 42°C and pH levels of 4.0 to 9.0. As a result, the Box-Behnken model yielded a growth equation. The API kit test was used to measure the biochemical properties of the selected bacteria.

Results: The result demonstrated that there were ten nitrogen-fixing bacteria (NFB) isolates with capacities of N_2 fixation and IAA production at 2.35–24.61 and 0.00–119.13 µg/ml. Among them, the NL3 strain was the best strain. The optimum condition to grow the NL3 strain was calculated as 5.44 days at pH 7.10 and temperature of 32.18°C to reach a bacterial density of 9.77929×10^8 CFU/ml. In addition, the NL3 strain was identified as an *Azotobacter* species. The genomic analysis revealed that the genome of *Azotobacter* sp. NL3 was nearly 5.4 Mb long and contained 45 N_2 fixation-relating genes and 7 IAA production-relating genes.

Conclusion: Not only can this study provide insight into the features of *Azotobacter* spp., but it also introduces a potent candidate that can improve soil health and crop yield by fixing N_2 and producing IAA in order to limit the use of N chemical fertilizer for a sustainable agriculture. Therefore, the selected bacterial strain should be further tested under the local field conditions and subsequently commercialized as a biofertilizer.

Keywords: *Azotobacter* sp., Acidic soil, Biochemistry, Genomics, Indole-acetic acid, N_2-fixing bacteria.

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1. INTRODUCTION

Nitrogen (N) is a crucial nutrient for crops, influencing and limiting their growth and yield [1,2]. It is generally used as N fertilizers in the form of $CO(NH_2)_2$ [2]. Moreover, the significant demand for food in response to overpopulation may lead to an increased utilization of nitrogen fertilizers [3]. However, the N fertilizer can be lost by up to 50% into the soil and environment [4,5], also called nitrate pollution [1], which can ultimately harm human health [6,7]. Therefore, the overuse of N fertilizers can lead to soil, underground water, and air contaminations [2,5,8,9]. Many proposed approaches addressed this situation, such as using slow-release N fertilizer [3], combining N fertilizer with mulch [10], and applying N_2-fixing bacteria (NFB) [11].

The pathway of N_2 fixation is reducing free N_2 in the atmosphere to ammonia under microaerobic conditions [1], which is facilitated by three types of nitrogenases (Mo-, V-, and Fe- nitrogenase) [1]. These enzymes are coded by *nif* genes [12, 13]. Some plants, especially the legume, which has been reported to intercrop with rice, maize, and wheat, have been reported to be able to fix N_2 by having a symbiotic relationship with NFB either in the soil or inside the root itself [1,5, 14]. One well-known NFB is *Astragalus sinicus* [12]. Singh *et al*. [2] reported that many N_2-fixing bacteria proved their abilities to act as a biocontrol and promote plant growth in sugarcane, such as *Bacillus megaterium* and *Bacillus mycoides*. Among these bacteria, *Azotobacter* spp. is a well-known candidate [5]. Moreover, these bacteria species can induce plant resistance to environmental stresses and produce plant growth-producing substances (PGPS), such as indole-acetic acid (IAA), gibberellins, and cytokinin [5]. Therefore, *Azotobacter* spp. has been developed as a biofertilizer [15]. Furthermore, bacterial IAA is such a popular PGPS that it can be used to evaluate the plant growth-promoting potential of a bacterium [16].

In Thai Nguyen Vietnam, the soil is classified as Kanhaplustult ultisoils [17, 18], with a low pH and a high concentration of nutrients, including N. Moreover, acidic soils can lead to great toxicities of Al, Fe, and Mn and low availability of N, phosphorus (P), Ca, potassium (K), and Mg [19, 20]. A study conducted in Thai Nguyen, Vietnam, found that prolonged nitrogen fertilization resulted in a modest rise in the soil's overall N content. However, it also decreased the levels of P and K [21], while the nutrient availability could be a constraint of crop growth and yield [4]. Given such difficulties in Thai Nguyen, Vietnam, finding an indigenous NFB strain to improve soil health and crop production is an ideal solution for sustainable agriculture here. Therefore, the study was conducted to isolate strong NFB strains in Thai Nguyen soils in order to evaluate their IAA production along with their morphological and biochemical characteristics.

2. MATERIALS AND METHODS

2.1. Isolation of N_2-fixing and IAA-producing Bacteria

Bacteria strains were collected from soils in communes of Luong Phu, Tuc Tranh, Vo Tranh, and Phan Me of Phu Luong District, Thai Nguyen Province, Vietnam.

The isolation was conducted on an Ashby medium containing 20.0 g Mannitol, 0.2 g K_2HPO_4, 0.2 g $MgSO_4$, 0.2 g NaCl, 0.1g K_2SO_4, and 5.0 g $CaCO_3$ in a litter of solution made up of distilled water. Bacteria strains that can live and propagate in the Ashby medium were able to fix N_2.

The N_2-fixing bacterial strains were cultured in an Ashby medium supplied with L-tryptophan 0.1%. Strains that can change the color of the Salkowski indicator were able to produce IAA, according to the method of Glikmann and Dessaux (1995).

2.2. Selection for N_2-fixing and IAA-producing Bacteria

The quantification of IAA production was based on the standard IAA solution reacting with the Salkowski indicator and measured for a certain ultraviolet (UV) absorbance. The bacteria were cultured in Ashby broth (supplemented with tryptophan 0.1%) at 30°C and shaken at 150 rpm for seven days.Subsequently, the culture was mixed with the Salkowski indicator and measured for UV absorbance.

The N_2-fixing capacity was based on the production of NH_4^+ in the bacterial culture. The bacterial strains were continuously cultured in Ashby broth at 30°C and shaken at 150 rpm for seven days. The culture was then mixed with the Nessler indicator and measured for UV absorbance.

2.3. Biological Evaluation for N_2-fixing and IAA-producing Bacteria

Bacteria colonies were observed under a Scanning

Electron Microscope (SEM) at a focal distance of 5 µm for morphology.

The effects of temperature and pH on the growth of the isolated strains were also assessed. The temperatures used were 28°C, 30°C, 32°C, 34°C, 36°C, 38°C, 40°C, and 42°C, while the variations of pH were 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, and 9.0. The pH was adjusted by the following buffer solution: Na_2HPO_4 and KH_2PO_4. The culture was conducted based on the above temperature or pH variation for five days. The cell density was measured according to an optical density (OD) at 610 nm. Hence, an optimal condition was established according to the Box-Behnken model with 17 experimental units and three replications. The optimized factors consisted of the following: culture duration (X1) with -1, 0, and +1 corresponding to 4, 5, and 6 days; culture temperature (X2) with -1, 0, and +1 corresponding to 30°C, 32°C, and 34°C; and culture pH (X3) with -1, 0, and +1 corresponding to 6.5, 7, and 7.5. From there, a multivariate model showing the cell density was established using the regression analysis. Analysis of variance (ANOVA) was applied to evaluate the established model and the interactions between factors affecting cell density. The expectation function method was used to optimize the density of NL3 cells obtained from the culture process using the Design-Expert software (DX 7.1.5).

The production of some enzymes, including phosphatase alkaline, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, D-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, D-galactosidase, ß-galactosidase, ß-glucuronidase, D-glucosidase, ß-glucosidase, N-acetyl-ß-glucosaminidase, D-mannosidase, and D-fucosidase, was tested using the API ZYM kit. The biochemical functions of the bacteria were evaluated using the API kit.

2.4. Genomic Analysis for N_2-fixing and IAA-producing Bacteria

To obtain a full genome of the selected bacteria, crude sequences were purified to remove adapters, low-quality nucleotides, and repetitive sequences.

3. RESULTS AND DISCUSSION

3.1. Isolation for N_2-fixing and IAA-producing Bacteria

In Table **1**, 10 strains can live in Ashby, which is labeled as NL3, NL21. NL22, NL23, NL24, NL213, NL215, NL226, NL235, and NL247. Moreover, their origin of isolation is shown in Table **1**. Among them, seven strains can synthesize IAA.

Their N_2-fixing and IAA-producing capacities have been evaluated and shown in Table **2**. The amount of NH_4^+ produced by the bacteria fluctuated from 24.61 µg/ml, while the IAA production was recorded as 11.33-119.13 µg/ml. Among them, the most significant amount of NH_4^+ and IAA belonged to the NL3 strains. Therefore, this strain was chosen for the following experiment. This is in accordance with previous studies, where some NFB strains can produce IAA [16, 22-24]. For example, in the study by Fang *et al*. [22], 131 strains were isolated from the roots of *Ageratina adenophora*. They can perform both N_2 fixation and IAA production, leading to significant improvement in plant growth as well. However, the IAA and N_2 fixation vary between various studies in different locations. Some NFB strains in South Sulawesi, Indonesia, showed IAA-producing results from approximately 300 to nearly 1,800 µg/ml [23]. Concurrently, in the study by Wagi and Ahmed [16], two *Bacillus* spp. can produce IAA of roughly 35.8–36.6 µg/ml. A *Curtobacterium* sp. strain in China can fix N_2 up to 13.38 µg/ml [24]. However, these above strains were proven to be capable of conducting other plant growth-promoting activities [25], such as P solubilization [22] and siderophores production [16]. Unfortunately, these characteristics were not investigated in our study. Therefore, they should be tested in future studies in which a field trial should be taken with a cultivar habiting in Thai Nguyen Province, Vietnam, such as tea [17], to assess the effects of the selected bacteria on soil health and plant productivity.

Table 1. Isolation result for N_2-fixing bacteria.

Strain	Location of Isolation	N_2-fixing Ability	IAA-producing Ability
NL3	**Luong Phu commune**	+	+
NL21	**Luong Phu commune**	+	+
NL22	Tuc Tranh commune	+	+
NL23	Tuc Tranh commune	+	+
NL24	Vo Tranh commune	+	+
NL213	Vo Tranh commune	+	-
NL215	Vo Tranh commune	+	-
NL226	Phan Me commune	+	-
NL235	Phan Me commune	+	+
NL247	Phan Me commune	+	+

Table 2. The capacity to produce IAA and fix N_2 of the isolated strains.

No.	Strain	IAA-producing Capacity (µg/ml)	N_2-fixing Capacity (µg/ml)
1	**NL3**	**119.13**	**24.61**
2	NL21	30.68	12.15
3	NL22	11.33	7.43
4	NL23	29.16	5.25
5	NL24	70.91	14.64
6	NL213	-	11.56
7	NL215	-	6.23
8	NL226	-	2.35
9	NL235	11.39	4.78
10	NL247	7.66	3.24

Table 3. Morphology of cells and colonies and Gram of the isolated strains.

No.	Strain	Colony Morphology	Cell Morphology	Gram	Motility
1	NL3	Round, translucent white to greenish white, convex, compact, glistened, and slimy	Short rod	-	+
2	NL21	Round, opaque white, convex, smooth, glistened, and slimy	Oval - round	-	+
3	NL22	Round, opaque white, wrinkled, crateriform, and rough surface	Oval - round	-	+
4	NL23	Opaque white, round with nucleus, smooth, convex, nonfilamentous	Oval	-	+
5	NL24	Round, translucent white, glistened, nonfilamentous, slimy, and raised	Short rod	-	+
6	NL213	Round, opaque white, glistened surface, nonfilamentous, slimy, and raised	Coma	-	+
7	NL215	Round, translucent white, glistened surface, filiform, and slimy	Short rod	-	+
8	NL226	Opaque white, round and without filament, rough surface	Oval	-	+
9	NL235	Translucent white, irregular, filiform, flat, and rough surface	Short rod	-	+
10	NL247	Opaque white, round, convex, filiform, lobate, and rough	Short rod	-	+

Additionally, the cell and colony features of the isolated bacterial strains are also listed in Table **3**. Although they were all generally white, Gram-negative, and mobile, their cell and colony morphological traits varied. In particular, the NL3 strain had short rod-shaped cells and colonies that were round, translucent white to greenish white, convex, compact, glistened, and slimy (Figs. **1** and **2**). This is in accordance with the study by Tang *et al*. [26] and Mahmud *et al*. [1], where the NFB were also commonly Gram-negative with convex colonies, especially the *Azotobacter* spp [27]. with motility [28].

3.2. Growth Evaluation for the NL3 Strain

The bacterial density of the NL3 strain ranged from 0.007 to 9.337 x 10^8 CFU/ml from 28°C to 42°C. The result peaked at 32°C and bottomed at 42°C (Table **4**). Conversely, the bacterial density fluctuated from 0.007 to 9.337 x 10^8 CFU/ml under pH from 4.5 to 9.0. The greatest result was found in the pH 7.0, whereas the lowest one was in the pH 4.5 (Table **5**). This is in accordance with the study by Mukhtar *et al*. [29], where it was reported that the *Azotobacter* spp. have survivable conditions of pH 5–9 and temperature of 25–40°C.

>Based on the Box-Behnken practical matrix of culture duration (X1), temperature (X2), and pH (X3), the function showing the bacterial density of the NL3 strain was formulated as follows: $Y = + 9.1 + 1.69*X1 + 0.066*X2 + 1.8*X3 + 0.37*X1*X2 + 1.45*X1*X3 - 0.47*X2*X3 - 2.08*X1^2 - 2.33*X2^2 - 3.66*X3^2$. The matrix is shown in Table **6**. As observed, all the factors affected the bacterial density of the *Azotobacter* sp. NL3 strain. However, when combined with each other or the factors above the limit, the influences could be negative.

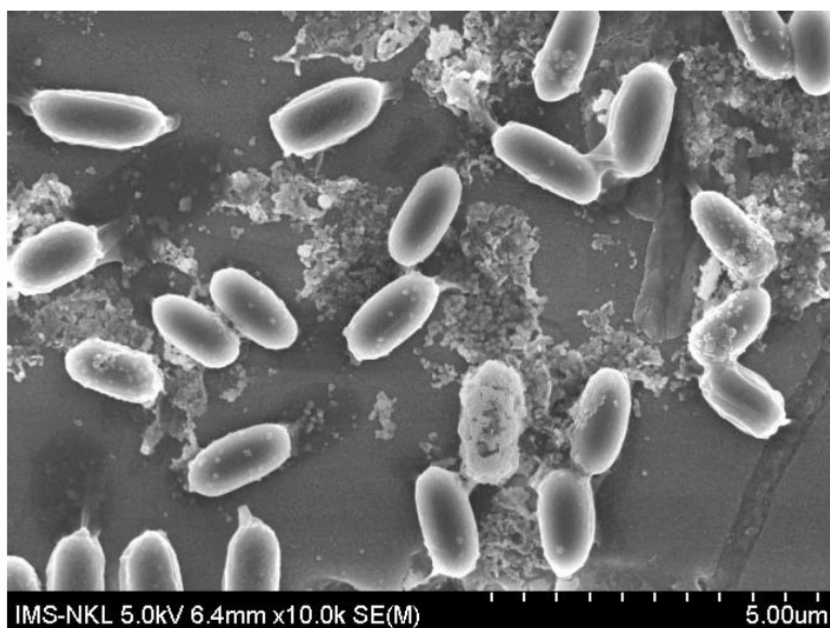


Fig. (1). SEM image of the NL3 strain at 5 µm focal distance.

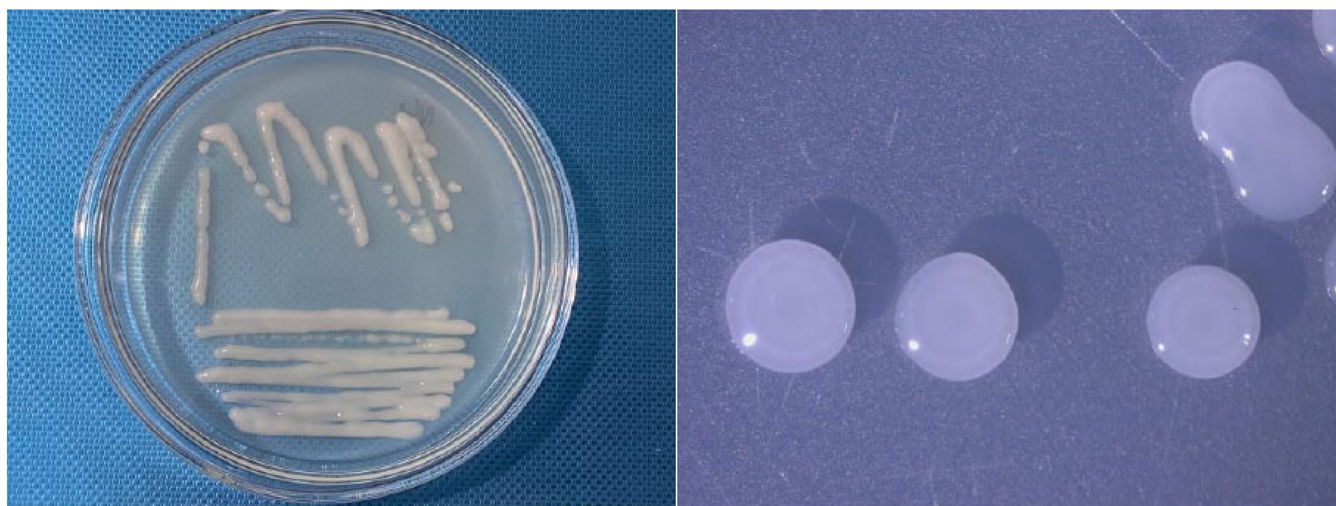


Fig. (2). Colonies of the NL3 strain.

Table 4. Influence of temperature on the NL3 strain.

No.	Strain	Bacterial Density (x 10^8 CFU/ml) After Five Days of Culture at a Certain Temperature							
		28°C	30°C	32°C	34°C	36°C	38°C	40°C	42°C
1	NL3	9.067	9.307	**9.337**	8.087	6.127	1.197	0.507	0.007

Table 5. Influence of pH on the NL3 strain.

Strain	Bacterial Density (x 10^8 CFU/ml) After Five Days of Culture at a Certain pH					
	pH = 4.0	pH = 4.5	pH = 5.0	pH = 5.5	pH = 6.0	pH = 6.5
NL3	-	0.007	0.757	2.087	8.957	9.297
Strain	**Bacterial density (x 10^8 CFU/ml) after five days of culture at a certain pH**					
	pH = 7.0	**pH = 7.5**	**pH = 8.0**	**pH = 8.5**	**pH = 9.0**	
NL3	9.337	9.197	7.117	1.617	0.097	

Table 6. The Box-Behnken practical matrix of three factors and the bacterial density of the NL3 strain under different culture conditions.

Experiment	Variables			NL3 Density (10^8CFU/ml)
	X1 Culture Duration (days)	X2 Culture Temperature (°C)	X3 Culture pH	
1	4.00	30.00	7.00	3.2251
2	6.00	30.00	7.00	5.654
3	4.00	34.00	7.00	2.9862
4	6.00	34.00	7.00	6.8982
5	4.00	32.00	6.50	1.294
6	6.00	32.00	6.50	1.9709
7	4.00	32.00	7.50	1.8614
8	6.00	32.00	7.50	8.3416
9	5.00	30.00	6.50	0.8959
10	5.00	34.00	6.50	1.6026
11	5.00	30.00	7.50	5.5644

(Table 6) contd.....

Experiment	Variables			NL3 Density (10^8CFU/ml)
	X1 Culture Duration (days)	X2 Culture Temperature (°C)	X3 Culture pH	
12	5.00	34.00	7.50	4.3798
13	5.00	32.00	7.00	9.1081
14	5.00	32.00	7.00	9.337
15	5.00	32.00	7.00	9.2375
16	5.00	32.00	7.00	8.9787
17	5.00	32.00	7.00	8.8592

Table 7. ANOVA for the NL3 growth model.

Source	Standard F	P values
Model	240.60	<0.0001
Lack of Fit	3.52	0.1276
R^2	0.9968	

Note: standard F: standard Fisher; Lack of Fit: standard for evaluating the incompatibility of the model with practice; R^2: regressive coefficient.

Table **7** demonstrates the significance and compatibility of the formulated model. The significance value of the model was P value < 0.0001 < 0.05. Therefore, the model was selected with a regressive coefficient of R^2 = 0.9968, showing that the practical data were compatible with the predicted model.

On the other hand, the expectation function method optimized the bacterial density of the NL3 strain. In total, 43 options were found, and the best one for the maximum NL3 density function was 5.44 days of culture at 32.18°C and pH = 7.10 (Fig. **3**), at which the maximum bacterial density was 9.77929 x 10^8 CFU/ mL (Fig. **4**). However, the optimum growth observed in the study by Mukhtar *et al*. [29] was at pH 8 and 30°C. This could be the different species or the different location of origin. In Thai Nguyen, the temperature is moderately high, and the soil is acidic [17, 30], leading to a lower optimum pH and higher optimum temperature of *Azotobacter* sp. in this study.

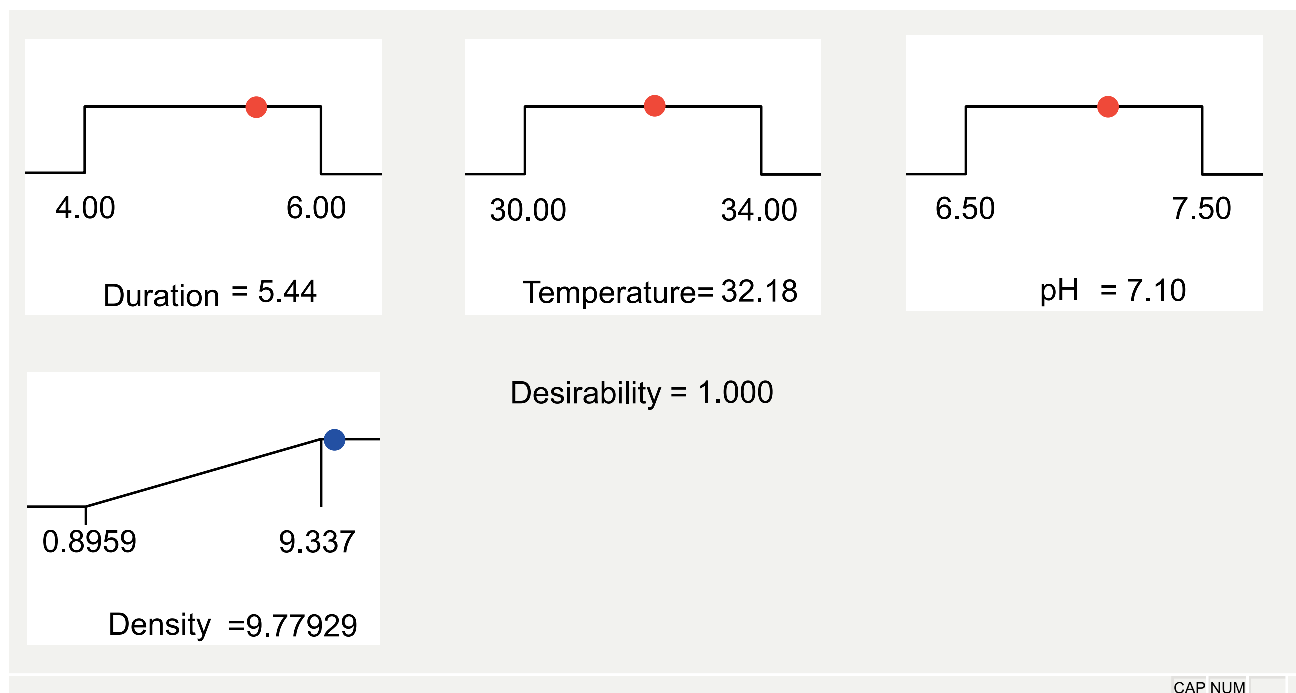
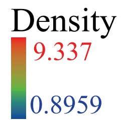


Fig. (3). The predicted model and the optimal condition for the maximum bacterial density of the NL3 strain.

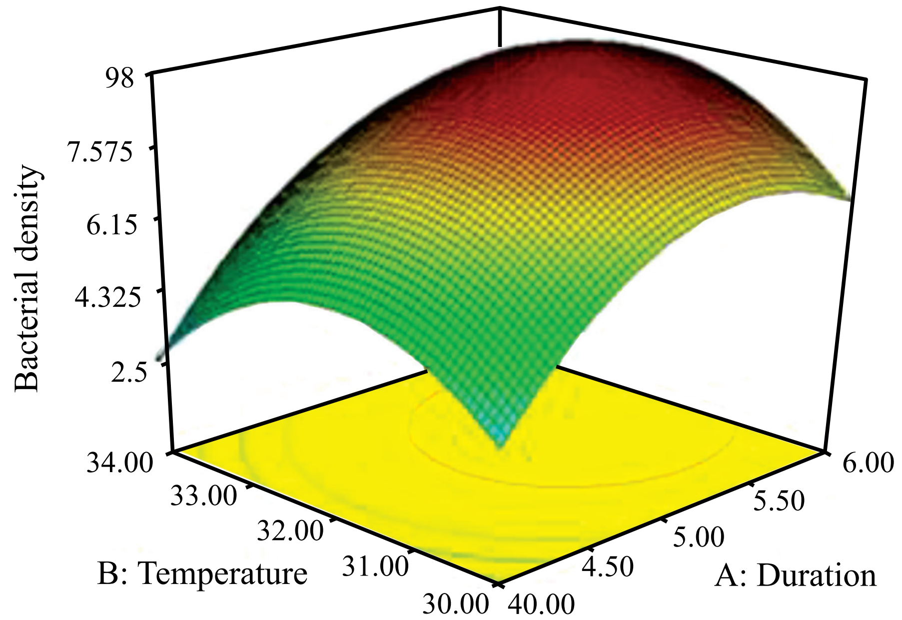
Design-Expert Software



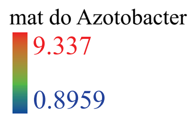
XI = A Duration
X2 = B Temperature

Actual Factor
C pH = 7.10

(a)



Design-Expert Software



XI = A Duration
X2 = C: pH

Actual Factor
Temperature = 32.15

(b)

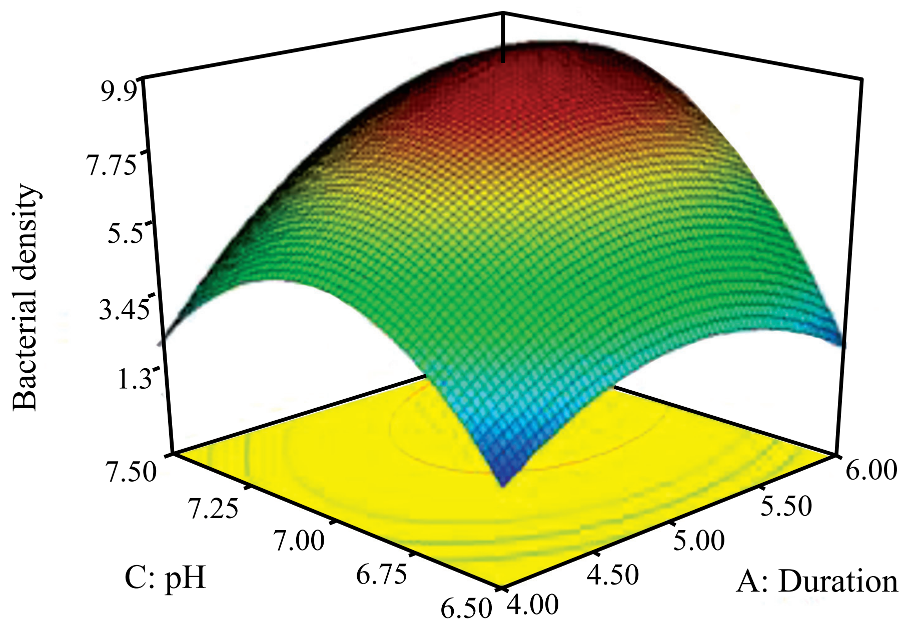


Fig. 4 contd.....

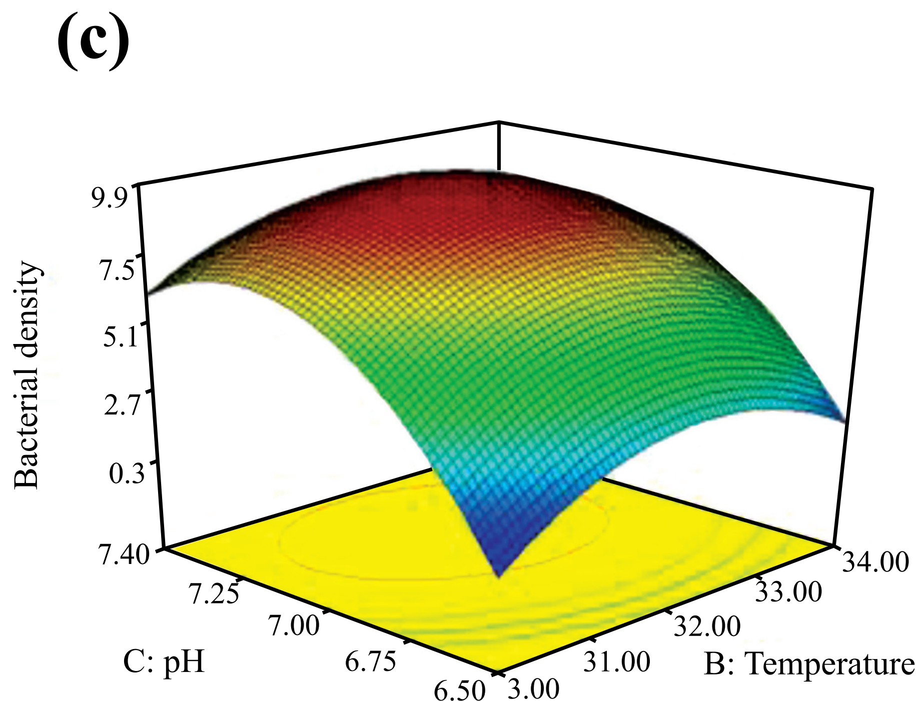


Fig. (4). Response surface of the NL3 bacterial density.
Note: (**a**) The interactive model between culture duration and temperature; (**b**) the interactive model between culture duration and pH; (**c**) the interactive model between culture temperature and pH.

3.3. Biochemical Function of the NL3 Strain

The NL3 showed a positive trait of producing alkaline phosphatase, esterase, lipase, leucine arylamidase, valine arylamidase, phosphatase acid, naphthol-AS-BI-phosphohydrolase, and D-glucosidase (Table **8**). Moreover, the NL3 strain was proven to be able to produce indole, catalase, and oxidase to assimilate esculin ferric citrate, D-glucose, D-mannitol, potassium gluconate, capric acid, malic acid, trisodium citrate, L-rhamnose, inositol, D-saccharose, lactic acid, glycogen, D-melibiose, D-sorbitol, valeric acid, potassium 2-ketogluconate, 3-hydroxybutyric acid, 4-hydroxybenzoic acid, and D-fructose and perform red methyl reaction, Voges–Proskauer reaction, and H_2S production (Table **9**). In the current study, the bacterial strain NL3 can perform glucose fermentation, H_2O_2 naturalization, citrate and indole utilization, and urease production [28].

3.4. Genomic Study of the NL3 Strain

The result of the before and after purification is described in Table **10**. The total number of sequences before and after purification showed that the Illumina sequencing score had a Q30 rate above 80%, which is qualified for the de novo assembly [31].

The assembly result is displayed in Table **11**, which illustrates that the total length of contigs was 5,378,928 bp and suitable for the NL3's genome size (roughly 5 Mb). The longest contig was 5,378,928 bp long, accounting for 100% of the total contig length. This is consistent with the study by Setubal *et al*. [32], where the length of an *Azotobacter* sp. was roughly 5.4 Mb.

Table 8. Enzyme production by the NL3 strain.

No.	Enzyme Production	NL3
1	Control	-
2	Alkaline phosphatase	+
3	Esterase (C4)	+
4	Esterase Lipase (C8)	-
5	Lipase (C14)	+
6	Leucine arylamidase	+
7	Valine arylamidase	+
8	Cystine arylamidase	-

(Table 8) contd.....

No.	Enzyme Production	NL3
9	Trypsin	-
10	D-chymotrypsin	-
11	Acid phosphatase	+
12	Naphthol-AS-BI-phosphohydrolase	+
13	D-galactosidase	+
14	ß-galactosidase	-
15	ß-glucuronidase	-
16	D-glucosidase	+
17	ß-glucosidase	-
18	N-acetyl-ß-glucosaminidase	-
19	D-mannosidase	-
20	D-fucosidase	-

Table 9. Some biochemical traits of the NL3 strain.

No.	Biochemical Index	Activity
1	Convert nitrate to nitrite	-
2	Produce indole	+
3	Ferment D-glucose	-
4	Assimilate L-arginine	-
5	Assimilate urea	-
6	Assimilate esculin ferric citrate	+
7	Assimilate gelatin	-
8	Assimilate 4-nitrophenyl-β D-glucopyranoside	-
9	Assimilate D-glucose	+
10	Assimilate L-arabinose	-
11	Assimilate D-mannose	-
12	Assimilate D-mannitol	+
13	Assimilate N-acetyl-glucosamine	-
14	Assimilate D-maltose	-
15	Assimilate potassium gluconate	+
16	Assimilate capric acid	+
17	Assimilate adipic acid	-
18	Assimilate malic acid	+
19	Assimilate trisodium citrate	+
20	Assimilate phenylacetic acid	-
21	Assimilate L-rhamnose	+
22	Assimilate D-ribose	-
23	Assimilate inositol	+
24	Assimilate D-saccharose	+
25	Assimilate Itaconic acid	-
26	Assimilate suberic acid	-
27	Assimilate sodium malonate	-
28	Assimilate sodium acetate	-
29	Assimilate lactic acid	+
30	Assimilate L-alanine	-
31	Assimilate potassium 5-ketogluconate	-
32	Assimilate glycogen	+
33	Assimilate 3-hydroxybenzoic acid	-
34	Assimilate L-serine	-
35	Assimilate salicin	-
36	Assimilate D-melibiose	+
37	Assimilate L-fucose	-
38	Assimilate D-sorbitol	+
39	Assimilate propionic acid	-

(Table 9) contd.....

No.	Biochemical Index	Activity
40	Assimilate valeric acid	+
41	Assimilate L-histidine	-
42	Assimilate potassium 2-ketogluconate	+
43	Assimilate 3-hydroxybutyric acid	+
44	Assimilate 4-hydroxybenzoic acid	+
45	Assimilate L-proline	-
46	Assimilate D-xylose	-
47	Assimilate D-fructose	+
48	Assimilate lactose	-
49	Catalase activity	+
50	Oxidase activity	+
51	Red methyl reaction	+
52	Voges–Proskauer reaction	+
53	Produce H_2S	+

Table 10. The sequencing quality of genes of the NL3 strain by the Illumina technique.

Sample	The Number of Read	Base Total (bp)	Length (bp)	%GC	% Q30
Before purification					
Forward sequencing R1	1,953,179	294,930,029	35-151	64.8	95.0
Reverse sequencing R2	1,953,179	294,930,029	35-151	64.8	94.3
After purification					
Forward sequencing R1	1,870,981	282,416,622	35-151	64.7	96.2
Reverse sequencing R2	1,870,981	282,416,622	35-151	64.8	95.6

Table 11. The de novo assembly of the NL3 strain.

Genomic Characteristics	Quantity
The number of contigs	1
The number of circles contigs	1
%GC (contigs >500 bp)	65.65
Total length of contigs (bp)	5,378,928
The longest contig length (bp)	5,378,928
N50 value (contigs >500 bp)	5,378,928
L50 value (contigs >500 bp)	1
CDS	5321
tRNA	64
rRNA	18
The number of protein functional coding genes:	-
Hypothesis protein	1233
Functional protein	4088
Protein with EC indicator	1232
Protein with GO arrangement	1059
Signaling proteins	935
Systematic protein	1710
Protein for PLfam, especially for the PATRIC genus	5200
Protein with the family-genus mission of PATRIC (PGfam)	5205
Protein with FIGfam mission	0

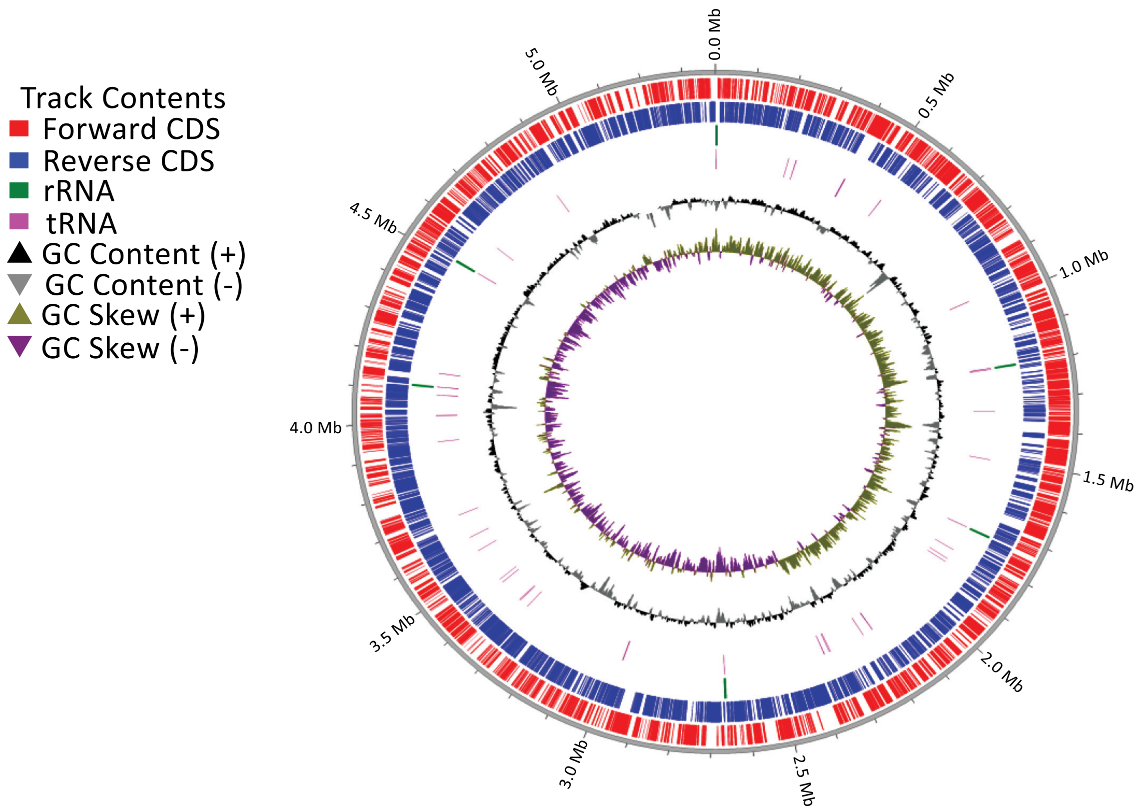


Fig. (5). Genomic map of the NL3 strain.

Table 12. Genes relating to N_2 fixation and IAA production of the NL3 strain.

No.	Function/Pathway
	Genes relating to N_2 fixation:
1	Nitrogenase (molybdenum-iron)-specific transcriptional regulator NifA
2	Nitrogenase (iron-iron) transcriptional regulator
3	Nitrogenase-associated protein NifO
4	Nitrogenase (iron-iron) transcriptional regulator
5	AnfO protein, required for Mo- and V-independent nitrogenase
6	AnfR protein, required for Mo- and V-independent nitrogenase
7	Nitrogenase (iron-iron) beta chain (EC 1.18.6.1)
8	Nitrogenase (iron-iron) alpha chain (EC 1.18.6.1)
9	Nitrogenase (iron-iron) delta chain (EC 1.18.6.1)
10	Nitrogenase (iron-iron) reductase and maturation protein AnfH
11	Nitrogenase (iron-iron) transcriptional regulator
12	Nitrogenase (molybdenum-iron)-specific transcriptional regulator NifA
13	4Fe-4S ferredoxin, nitrogenase-associated
14	Nitrogenase FeMo-cofactor synthesis FeS core scaffold and assembly protein NifB
15	Nitrogenase-associated protein NifO
16	Nitrogenase FeMo-cofactor synthesis molybdenum delivery protein NifQ
17	Nitrogenase (molybdenum-iron) reductase and maturation protein NifH
18	Nitrogenase (molybdenum-iron) alpha chain (EC 1.18.6.1)
19	Nitrogenase (molybdenum-iron) beta chain (EC 1.18.6.1)
20	NifT protein
21	Nitrogenase FeMo-cofactor scaffold and assembly protein NifE
22	LRV (FeS)4 cluster domain protein clustered with nitrogenase cofactor synthesis

(Table 12) contd.....

No.	Function/Pathway
23	Nitrogenase FeMo-cofactor scaffold and assembly protein NifN
24	Nitrogenase FeMo-cofactor carrier protein NifX
25	NifX-associated protein
26	Uncharacterized protein RPC_4456
27	Iron-sulfur cluster assembly scaffold protein NifU
28	Probable iron-binding protein from the HesB_IscA_SufA family in Nif operon
29	Cysteine desulfurase (EC 2.8.1.7) => NifS
30	Nitrogenase stabilizing/protective protein NifW
31	Nitrogenase vanadium-cofactor synthesis protein VnfY
32	NifZ protein
33	Homocitrate synthase (EC 2.3.3.14)
34	Nitrogenase (vanadium-iron) beta chain (EC 1.18.6.1)
35	Nitrogenase (molybdenum-iron) reductase and maturation protein NifH
36	Nitrogenase (vanadium-iron) alpha chain (EC 1.18.6.1)
37	Nitrogenase vanadium-cofactor synthesis protein VnfX
38	Nitrogenase (vanadium-iron) delta chain (EC 1.18.6.1)
39	4Fe-4S ferredoxin, nitrogenase-associated
40	Nitrogenase vanadium-cofactor synthesis protein VnfN
41	Nitrogenase vanadium-cofactor synthesis protein VnfE
42	Electron transfer flavoprotein, beta subunit FixA
43	Electron transfer flavoprotein, alpha subunit FixB
44	Electron transfer flavoprotein-quinone oxidoreductase FixC
45	Ferredoxin-like protein FixX
Gens relating to IAA production:	
1	Indole-3-acetate_biosynthesis_I
2	Indole-3-acetate_biosynthesis_III
3	Indole-3-acetate_biosynthesis_IV
4	Indole-3-acetate_biosynthesis_V
5	L-tryptophan_degradation_VII
6	L-tryptophan_degradation_X
7	Methyl_indole-3-acetate_interconversion

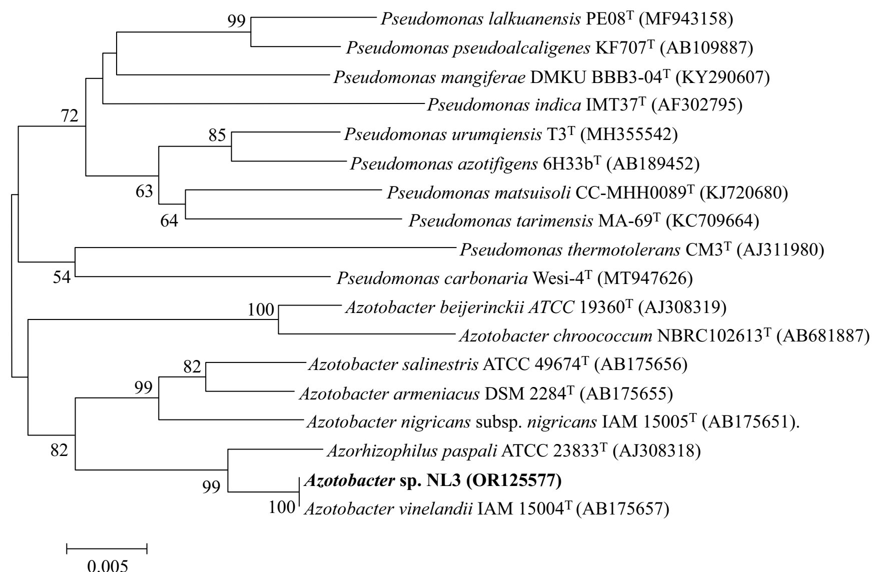


Fig. (6). Neighbor-joining tree of strain NL3 and closely related species based on 16S rRNA gene sequence. Numbers at nodes indicate levels of bootstrap support (%) based on a neighbor-joining analysis of 1,000 resampled datasets; only values ≥ 50% were given. Bar, 0.005 substitutions per site.

The genomic map of the NL3 strain is built in Fig. (**5**). The analysis results show that from the center outward, circle 1 illustrates the tilt of the GC. Circle 2 showed the GC content (peaks outside or inside of the circle indicate values that were higher or lower than the average G+C content, respectively). Circle 3 represents the ncRNA gene. Circles 4, 5, and 6 represent CDS, with colors according to the COG, KEGG, and GO categories, respectively. Circle 7 represents the functionally predicted protein-coding sequences.

According to Fig. (**6**), the NL3 strain was identified as an *Azotobacter* sp. with an accession number of OR125577, which was closely related to the *Azotobacter vinelandii* IAM 15004[T] strain.

3.5. Primary Analysis of Genetic Structures for N_2 Fixation and IAA Production of the Azotobacter sp. NL3

Based on the genome of the NL3 strain, 45 genes were found relating to N_2 fixation by the NL3 strain. The result is presented in Table **12** and Fig. (**7**) with *nif* and *fix* genes that directly participated in N_2-fixation. Furthermore, genomic analysis of the NL3 strain also provided 07 genes participating in IAA metabolism and referred in the MetaCyc database (Table **12** and Fig. **8**).

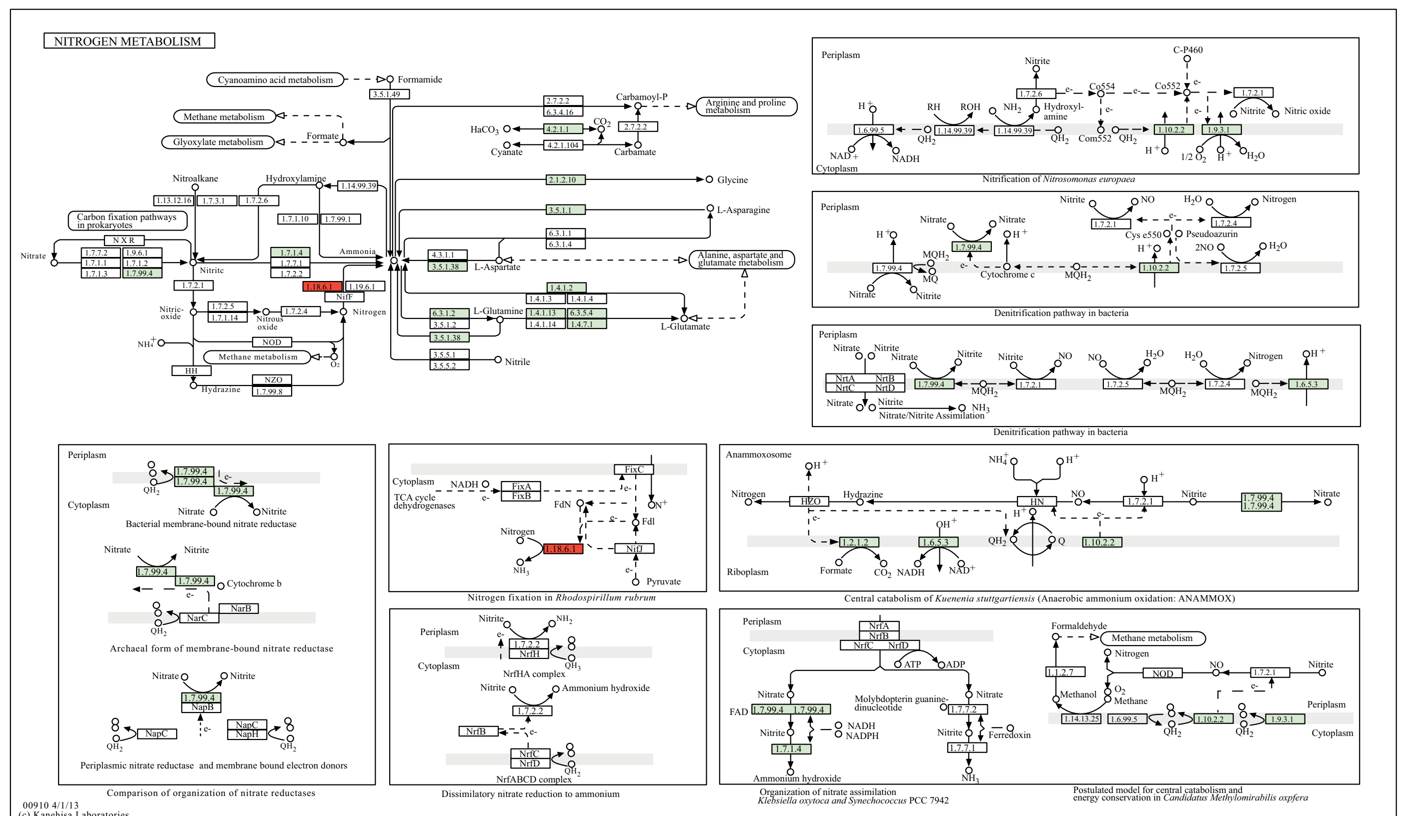


Fig. (7). N_2 fixation of the *Azotobacter* sp. NL3 strain.

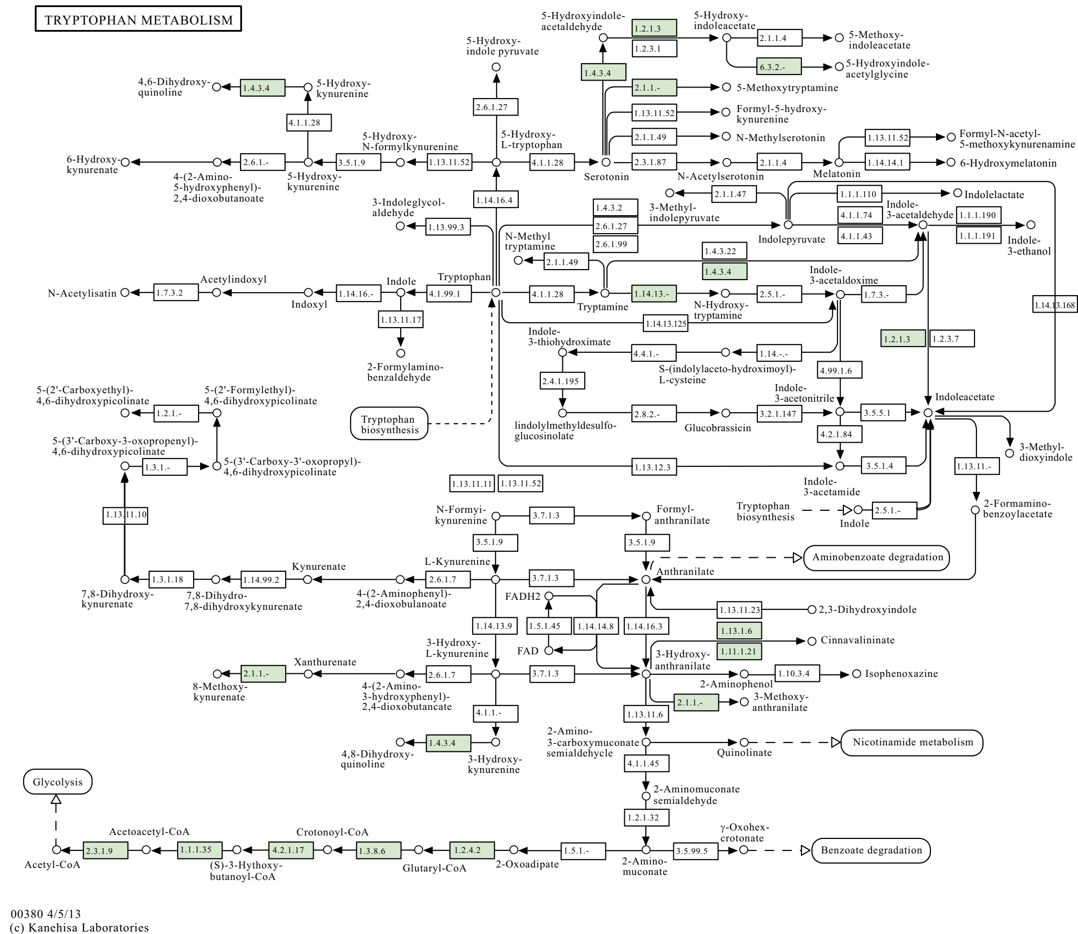


Fig. (8). IAA production of the *Azotobacter* sp. NL3 strain.

CONCLUSION

From acidic soils in some communes of Thai Nguyen province, Vietnam, ten isolates of NFB were found. Among them, the NL3 strain performed the greatest N_2 fixation and IAA production (roughly 24.61 and 119.13 µg/ml, respectively). The optimum pH and temperature were correspondingly 7.10 and 32.18°C for the maximum bacterial density of 9.77929×10^8 CFU/ml according to the Box-Behnken-based growth function as follows: $Y = + 9.1 + 1.69*X1 + 0.066*X2 + 1.8*X3 + 0.37*X1*X2 + 1.45*X1*X3 - 0.47*X2*X3 - 2.08*X1^2 - 2.33*X2^2 - 3.66*X3^2$. Furthermore, the NL3 strain was identified as an *Azotobacter* sp. with a nearly 5.4 Mb long genome containing 45 N_2-fixing genes and 7 IAA-producing genes. The newly isolated *Azotobacter* sp. is promising to improve soil fertility and crop productivity and reduce the use of chemical fertilizer on acidic soil for sustainable agriculture. However, the study did not measure the plant growth promotion and soil remediation by the selected bacteria. We will conduct those in the future study. Nevertheless, there are difficulties in selecting appropriate carriers for bacteria to survive under field conditions, determining reasonable costs for farmers' use, and raising farmers' awareness of the importance of using biofertilizers rather than chemical fertilizers.

LIST OF ABBREVIATIONS

OD	=	Optical Density
SEM	=	Scanning Electron Microscope
NFB	=	Nitrogen-fixing Bacteria

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The data that support the findings of this study are available from the corresponding author, [H.A], on special request.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest, financial or otherwise.

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Declared none.

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