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Isolation and Characterization of *Bacillus velezensis* from Lake Bogoria as a Potential Biocontrol of *Fusarium solani* in *Phaseolus vulgaris* L.

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Abstract: The common bean (*Phaseolus vulgaris* L.) is a significant vegetable crop, grown because it is a rich source of protein, carbohydrates, and vitamin B complex. *Fusarium solani* and *Rhizoctonia solani* are the most widely known pathogens contributing to large yield losses for this crop. The use of cultural and chemical control practices has been ineffective. Therefore, a sustainable, affordable, and effective control method is urgently required. In this study, we aimed to isolate and characterize *Bacillus velezensis* from Lake Bogoria as a potential biocontrol agent for *Fusarium solani*. Bacteria were isolated from soil and sediments using the serial dilution technique. Molecular characterization was performed using the 16S rRNA gene. A total of 13 bacteria were isolated from soil and sediments. Based on the partial sequences, BLAST analysis showed two isolates, B20 (*Bacillus velezensis strain* QH03-23) and B30 (*Bacillus velezensis strain JS39D*), belonging to *Bacillus velezensis*. Other isolates were identified as *Bacillus tequilensis*, *Brevibacillus brevis*, *Bacillus subtilis*, *Bacillus amyloliquefaciens*, and *Bacillus licheniformis*. The effectiveness of their antifungal properties was determined via co-culturing, and we found mycelial inhibition rates of 28.17% (for B20) and 33.33% (for B30) for the *Fusarium solani* isolates. The characterization of the *Bacillus velezensis* strain revealed that they were Gram-positive and grew well at pH 7.0 and 8.5, although growth was recorded at pH 5.0 and 10.0. In terms of temperature, the optimal temperature conditions were 30−35 °C, with an optimum salinity of 0–0.5 M NaCl. When these isolates were tested for their ability to produce secondary metabolites, they were found to produce phosphate, pectinase, chitinase, protease, indole -3- acetic acid (IAA), and hydrogen cyanide (HCD), making them potential biocontrol agents.

Keywords: isolation; *Bacillus velezensis*; *Fusarium solani*; biocontrol; characterization

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1. Introduction

Phaseolus vulgaris L. is a yearly herbaceous plant, grown for its edible dry seeds [1]. Depending on their uses, common beans can be classified as dry beans, snap beans, and shell beans. The leaves are used as vegetables and animal feed. According to their botanical classification as members of the Fabaceae legume family, they obtain nitrogen through a symbiotic relationship with Rhizobia, a nitrogen-fixing bacterium of beans [1]. Common beans' dry seeds and leaves contain antioxidants, phenolic compounds, flavonoids, vitamins B complex, and essential metal ions, such as copper, zinc, and calcium. They have also exhibited anticancer and anti-inflammatory effects and can increase capillary resistance [2]. About half a million tons (Mt) of beans are harvested each year in Kenya.

A major difficulty in growing beans is the presence of pests and diseases. Cutworms, bean flies, red spider mites, aphids, pod borers, whiteflies, and thrips are some problematic crop pests. *Fusarium solani* fungus is an important soil-borne pathogen of common beans that causes fusarium wilt [2,3]. This fungus can remain viable for several years in the

soil due to its ability to produce chlamydospores [1]. *Fusarium wilt* is one of the most difficult diseases to control due to its ability to inhabit the soil and penetrate the plant through the root systems. It can infect more than 600 species of legumes [4]. Therefore, this has increased its survival rate and growers' inability to manage it. Water stress, soil acidification, and calcium deficiency also favor fungal development. Given the lack of a fully effective control mechanism for *Fusarium solani*, several studies have been conducted to develop biological approaches for managing this pathogen [2,3]. Several *Bacillus species* are known to act as antagonists of phytopathogenic fungi and have been used in biological control programs [5–7]. As described in [8], *Bacillus velezensis* has been used as a potential biocontrol agent for fusarium head blight. Furthermore, the research presented in [3] indicates that *Bacillus velezensis* is responsible for controlling bitter apple rot caused by *Colletotrichum gloeosporioides*.

There is a need for the biocontrol of this pathogen due to the prolonged use and reduced efficacy of synthetic fungicides. These chemicals also pollute the environment, leaving toxic residues that broaden the range of resistant pathogens [9]. Therefore, biological control has been reported as an alternative method to control *Fusarium* wilt [7,10–12].

In our study, we aimed to isolate and characterize *Bacillus velezensis* from Lake Bogoria as a potential biocontrol agent of *Fusarium solani* in *Phaseolus vulgaris* L. Lake Bogoria is an example of a Kenyan soda lake, known for its hot springs and thus providing a habitant for extremophilic bacteria and fungi. Soda lake microorganisms have been used for decades, but very little is known about their effectiveness in combating and controlling *Fusarium solani*. The use of *Bacillus velezensis* as a biological control agent may have several advantages, such as being suitable for the environment and providing efficient management [13,14] The bioactivity of *Bacillus velezensis* isolated from Lake Bogoria as a potential biocontrol agent against *Fusarium solani* represents a novel discovery and will open up more opportunities for research in agriculture.

2. Results

2.1. Isolation and Morphological Characteristics

A total of 13 bacterial isolates were obtained from Lake Bogoria.

Morphological characterization was performed using microscopic techniques to determine colony and cell morphology. The colony morphology characteristics included forms ranging from circular to irregular, as well as various elevations (Table 1). The isolates also exhibited differences in their margins, ranging from circular to irregular, wavy, lobate, smooth, and filamentous. The color of the isolates ranged from white to cream, cream-white, teal, blue, and cream-yellow. Their sizes ranged from small to medium and large.

Table 1. Morphological characterization of bacteria isolated from Lake Bogoria.

	Colony Morphology						Cell Morphology	
Isolate	**Form**	**Elevation**	**Margin**	**Size**	**Color**	**Surface**	**Shape**	**Gram Staining**
B7	Circular	Raised	Circular	Medium	Cream	Smooth	*Streptococcus*	Positive
B11	Circular	Raised	Irregular	Small	White	Dull	*Diplococcus*	Positive
B12	Circular	Flat	Circular	Small	Cream	Smooth	*Coccus*	Negative
B17	Circular	Raised	Circular	Small	Teal	Smooth	*Coccus*	Negative
B19	Irregular	Raised	Circular	Small	Blue	Smooth	*Coccus*	Negative
B20	Penctiform	Flat	Wavy	Medium	Cream-white	Smooth	*Coccus*	Positive
B21	Irregular	Flat	Lobate	Medium	Cream	Rough	*Bacillus*	Positive
B26	Circular	Raised	Circular	Small	Cream	Smooth	*Streptococcus*	Positive
B29	Irregular	Raised	Irregular	Medium	Cream-white	Smooth	*Coccus*	Positive
B30	Irregular	Raised	Circular	Medium	Cream-white	Smooth	*Streptobacillus*	Positive
B32	Irregular	Flat	Filamentous	Large	Cream	Dull	*Streptococcus*	Positive
B38	Circular	Raised	Smooth	Medium	Cream-yellow	Smooth	*Coccus*	Positive
B39	Circular	Raised	Smooth	Medium	Cream-white	Smooth	*Bacillus*	Positive

The cell morphology of the pure isolates was assessed in terms of their shape, ranging from rods to cocci. In addition, the surface of the isolates was determined and characterized as smooth, dull, or rough. The Gram staining technique showed that 10 bacterial isolates were Gram-positive and 3 bacterial isolates were Gram-negative (Table 1).

2.2. Molecular Characterization

From the partial sequences, the BLAST analysis showed two isolates B20 (*Bacillus velezensis* strain QH03-23) and B30 (*Bacillus velezensis* strain JS39D) as *Bacillus velezensis* strains. Among the other isolates were *Bacillus tequilensis, Brevibacillus brevis, Bacillus subtilis, Bacillus amyloliquefaciens,* and *Bacillus licheniformis* (Figure 1). Three isolates belonged to Gammaproteobacteria in the proteobacteria phylum with a 99.59% to 100% similarity index. Among the proteobacteria groups were the *Alcaligenaceae bacterium*, *Pseudomonas* sp., and *Pseudomonas aeruginosa* (Figure 1).

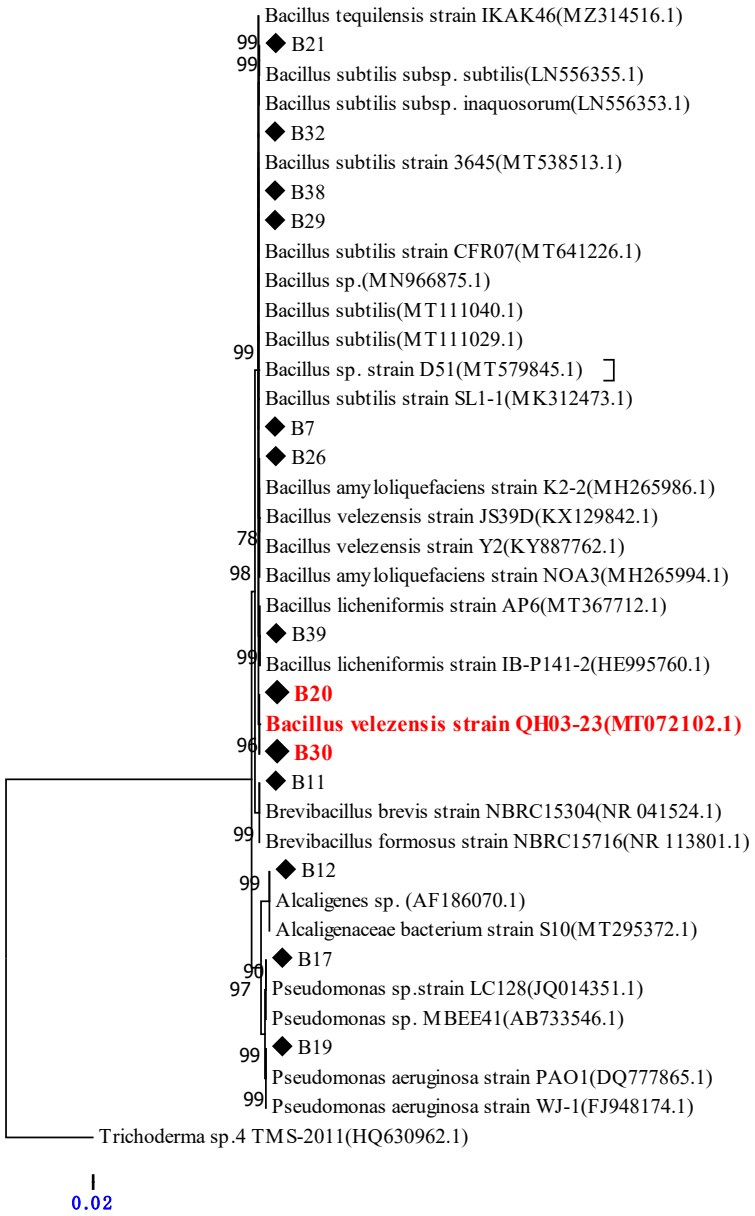

Figure 1. Phylogenetic tree of bacterial isolates from Lake Bogoria based on 16S rRNA sequences. (B) Bogoria isolates. The evolutionary history was inferred using the maximum likelihood method based on the Kimura 2-parameter model. A scale bar of 0.02 was used.

2.3. Evaluation of *Bacillus velezensis* against *Fusarium solani*

In Table 2, the ANOVA analysis showed a significant ($p < 0.05$) variation in the diameter of the *F. solani* mycelium after the tested bacterial treatment. Isolate B30 (33.33%) had the highest inhibition rate compared to B20 (28.17%) (Figure 2).

Table 2. Antifungal activity of *Bacillus velezensis* strains (B20 and B30) on *Fusarium solani* mycelium growth after 14 days.

Lakes	Isolate Code	Mycelium Length (cm)	Inhibition Rate %
Bogoria	Control	8.4 ± 0.00^{a}	0.00
	B20	6.03 ± 0.25^{b}	28.17
	B30	5.60 ± 0.20^{c}	33.33

The mean values with the same superscript letter(s) within the same column are not significantly different by the Fisher test. According to the Fisher's LSD test ($p < 0.05$), different superscript letters ($^{a, b, c}$) indicate significantly different means within a column 2.4.

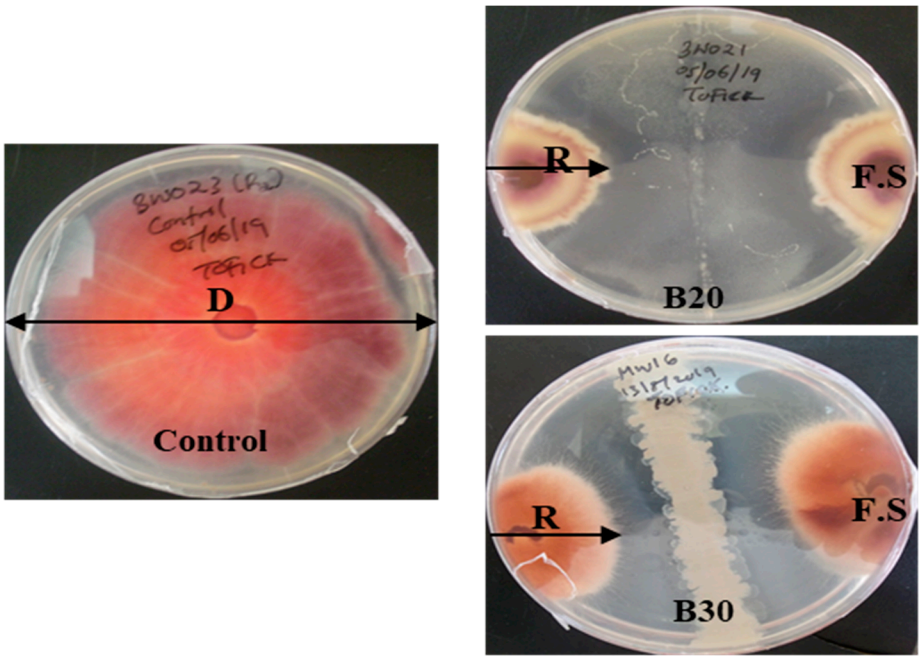

Figure 2. Antibiosis assay of *Bacillus velezensis strains* (B20 & B30) against *Fusarium solani* after 14 days of incubation at 30 °C ± 2.0 using the co-culturing technique: (D) diameter of pathogen mycelium in control, (R) radius of pathogen mycelium co-cultured with bacterial isolate, (F.S) *Fusarium solani*, (B20) *Bacillus velezensis strain QH03-23*, and (B30) *Bacillus velezensis strain JS39D*.

2.3.1. Growth at Different pH

Figure 3 shows a varied growth of Isolates B20 and B30 at different pH. Although the isolates were obtained from an alkaline environment, both isolates could grow at pH 5.0 and 7.0. Isolate B20 recorded the highest O.D. at pH 8.5, while isolate B30 was at pH 10.0. The growth trend at pH 5.0, pH 7.0, and pH 8.5 increased significantly for isolate B20 compared to isolate B30.

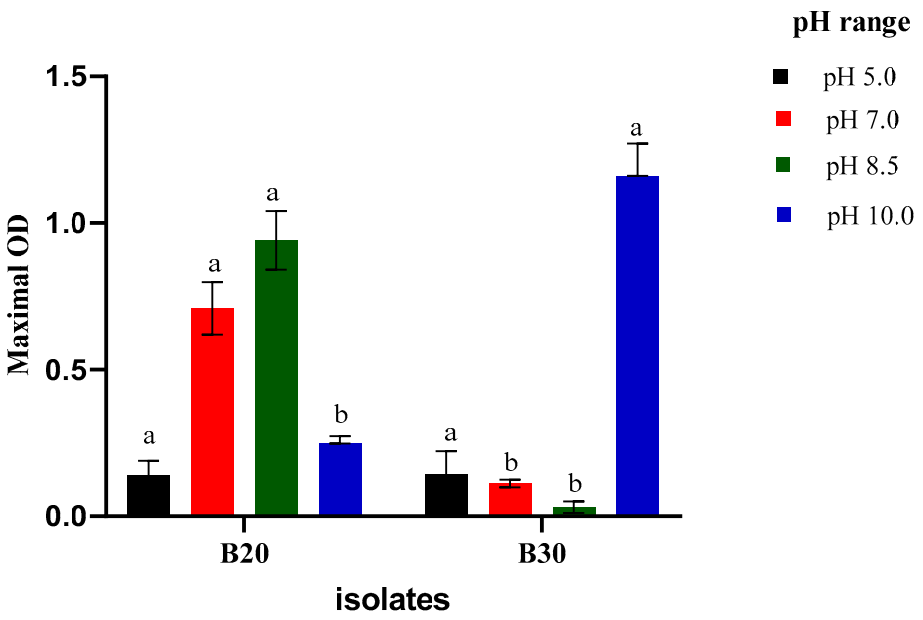

Figure 3. The growth of isolates B20 and B30 at different pH. The results are the mean values of three replications. The vertical bars represent standard errors (S.E.), and the different letter above the errors bar indicate the separation of means according to different letter means significant differences according to Fisher's LSD test at $p < 0.05$. The same letters per pH indicates no significant differences and different letters per pH indicates significant differences according to Fisher's LSD test.

2.3.2. Growth at Different Salinity

The isolates B20 and B30 showed varied growth at different concentrations of sodium chloride concentration. The salt concentration of 0.0 M recorded the highest O.D. as an indicator of the highest growth, followed by 0.5 M NaCl, and gradually decreased toward 2.0 M with minimal growth (Figure 4). Additionally, the growth trend decreased with an increase in sodium chloride concentration.

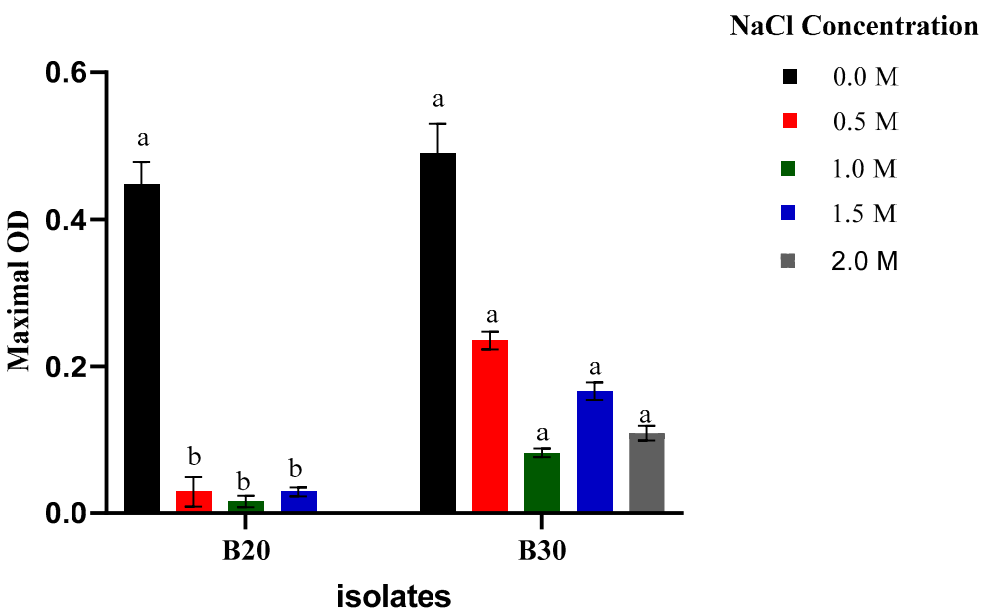

Figure 4. The growth of Isolates B20 and B30 at different salt concentrations. The results are the mean values of three replications. The vertical bars represent standard errors (S.E.), and the letter above the errors bar indicate the separation of means according to Fisher's LSD test at $p < 0.05$. The same letters per salt concentration indicates no significant differences and different letters per salt concentration indicates significant differences according to Fisher's LSD test.

2.3.3. Growth at Different Temperatures

The isolates B20 and B30 grew at a wide range of temperatures (20–60 °C). Optimal growth was observed at 30–40 °C (Figure 5). However, optical growth was observed at 35 °C.

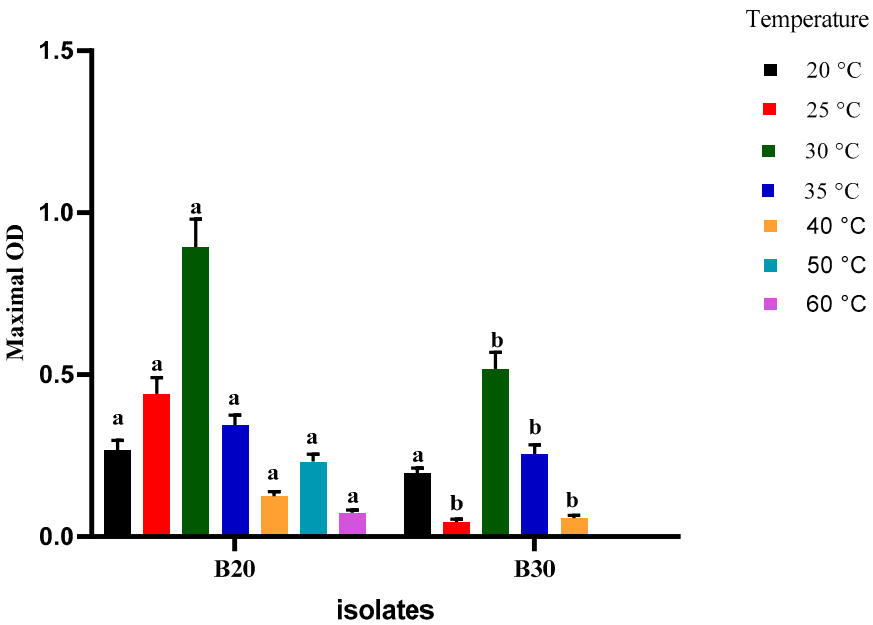

Figure 5. The growth of isolate B20 and B30 at different temperatures. The results are the mean values of three replicates. The vertical bars represent standard errors (S.E.), and the letter above the errors bar indicate the separation of means according to different letter means significant differences according to Fisher's LSD test at $p < 0.05$. The same letters per temperature indicates no significant differences and different letters per temperature indicates significant differences according to Fisher's LSD test.

2.4. Enzymatic Bioassay of Isolate B20 and B30

The isolates B20 and B30 on skim milk agar showed a clear zone of inhibition formed around the isolates, indicating a protease-producing strain (Figure 6). B20 and B30 did not show a clear zone of inhibition for pectinase (Table 3).

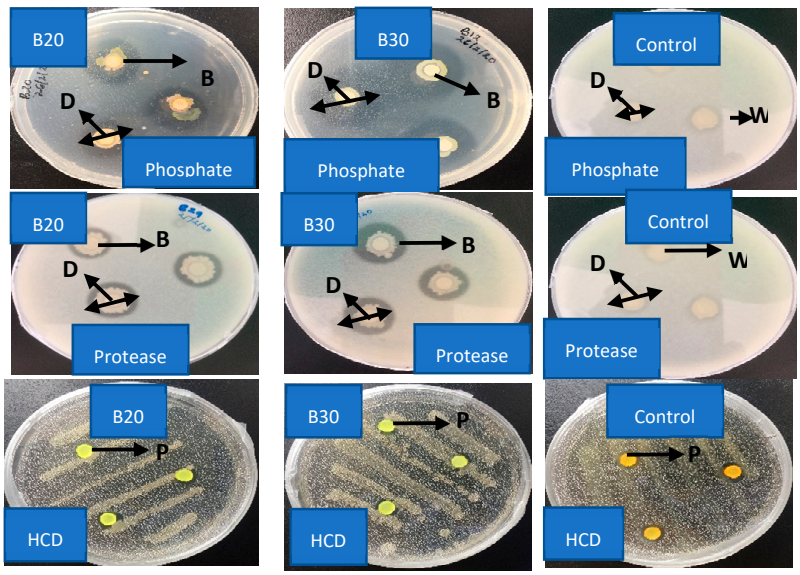

Figure 6. Enzymatic activity of isolates B20 and B30: (B) disc with bacteria, (D) diameter of zone of inhibition, (P) disc with picric alkaline, and (W) disc with sterile water.

Table 3. Functional characteristics of Isolates B20 and B30 from Lake Bogoria against *Fusarium solani*.

Isolate Code	Protease	Pectinase	Phosphate	Chitinase	HCD	Indole-3-Acetic Acid
Control	0.00 ± 0.00^{c}	0.00 ± 0.00^{a}	0.00 ± 0.00^{c}	0.00 ± 0.00^{b}	−ve	−ve
B20	1.23 ± 0.03^{b}	0.00 ± 0.00^{a}	1.93 ± 0.03^{a}	0.00 ± 0.00^{b}	−ve	+ve
B30	1.70 ± 0.06^{a}	0.00 ± 0.00^{a}	1.40 ± 0.00^{b}	1.37 ± 0.03^{a}	−ve	+ve
CV	6.82		3.00	7.32		
LSD	0.13	0.00	0.07	0.07		
p>	0.0001		0.0001	0.0001		

The mean values with the same superscript letter(s) within the same column are not significantly different by the Fisher test. According to the Fisher's LSD test ($p < 0.05$), different superscript letters (^{a}, ^{b}, ^{c}) indicate significantly different means within a column. Hydrogen cyanide (HCD).

3. Discussion

Soil-borne fungal diseases cause worldwide economic losses in the agriculture sector. Farmers over the past years have relied on the use of chemical fungicides, which can potentially be harmful to the environment and human health. Additionally, the use of fungicides is linked to soil infertility due to pH change and low yield. In this study, we highlight the alternative use of *Bacillus velezensis* as an eco-friendly solution for the control of soilborne diseases. In recent years, the use of *Bacillus* as biological control has been reported. A member of this genus is *B. velezensis,* a species considered valuable due to its potent bioactive molecule. In this context, two *B. velezensis* were recovered from the soil and sediment of Lake Bogoria, which is thermophilic because of its high-temperature range. The lake is also classified as a highland lake by [15], with a surface more than 990 m above sea level.

The isolation of bacteria through serial dilution technique clearly showed that lake Bogoria harbors diverse microbes of different morphological characteristics. The study indicates that microbes occur in various ecological environments, such as soil and sediment of the alkaline ecosystem; hence, had the potential to survive a harsh environment. Several studies have indicated that Lake Bogoria is rich in the microbial community. For example, Kambura Et al., [16] reported lake's microbial community, ranging from *Bacillus* to Gammaproteobacteria species. Furthermore, the study by [17] indicates that Lake Bogoria has a wide range of *Bacillus* species with antibiotic-producing properties. The lake, which is saline and thermophilic, has a wide range of enzyme-producing bacteria that have been exploited, identified, and used in various biotechnological fields [18–20]. Soil microbial communities are responsible for the cycling of nutrients, stabilizing the ecosystem, and maintaining the structure of the soil. Approximately 90% of the bacteria are found in the soil, contributing to the soil's nutrient content compared to sediment. Grasslands have also been shown to have more diverse bacterial communities due to biomass stability than water and sediments [21]. Studies have shown a relationship between microbes and environmental factors, geographical location [22,23], pH [22,23], nutrients [21,23], and temperature.

In our study, *B. velezensis* was identified based on partial 16S rDNA genes where two different strains were obtained. They include Isolate B20, closely related to *Bacillus velezensis* strain QH03-23 (MT072102.1), and B30, related to *Bacillus velezensis* strain JS39D (KX129842.1), with a similarity index of 98.71–100%. Additionally, other *Bacillus* spp. such as *B. subtilis*, *B. amyloliquefaciens*, *B. licheniformis* and *Brevibacillus* were identified. Others belong to the class of *Gammaproteobacteria* in the phylum *proteobacteria* with a range of 98.71–100% similarity index, such as *Alicagenes* sp and *Pseudomonas* spp. These results are consistent with previous studies on L. Bogoria, where phylogenetic analysis of the bacterial 16S rRNA gene sequence of the bacteria sequence showed Firmicutes and *Gammaproteobacteria* as the dominant bacteria domains [17,18,24,25].

In vitro tests, through co-culturing experiments, clearly showed that B20 and B30 could inhibit the fungal radial growth of *Fusarium solani*. Interestingly, B30 (*B. velezensis* Strain JS39) produced the highest inhibition rate compared to B20 (*B. velezensis* Strain

QH03-23). Numerous strains of this species have been reported to suppress the growth of microbial pathogens, including bacteria, fungi, and nematodes. Genomic analysis has revealed that *B. velezensis* possesses strain-specific clusters of genes related to the biosynthesis of secondary metabolites, which play significant roles in pathogen suppression and plant growth promotion. Specifically, *B. velezensis* exhibits a high genetic capacity to synthesize cyclic lipopeptides such as surfactin, bacillomycin D, fengycin, and bacillibactin, and polyketides such as macrolactin, bacillaene, and difficidin [26]. Secondary metabolites produced by *B. velezensis* can also trigger trigger-induced systemic resistance in plants, a process by which plants defend themselves against recurrent attacks by virulent microorganisms. According to [14], putative butanediol dehydrogenase, a critical enzyme in butanediol production, is known to induce systemic resistance (ISR) in plants. Therefore, the presence of this gene suggests that *B. velezensis* may contribute to plant defenses through ISR, allowing plants to prepare themselves for faster and stronger defenses against pathogens [13]. Surfactins are known to have antimicrobial properties that act against both bacteria and fungi. They can be inserted into bacterial cell membranes, solubilizing the fluid phospholipid bilayer and creating pores and ion channels [26,27]. Surfactins have also been shown to interfere with protein processing and secretion. For antifungal activity, surfactins can inhibit glucan synthase, which is involved in cell wall synthesis, and induce apoptotic markers [26]. Furthermore, surfactins are believed to play a key role in ISR triggering [28]. They may also contribute to swarming motility [29], which can help achieve effective rhizosphere colonization and facilitate plant growth-promoting traits while simultaneously inhibiting competing microorganisms through antimicrobial activity. The use of *B. velezensis* as a potential control against *F. solani* has been reported by [3,8,13,30–32]. Therefore, more exploitation must be evaluated to quantify its mode of action. Additionally, *B. velezensis* is attracting attention as a valuable biocontrol agent. Consequently, to develop and formulate biobased products, it is increasingly important to understand the antifungal potential of biosynthesis of *B. velezensis*. Furthermore, the elucidation of genes responsible for bioactive secondary metabolites and the ability to control such genes are important and are additional steps to increase the production of metabolites by beneficial microbes and facilitate metabolic engineering. *B. velezensis* may be represented as a practical and powerful biocontrol agent that can be used as an effective alternative to synthetic agrochemicals.

The morphological results of the isolated bacteria indicate that they are Gram-positive and rod-shaped; they are also cream-white, though there is variation in their texture, elevation, form, and size due to different strains. The results are consistent with the earlier morphological description of *B. velezensis* strains of bacteria as aerobic, Gram-positive, rod-shaped, and spore-forming bacteria [3].

In our study, physiochemical characterization of the isolates B20 and B30 showed that they could grow in a wide pH range. The highest growth was observed at pH 7.0, 8.5, and 10.0, respectively. However, the results also indicated that isolates B20 and B30 could grow at an acidic pH of 5.0. The results were consistent with a previous study by [33] which indicated that a pH range of 5.7 to 9.0 favors the growth of alkaliphiles. However, the pH range of 7.0 to 11.0 serves as a selective optimum pH. Soda lakes have a high pH range. Therefore, microbes can only survive by maintaining their cytoplasm at the same pH as their mesophilic relatives. The mechanism for achieving this is secondary proton uptake mediated by membrane-associated antiporters. Alkaliphiles can also maintain an internal pH > 7, 5 regardless of environmental pH. Most bacteria activate the sodium-ion pump at this high pH, which lowers the internal pH by transporting hydrogen ions into the cells. According to [34], alkaliphiles have cell membranes with a special composition of tissues that protect them from highly alkaline conditions. The study was consistent with previous findings [35], which reported a wide pH range (4.5–10.0) where *bacillus* can grow. It also agrees with the findings of [35], who reported varied bacterial growth at different pH.

For Sodium chloride concentration, our study showed that isolates B20 and B30 could tolerate different concentrations of salinity conditions. They had a maximum growth at

0.0 M NaCl. This is because they were isolated from soil and sediment samples. Isolate B30 was able to grow at 2.0 M NaCl. These isolates can be classified as halophiles. The isolate B20 grew at all salt concentrations, hence known as halotolerant. The ability of the isolates to grow at different salt concentrations indicates that they are tolerant to salinity and as an adaptive strategy to survive in adverse growth conditions. This is in agreement with earlier studies on *Bacillus* spp. [36]. Soda lake bacteria can survive millions of years in the fluid inclusions of salt deposits, including evaporates, by adapting to these potentially deadly ecosystems. To prevent water loss from their cell membrane, the halophile compensates for offsets the high salt in the environment by accumulating potassium and glycine-betaine compounds. This mechanism balances the salt concentration inside the cell and the environment, preventing the cell from bursting due to the diffusion of water. The ability of isolates to grow at different salt concentrations has also been reported by [15,37–39] for Lake Bogoria isolates.

The isolate B20 and B30 grew at a wide range of temperatures. The optimal temperature was recorded at 35 °C. Compared to the temperature recorded at sampling points, most bacterial isolates were obtained in the soil sample with a lower temperature than in the sediments. Although 35 °C was recorded as the optimal temperature, some bacteria survived up to 60 °C. The growth of bioactive bacteria at varied temperatures indicates that Bogoria harbors diverse microbes that can adapt to a wide temperature range. This property is ideal for biocontrol since different environments have different temperatures and can adapt to them, making them good biocontrol agents. The study agrees with previous studies that show the ability of Lake Bogoria bacterial isolates to adapt at different temperatures [16,19,20,38].

In our study, enzymatic assay for isolates B20 and B30 showed varied presence and absence of protease, phosphatase, chitinase, pectinase, indole -3-Acetic Acid (IAA), and hydrogen cyanide (HCD). This feature is highly suitable helping suppress the disease and promote plant growth and productivity [40]. Additionally, our results emphasized that selected antagonist bacteria with high antifungal activity shared excellent attributes. Most of these bacterial isolates exerted different biocontrol mechanisms such as the production of cell wall degrading enzymes, cyanide (HCD) production, and plant growth promotion traits. Producing lytic enzymes is among the major mechanisms' biocontrol agents employed to control fungal pathogens. Our results showed that B20 and B30 displayed protease and phosphate production, while chitinase was observed in B30 only, and IAA in B20. There was no production of pectinase and HCD for both isolates. Chitinase isis important because the microbes attack the fungal cell wall and cause lysis by degrading chitin [3,41]. Similar results were found for *pseudomonas fluoresces*, which produced chitinase against the growth of Sclerotina sclerotium [42]. The production of IAA is characterized by a strong affinity for iron which is one of the important mechanisms used by biocontrol [32,43,44].

Additionally, IAA contributes to the increasing root surface area and length and, therefore, better uptake of nutrients. Phosphate solubilization is considered potential bioinoculant to increase crop production by utilizing phosphate in the soil to soluble form for plant use. In our study, both B20 and B30 were able to solubilize the phosphate. The results concur with previous findings [45,46] that *Bacillus* spp. could potentially enhance plant growth by producing IAA, NH3, and ACC deaminase.

4. Materials and Methods

4.1. Sampling Site and Sample Collection

Lake Bogoria is located in the northern part of the Kenyan Rift Valley (0°20′ N and 36°15′ E). It is approximately 35 km long and 3.4 km wide. It is a drainage sink for water over 700 km^2, and its surface has increased by 990 m. The lake is alkaline and has a higher concentration of Na^+, HCO^{3-}, and $CO_3{}^{2-}$ ions [15]. It is sometimes home to one of the world's largest populations of lesser flamingos. It is famous for geysers and hot springs, which erupt up to 5 m high. Seven soil and sediments were collected from the shores of the lake using a purposive sampling technique. Temperature, pH, total dissolved salts, and

conductivity were measured at the sampling location. The collected samples were put in a sterile bottle, labeled, and preserved in a cool box (4 °C). They were transported to the Institute of Biotechnology Research Laboratory in Jomo Kenyatta University of Agriculture and Technology (JKUAT, Nairobi, Kenya), for further analysis.

4.2. Isolation and Morphological Characterization of Lake Bogoria

The isolation of *Bacillus velezensis* strains from soil and sediment was carried out using methods described by [47,48]. In brief, 1 g of soil and sediment were weighed and then homogenized in a sterile test tube containing 9 mL of sterile physiological saline (0.85% NaCl). The resulting soil and sediment suspensions were then vigorously vortexed at 150 rpm for 1 min. A five-fold serial dilution of soil and sediment suspension with physiological saline (0.85% NaCl) was done in 1 mL to 9 mL. The dilutions were: 10^0, 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}. An aliquot of 30 µL from dilutions 10^{-3} and 10^{-4} was cultured according to [49] on modified nutrient agar-Himedia containing cycloheximide (antifungal) and 35 g/L (*w/v*) of NaCl. The plates were incubated at 39.5 °C for 24 h, followed by a subculture of the bacterial colonies using an isolation medium (modified nutrient agar) until pure colonies were obtained. The isolates were cryopreserved at −86 °C in an isolation medium (Nutrient Agar) supplemented with 20% glycerol for further analysis. Cultural morphological features were observed as follows: form, elevation, margin, size, pigmentation, surface, texture, and opacity, following standard microbiological techniques described by [50]. Cell morphology was done by Gram staining technique as described by [51,52] and light microscope used to determine the shape and Gram reaction (Gram-positive or negative) of the bacterial cells.

4.3. Molecular Characterization

4.3.1. Genomic DNA Extraction

Genomic DNA extraction was done using a bacterial DNA isolation kit (Norgen Biotek Corp., Thorold, ON, Canada) described by the manufacturer. A spectrophotometer was used to measure the DNA concentration and purity. The fragments were separated by electrophoresis on 0.8% agarose gel stained with ethidium bromide (Sigma-Aldrich, St. Louis, Missouri, United States) gel for 1 h. The DNA template was then stored at −20 °C for further analysis.

4.3.2. PCR Amplification of 16S rRNA Genes

To amplify the 16S rRNA genes, genomic DNA from each bacterial isolate was used as a template. A pair of 27F Forward (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R Reverse (5′-CGGCTACCTTGTTACGACTT-3′) bacterial primers were used to amplify the 16S rRNA gene in response to the *Escherichia coli* gene sequence. The amplification was performed using Peqlab Primus 96 PCR equipment. It was amplified in a 40 µL mixture comprising 20 µL of Master mix, 18.2 µL of PCR water, 0.4 µL of 27F forward primer, 0.4 µL of 1492R reverse primer, and 1 µL of template DNA (750 ng/L) DNA.

The following temperature cycling profiles were applied for the reaction mixtures: A 10 min enzyme activation at 96 °C for a single cycle, which was followed by 35 cycles of 45 s of denaturation at 95 °C, 45 s of primer annealing at 53 °C, 1 min of the chain of elongation at 72 °C, and 10 min of the chain of final extension at 72 °C [17]. The presence and size of PCR amplicon were verified on 1.2% agarose gel and visualized under U.V. light [17]. PCR amplicons were purified using the QIAquick PCR amplification kit protocol (Qiagen) according to the manufacturer's instructions. The PCR amplicon was sent to Macrogen for sequencing.

4.3.3. Phylogenetic Analysis of Bioactive Isolates

The Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (NCBI) site was used to compare the 16S rRNA of the bacterial isolates. The BLAST findings selected 16S rRNA gene sequences with the highest similarity index.

The sequences were aligned using CLUSTAL W 2.0. and incorporated in MEGA 7 for pairwise and multiple sequence alignment [53]. Phylogenetic analysis was conducted using MEGA 7, where the Kimura-2 parameter model was used to calculate the evolutionary distances and construct the phylogeny tree. The evolutionary distance was calculated using the maximum composite likelihood techniques [53]. A bootstrap analysis of 1000 replicates denote the evolutionary history of the already analyzed taxa.

4.4. Evaluation of Bacillus velezensis against Fusarium solani

The antifungal efficacy of selected bacteria isolates B20 and B30 was examined in vitro by inhibiting the growth of the phytopathogenic fungus, *Fusarium solani* using co-culturing as described by [10] on the PDA medium. A 5-day active growth fungal agar plug (8 mm diameter) was cut from a plate of potato dextrose agar (PDA) with *Fusarium solani*. The plugs were placed at the end of the plate, and bacteria (10^8 CFU/mL) were streaked perpendicularly across the plate in a PDA medium. In all antagonistic studies, a plate with only a plug placed at the center was used as a control. The experiment and the control were arranged in a randomized design with three replicates per experiment. The Petri dishes were incubated at 30 °C for 7 days. The mycelium percentage inhibition rate (I.R.) of *Fusarium solani* was calculated using the formula described by [10].

Formula; IR% = $[(C2 - C1)/C2] \times 100$ N/B I.R.: Inhibition rate, C2: colony diameter of the pathogen in control, and C1: colony diameter of the pathogen co-cultured with the bacterial isolate. The mean was obtained and recorded as the final data.

4.5. Physiochemical Characterization

4.5.1. Growth at Different Sodium Chloride Concentrations

Isolates B20 and B30 were cultured in Luria Bertani Broth (L.B.) Himedia (10.0 g of tryptone, 5.0 g of yeast extract, 10.0 g of NaCl) at various NaCl concentrations per liter (0.0 M, 0.5 M, 1.0 M, 1.5 M, and 2.0 M NaOH), according to the manufacturer. The optical density (O.D. 600 nm) of the Isolate B20 and B30 were recorded after 48 h at 30 °C in a shaking incubator at a speed of 120 rpm/min. An uninoculated L.B was used as a blank tube.

4.5.2. Growth at Various Temperatures

Isolates B20 and B30 were cultured on L.B. broth at varying temperatures of 20 °C, 25 °C, 30 °C, 35 °C, 40 °C, 50 °C, and 60 °C. They were incubated in a shaking incubator of 120 rpm at 30 °C. The growth of the isolates was determined by measuring optical density at 600 nm after 48 h.

4.5.3. Effect of pH on the Growth of the Isolates

Isolates B20 and B30 were cultured in L.B. broth at a varying pH of 5.0, 7.0, 8.5, and 10.0. A tube with uninoculated L.B. was used as blank. The tubes were incubated in the shaking incubator at a speed of 120 rpm/min at 30 °C for 48 h. The optical density (O.D.) at 600 nm was measured and recorded.

4.6. Enzymatic Characterization

4.6.1. Protease Activity

The proteolytic activity of the bacterial strains B20 and B30 was assessed following the protocol described by [54] on skim milk agar-Himedia (28.0 g S.M. powder, 5.0 g Tryptone, 2.5 g yeast extract, 1.0 g Dextrose or glucose, and 15.0 g Agar) per liter (3%) (*v/v*) medium. The bacteria strains B20 and B30 were cultured overnight, and 5 µL of the bacterial suspension was soaked on a filter-paper disc, allowed to dry, and placed on a Petri-dish containing skim milk agar. Sterilized water was used as a control. The experiment was performed in triplicate and incubated at 30 °C for 24 h. Proteolytic activity was detected by forming clear zones or a halo around the bacterial spots, and the inhibition zone diameter was measured and recorded.

4.6.2. Chitinase Activity

The isolates B20 and B30 were screened for chitin hydrolysis by spotting on the center of 1% CCA (1% Colloidal Chitin, 0.2 g $NaNO_3$, 0.1 g K_2HPO_4, 0.1 g $MgSO_4$, 0.1 g $CaCO_3$, 0.001 g $FeSO_4 \cdot 7H_2O$, 0.05 g KCL) media at pH 7.0. Sterilized water was used as a control. The plates were conducted in triplicates and incubated at 30 °C for 72 h. Chitinase activity was detected by forming a clear zone around the bacterial spots.

4.6.3. Pectinase Activity

Pectinase enzyme assay was based on the determination of reducing sugars produced as a result of enzymatic hydrolysis of pectin by the dinitrosalicylic acid reagent (DNS) method [55]. A total of 1.5 mL of freshly grown culture was taken for enzyme assay and centrifuged at 10,000 rpm for 5 min. The enzyme's source was the supernatant (100 µL) from the culture broth. In addition, the substrate was prepared by mixing 0.5% (*w/v*) citrus pectin in 0.1 M of pH 7.5 phosphate buffer.

From the prepared substrate, 900 µL was added to three clean labeled test tubes; one for the enzyme, one for the enzyme blank, and one for the reagent blank. Then, 100 µL of the crude enzyme was added to the test tube labeled as an enzyme, and 100 µL of distilled water was added to the test tube labeled as regent blank while the test tube labeled as enzyme blank remained as it was. Then, the test tubes were incubated at 50 °C for 10 min in the water bath. After incubation, 2000 µL of dinitrosalicylic acid reagent (DNS) was added to all test tubes to stop the reaction. Meanwhile, in a test tube labeled enzyme blank, 100 µL of the crude enzyme was added after the DNS. Then, all the test tubes were placed in a boiling water bath (92 °C) for 10 min. Finally, the tubes were cooled, and optical density (O.D.) was measured using a spectrophotometer at 540 nm. Enzyme activity was measured against enzyme blank and reagent blank. The enzyme unit was defined as the amount of enzyme that catalyzes µmol of galacturonic acid per minute (µmol min^{-1}) under the assay conditions.

Relative activity was calculated as the percentage enzyme activity of the sample concerning the sample for which maximum activity is obtained:

$$\text{Relative Activity} = \frac{\text{Activity of sample (U)} \times 100}{\text{Maximun enzyme activity (U)}} \quad (1)$$

4.6.4. Hydrogen Cyanide (HCD) Production Ability

The application of the Lorck method allowed the qualitative detection of hydrogen cyanide (HCD) [56]). Fresh B20 and B30 isolates were cultured on a Petri plate with nutrient agar supplemented with glycine (4.4 g/L: *w/v*). Filter paper discs sterilized (9 cm in diameter) were soaked in a picric alkaline solution (2% sodium carbonate and 0.5% picric acid) and then placed on the top of each Petri dish. Positive infected control plates were used for comparison. They were incubated at 25 °C for four days. If HCD generation is produced, the color will shift from yellow to a pale reddish brown.

4.6.5. Phosphate Solubilization Ability

The phosphate solubilization assay was performed qualitatively as described by [57]. The bacteria strains B20 and B30 were cultured on Pikovskaya's medium. For comparison, uninoculated plates were used. The plates were incubated at 30 °C for 7 days. The presence or absence of a clear zone around the colonies was checked.

4.6.6. Indole-3-Acetic Acid (IAA) Production Ability

The ability of isolates B20 and B30 to produce indole-3-acetic acid was evaluated using the colorimetric approach [10,44]. Isolates of bacteria were plated in 20 mL of Luria-Bertani Broth supplemented with L-tryptophan (2.0 g/L; *w/v*). In addition, 1 mL of the culture supernatant was treated with 2 mL of Salkowski's reagent and 3 drops of orthophosphoric

acid. The medium without inoculation was used as a negative control. Positive IAA production is represented by red [44].

4.7. Data Analysis

Molecular data were analyzed using MEGA 7, where the maximum composite likelihood method was used to analyze phylogenetic data. Antifungal activity and enzymatic assay were subjected to one-way analysis of variance (ANOVA) using SAS software. The data was recorded in triplicates, and means were separated using Fisher's Least Significant Difference (LSD) test at $p \geq 0.05$. GraphPad-Prism version 6.0 was used for physiochemical characterization to present the data in graph format.

5. Conclusions

The study was able to identify *Bacillus velezensis* strains from Lake Bogoria and demonstrated that they inhibited the growth of the mycelia of *Fusarium solani* under in vitro conditions. The isolates also varied in physiochemical characteristics regarding temperature, salinity, and pH. The fungal mycelia reduction could be because of several actions such as chitinase, HCD, protease, and phosphate IAA among others. However, the detailed mode of action should be investigated. The strains of *Bacillus velezensis* may represent a prospective candidate for biocontrol in managing diseases caused by *Fusarium solani*. However, in vivo and field trials should be conducted to assess the potential of *Bacillus velezensis* strains in managing diseases caused by *Fusarium solani.*

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