ISOLATION, IDENTIFICATION AND OPTIMIZATION OF MASS PRODUCTION OF *Bacillus velezensis*

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Abstract: *Bacillus velezensis strain BL-1 was successfully isolated from the soil samples collected from Quang Xuong district, Thanh Hoa province, Vietnam. The BL-1 strain was identified as Bacillus velezensis based on morphological and phylogenetic analysis of its 16S rRNA gene. Our study proposed a protocol for mass production of B. velezensis BL-1 using a soybean flour molasses-based medium which provided a high efficiency. The optimized fermentation conditions were an incubation for 48 hours at 35°C with a shaker at speed of 120 rpm, pH 7.0, and inoculum volume of 2% (v/v).*

Keywords: *Bacillus velezensis, isolation, mass production*.

1. Introduction

Developing organic agricultural production is an inevitable direction in Vietnam and other countries in the world to ensure sustainable, harmonious, nature-friendly, and safe agriculture. Since biopesticides and microbiological organic fertilizers play an important role in the success of organic agricultural production, investigation, and application of microorganisms have become more widespread [8].

The bacterial genus *Bacillus* has a wide range of physiological properties and their ability to produce a various range of enzymes, antibiotics and metabolites have made them potential agents for agricultural applications. *Bacillus velezensis*, a typical of *Bacillus* species, is an endospore-forming, free-living soil bacterium with potentials as a plant growth-promoting agent, a biopesticide against a broad spectrum of microbial pathogens of plants, and an important source of microorganisms for producing organic fertilizers [10].

Many strains of *B. velezensis* have widely been reported to benefit plant growth by nutrient uptake and secreting secondary metabolites such as indole-3-acetic acid to promote the system development of plant roots [4] [6]. Numerous strains of this species have been reported to suppress the growth of microbial pathogens, including bacteria, fungi, and nematodes [10]. In Vietnam, researches on the isolation and selection of *Bacillus* sp. for agricultural applications have been carried out widely in recent years. Three strains of bacteria belonging to the species *Bacillus amyloliquefaciens*, *Bacillus velezensis,* and *Bacillilus subtilis* were isolated and characterized with capacities of degrading insoluble nutrients in the soil, decomposing cellulose quickly, and growing well in the composting process in Binh Thuan province [12]. *B. velezensis* showed high inhibitory efficiency against fungal pathogen *Phytophthora* sp. [13].

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In the present study, we attempt to isolate and identify a strain of *B. velezensis* that performs promising properties for agricultural application in Thanh Hoa province, Vietnam. The optimal conditions for mass production of strain *B. velezensis* are also examined to facilitate the production of microbiological fertilizers.

2. Methods

2.1. Soil sample collection and isolation of Bacillus sp.

Fifteen grams of soil sample from the root zone at depth of 5-10 cm were collected using a sterile inoculating spoon in Quang Xuong district, Thanh Hoa province, Vietnam. Each sample was packaged in a sterile bottle, labeled appropriately. The samples were stored at 4-8^{o}C until ready for processing. Ten grams of each soil sample were suspended in 90 mL of sterilized distilled water (SDW) in universal bottles. The soil suspension was heat-shocked at 60^{o}C for one hour in a water bath to kill non-spore-forming organisms [14]. The samples were shaken for 10 minutes (min) at 150 rpm and left to stand for 10 minutes afterward. Then 3 serial 10-fold dilutions were made. Finally, 1 mL of the serially-diluted suspensions was spread on Nutrient Agar (peptone 5 g/L, yeast extracts 3 g/L and agar 20 g/L, pH 7,0-7,2). Petri plates were sealed and incubated at 37^{o}C for 24h and examined for colony morphology. The colonies that exhibited typical characteristics of *Bacillus* species (round or irregular thick and opaque; cream-colored colonies) were sub-cultured onto nutrient agar plates for further screening to obtain pure cultures [1].

2.2. Identification of Bacillus sp. by 16S rRNA gene sequence analysis

Genes of 16S rRNA were sequenced and blasted for identification of the bacterial species. The bacterial cells were harvested for chromosomal DNA isolation and purification. The 16S rRNA gene was amplified via the polymerase chain reaction (PCR) following the protocol provided by Bio-techem CO., LTD (www.biotechem.com.vn). After the amplification was completed, the fragment was detected by agarose gel electrophoresis, recovered, and sequenced. The 16S rRNA sequences were confirmed and compared using a BLAST nucleotide search provided by the National Center for Biotechnology Information (NCBI) GenBank (U.S. National Library of Medicine, Bethesda, Maryland, USA). The phylogenetic tree was constructed using the maximum likelihood method by MEGA 5.1 software [11].

2.3. Identification of the optimal conditions for mass production of Bacillus sp.

For optimal carbon source: To standardize the medium for the optimum growth of *Bacillus* sp., a preliminary shake flask culturing was conducted to select the most suitable carbon source. The conical flask was added with 10 g of carbon source (either Glucose, Sucrose, Lactose or Glycerol), 10 g yeast extract, 1.5 g NaCl, K2HPO4 1.5 g, MgSO4 1.5 g per liter, pH 7.0 and incubated on a shaker at speed of 120 rpm, 35^{o}C for 48 hours. The effective bacterial growth was tested via serial dilutions, spreading diluted suspensions of

Bacillus sp. culture on plates with media containing different sources of carbon, and incubated for 24 hours.

For optimal nitrogen source: Three nitrogen sources including Soybean flour, Yeast extract, and Peptone were examined for the selection of the best one. The batch process was started with an initial volume of 2 L of either sterile molasses soybean flour (molasses 10%, soybean four 2%), molasses yeast extract (molasses 10%, yeast extract 1%), or molasses peptone (molasses 10%, peptone 1%) media in pH 7. They were inoculated with a 25 mL shake flask pre-inoculated *B. velezensis* BL-1 culture at 35°C on a shaker at 120 rpm to agitate the culture. The optimum aeration was standardized using an aquarium air pump, 8-Liter/ min (VIPSUN, China). The effective bacterial growth in each tested medium was tested by serial dilution and spreading *B. velezensis* BL-1 culture on plates for 24 hours.

For optimal duration of batch fermentation: Batch cultures were carried out in a fermentor with 5 mL capacity. Two liters of Soybean flour molasses medium was sterilized, then 40 mL of this pre-inoculated bacterial culture broth (10^6 CFU/mL) was inoculated into 2 L of King's B broth or soybean flour liquid, followed by incubation at 35°C on a shaker at 120 rpm to agitate the culture, under aerobic condition (pumping fresh air) for 2 days. The viable cell density of bacteria was counted at 12, 24, 48 hours after incubation by serial dilution and spread planting of *B. velezensis* BL-1 culture for 24 hours.

2.4. General procedure for production of Bacillus sp.

Bacillus sp. were streaked onto King's B medium (peptone 15 g/L, K_2HPO_4 1.5 g/L, $MgSO_4$ 15 g/L, glycerol 10 mL/L, agar 20g/L, pH 7,0 – 7,2) and incubated at 35°C for 2 days.

Bacterial liquid cultures were carried out in a 250 mL flask with a working volume of 100 mL of King'B broth. Transfer a loopful of *Bacillus* sp. culture to 100 mL of sterilized King's B broth in a 250 mL conical flask and incubate on a shaker at 120 rpm, 35°C for 48 hours. The number of cells was counted as colony-forming units (CFUs) by making serial dilutions, spreading suspensions on King's B medium plate, and incubating at 35°C for 1 day. Cell density was adjusted to the optimum for subsequent use.

Batch cultures were carried out in a fermentor of 5 L capacity, prepare 2 L of King's B broth or soybean flour molasses broth, and sterilize. Then, 40 mL of this pre-inoculated bacterial culture (10^6 CFUs/mL) was inoculated into 2 L of King's B broth or soybean flour liquid, followed by incubation at 35°C, under aerobic condition (pumping fresh air) for 2 days. The viable cell density (CFUs/mL) of the culture was determined and adjusted for use.

Sterilize substrate (talc powder) at 121°C, 1 atm for 30 min in two successive days. Transfer 1 kg of the sterilized substrate into a polythene bag or sterile container under aseptic conditions and add bacterial broth culture to reach the cell density of 1 - 2 x 10^8 CFUs/g. Mix thoroughly and shade dry to bring its moisture content to less than 20%. The formulation was packed into a polythene bag and could be stored for 3 - 4 months under room temperature (RT).

3. Results and discussion

3.1. Morphological characterization

Colonies of bacterial isolate BL-1 were flat on King'B solid medium, with a serrated edge, dirty white, rough surface, opaque, viscous, and 3–5 mm in diameter at 30°C for 1 day after inoculation (Fig. 1A). Gram staining showed blue-purple stained cells, indicating that the isolate BL-1 was Gram-positive (Fig. 1B). The results indicated that isolate BL-1 had close morphology with the genus *Bacillus*, which made the preliminary reference for the next step of identification by sequence homology among published reference sequences with the BLAST tool.

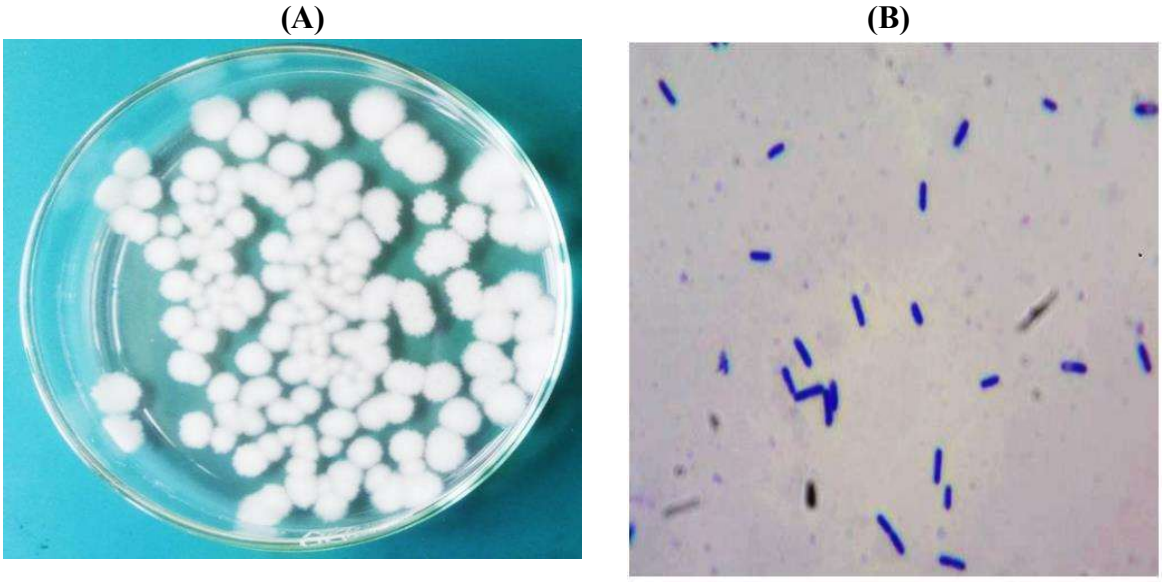

***Figure 1**. Colony growth of BL-1 on King'B medium after 1 day inoculation (A) and Gram-positive cells under the microscope (**B**)*

3.2. Genetic identification of isolate BL-1 based on phylogenetic analysis of 16S rRNA

A successful PCR yields a single band of the expected size of 16S rRNA gene of isolate BL-1 that was purified and then sequenced (Fig. 2A). The size of 16S rRNA gene of isolate BL-1 was determined as 1437 bp. The gene sequence of BL-1 was compared with sequences deposited in the 16S rRNA database, which resulted in identification at the species level with an identity at 100% with a known *Bacillus velezensis* strain EN01 (CP053377.1) (Fig. 2A, B).

The genetic distance scale is the reference of each branch length in the phylogenetic tree (Fig. 3). The location of the nodes represents the genetic relationship. The shorter the branch length between two end nodes means the relationship is closer. Based on the consistency between the results of the 16S rRNA sequence analysis and the physiological characterization, isolate BL-1 was indicated as a strain of *Bacillus velezensis* BL-1.

(A) 16S rRNA gene sequence of isolate BL-1 (1437bp)

CCCCAATCATCTGTCCCACCTTCGGCGGCTGGCTCCATAAAGGTTACCTCAC
CGACTTCGGGTGTTACAAACTCTCGTGGTGTGACGGGCGGTGTGTACAAGG
CCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCC
AGCTTCACGCAGTCGAGTTGCAGACTGCGATCCGAACTGAGAACAGATTTG
TGGGATTGGCTTAACCTCGCGGTTTCGCTGCCCTTTGTTCTGTCCATTGTAG
CACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCA
CCTTCCTCCGGTTTGTCACCGGCAGTCACCTTAGAGTGCCCAACTGAATGCT
GGCAACTAAGATCAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTC
ACGACACGAGCTGACGACAACCATGCACCACCTGTCACTCTGCCCCCGAAG
GGGACGTCCTATCTCTAGGATTGTCAGAGGATGTCAAGACCTGGTAAGGTT
CTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCC
GTCAATTCCTTTGAGTTTCAGTCTTGCGACCGTACTCCCCAGGCGGAGTGCT
TAATGCGTTAGCTGCAGCACTAAGGGGCGGAAACCCCCTAACACTTAGCAC
TCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTCGCTCCCCAC
GCTTTCGCTCCTCAGCGTCAGTTACAGACCAGAGAGTCGCCTTCGCCACTGG
TGTTCCTCCACATCTCTACGCATTTCACCGCTACACGTGGAATTCCACTCTC
CTCTTCTGCACTCAAGTTCCCCAGTTTCCAATGACCCTCCCCGGTTGAGCCG
GGGGCTTTCACATCAGACTTAAGAAACCGCCTGCGAGCCCTTTACGCCCAA
TAATTCCGGACAACGCTTGCCACCTACGTATTACCGCGGCTGCTGGCACGT
AGTTAGCCGTGGCTTTCTGGTTAGGTACCGTCAAGGTGCCGCCCTATTTGAA
CGGCACTTGTTCTTCCCTAACAACAGAGCTTTACGATCCGAAAACCTTCATC
ACTCACGCGGCGTTGCTCCGTCAGACTTTCGTCCATTGCGGAAGATTCCCTA
CTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCCGAT
CACCCTCTCAGGTCGGCTACGCATCGTCGCCTTGGTGAGCCGTTACCTCACC
AACTAGCTAATGCGCCGCGGGTCCATCTGTAAGTGGTAGCCGAAGCCACCT
TTTATGTCTGAACCATGCGGTTCAGACAACCATCCGGTATTAGCCCCGGTTT
CCCGGAGTTATCCCAGTCTTACAGGCAGGTTACCCACGTGTTACTCACCCGT
CCGCCGCTAACATCAGGGAGCAAGCTCCCATCTGTCCGCTCGAC

(B) Blast results

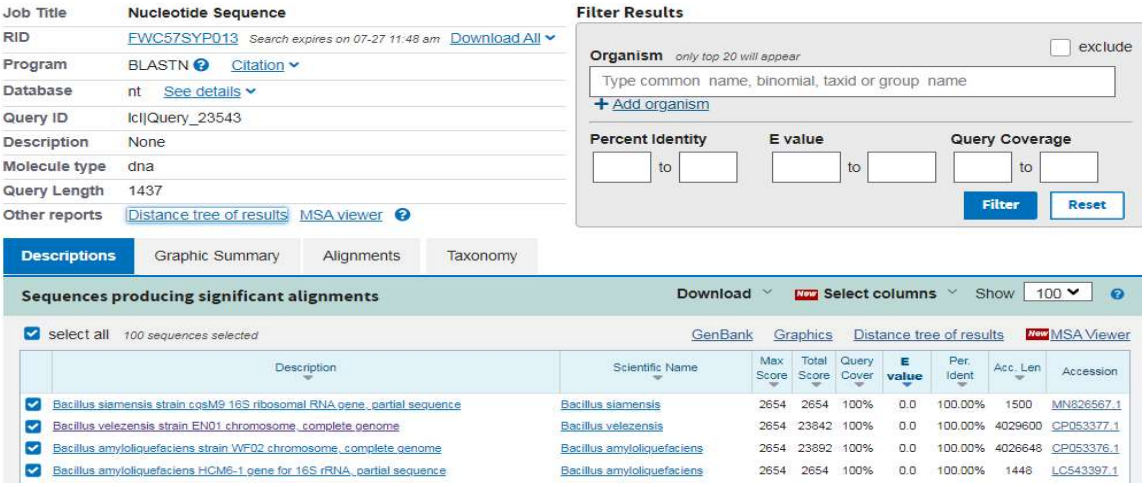

***Figure 2**. Identification of isolate B. velezensis strain BL-1. (**A**) The 16S rRNA gene sequence of isolate BL-1; (**B**) The 16S rRNA sequences were confirmed and compared through a BLAST nucleotide search provided by NCBI GenBank.*

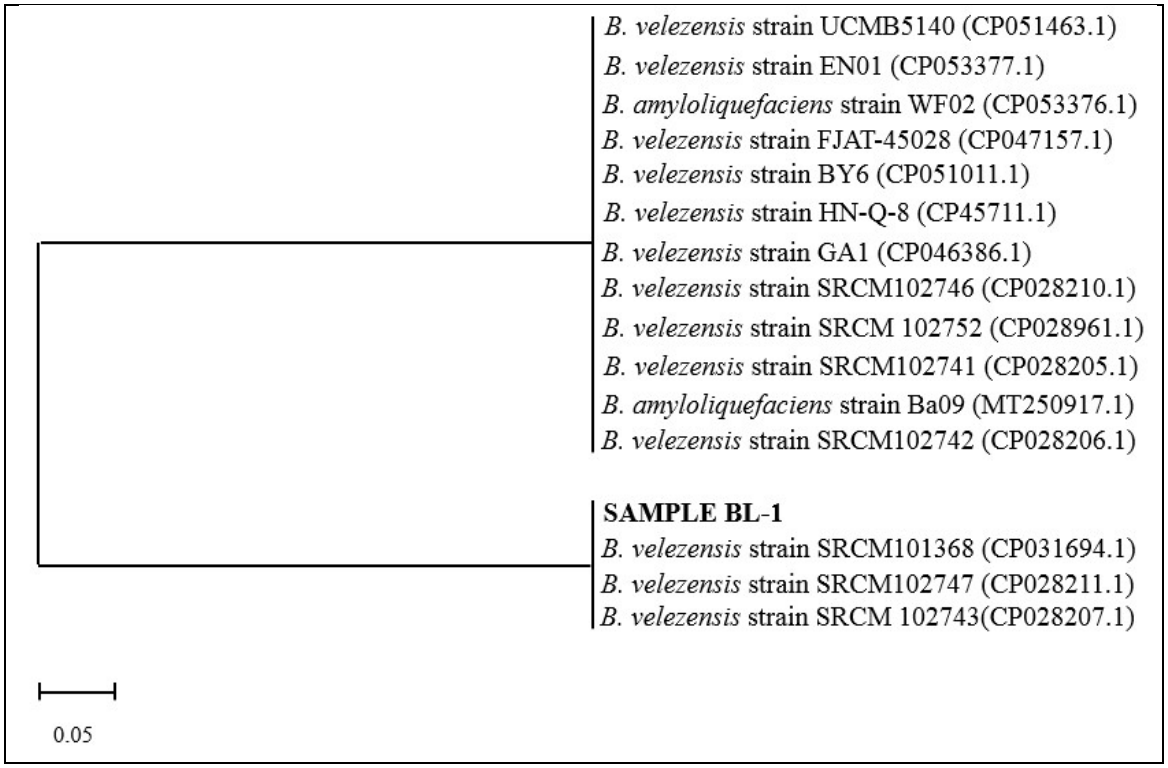

Figure 3. *Phylogenetic tree based on the partial 16S rRNA gene of BL-1 and other homologous Bacillus strains. The bar represents 0.05 substitutions per site*

3.3. Identification of the optimal conditions for BL-1 strain

3.3.1. Identification of the optimal carbon source in shake flask culturing for BL-1 strain

Four carbon sources (either Glucose, Sucrose, Lactose or Glycerol) were examined for the optimum growth of strain *B. velezensis* BL-1. Among the fourth tested carbon sources for culturing *B. velezensis* BL-1, number of the viable cells cultured on sucrose medium was 4.24 x 10^8 CFU/mL, on average, which was significantly higher than that on other media. Therefore, sucrose was selected as an ideal carbon source for further scale-up production of *B. velezensis* BL-1 (Table 1). Many experiments have proven that in terms of the growth of *Bacillus subtilis*, sucrose was one of the optimal carbon sources in culture broths [15].

Table 1. *Culture profile of Bacillus velezensis BL-1 using different carbon source*

Carbon source	Total viable cells (CFU/mL)
Glucose	3.37 x 10^8 ± 0.08 x 10^8
Glycerol	1.08 x 10^8 ± 0.02 x 10^8
Sucrose	4.24 x 10^8 ± 0.06 x 10^8
Lactose	2.23 x 10^8 ± 0.03 x 10^8

Note: Data present means ± standard error of the mean (SEM)

3.3.2. Identification of the optimal nitrogen source in batch cultivation

The effective bacterial growth in each of 3 nitrogen sources including Soybean flour, Yeast extract, and Peptone was tested by serial dilution and spreading *B. velezensis* BL-1 culture on plates for 24 hours. The results revealed that soybean flour was the most optimal nitrogen source which performed high efficiency (7.07×10^8 CFU/mL) in a low cost, and was suitable for mass production of *Bacillus velezensis* BL-1 (Table 2). The present result differed from the study of Peighmi-Ashnari et. al.(2009) which reported that molasses and yeast extract based media to be the most suitable for rapid growth and high cell yield of *B. subtilis* [9].

Table 2. *Culture profile of Bacillus velezensis BL-1 using different nitrogen sources*

Nitrogen source	Total viable cells (CFU/mL)
Soybean flour	$7.07 \times 10^8 \pm 0.20 \times 10^8$
Yeast extract	$3.41 \times 10^8 \pm 0.09 \times 10^8$
Peptone	$4.51 \times 10^7 \pm 0.05 \times 10^8$

Note: Data present means ± standard error of the mean (SEM)

3.3.3. Identification of the optimal duration for batch fermentation

The optimal time length for batch fermentation of *B. velezensis* BL-1 was examined by performing bacteria cultrure for 2 days in medium of soybean flour (2%) flus molasses (10%). The result indicated that the viable cell reached the highest number at 48 hours of incubation (5.2×10^8 CFU/mL). This also suggested that the optimal fermentation conditions for *B. velezensis* BL-1 were 48 hours incubation at 35°C, pH 7.0, and 2% of inoculum size (Table 3). Nakkeran et. al. (2006) reported that B. subtilis growth was good at temperature of 28±2°C [7]. Whereas, Korsten and Cook (1996) reported that temperature of 30-37°C and pH of 7-8 good for the growth and multiplication of *B. subtilis* [5].

Table 3. *Bacterial growth in the batch fermentation using soybean molasses-based medium*

Medium	Total viable cells (CFU/mL) after incubation (hours)		
	12	24	48
Soybean flour (2%) + Molasses (10%)	$6.8 \times 10^7 \pm 0.17 \times 10^7$	$3.4 \times 10^8 \pm 0.28 \times 10^8$	$5.2 \times 10^8 \pm 0.31 \times 10^8$

Note: Data present means ± standard error of the mean (SEM)

4. Conclusions

In the present study, a spore-forming and rod-shaped isolated BL-1 strain was successfully isolated from soil and identified as *Bacillus velezensis* based on morphological and phylogenetic analysis of 16S rRNA. We also proposed a protocol for *Bacillus velezensis* BL-1 mass production using soybean flour and molasses as an optimal nitrogen and carbon source based on batch culture fermentation technique. The optimal duration for

batch fermentation of *Bacillus velezensis* BL-1 using medium of soybean flour (2%) plus molasses (10%) was a 48-hour incubation, which offered the highest yield of bacterial growth (> 5 x10^8 CFU/mL).

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