ESKİŞEHİR TEKNİK ÜNİVERSİTESİ BİLİM VE TEKNOLOJİ DERGİSİ
C- YAŞAM BİLİMLERİ VE BİYOTEKNOLOJİ

Eskişehir Technical University Journal of Science and Technology
C -Life Sciences and Biotechnology

Estuscience – Life, 2025, 14 [1] pp. 14-24, DOI: 10.18036/estubtdc.1413386

RESEARCH ARTICLE

pH INFLUENCE ON SHELF LIFE OF LIQUID PGPR FORMULATIONS WITH *Bacillus subtilis* STRAINS

Sevgi İŞLEK [1,*], Kemal KARACA [2], Rengin ELTEM [3]

[1] Graduate School of Natural and Applied Sciences, Department of Bioengineering, Ege University, 35040 Bornova, Izmir, Turkey
sevgi.islek95@gmail.com - *0000-0001-7743-7014*

[2] Graduate School of Natural and Applied Sciences, Department of Bioengineering, Ege University, 35040 Bornova, Izmir, Turkey
kemalkaraca1@gmail.com - *0000-0003-2193-2854*

[3] Faculty of Engineering, Department of Bioengineering, Ege University, 35040 Bornova, Izmir, Turkey
rengin.eltem@ege.edu.tr - *0000-0002-0642-7676*

Abstract

Plant Growth Promoting Rhizobacteria (PGPRs) are bacteria that promote plant growth through both direct and indirect mechanisms. The formulation of PGPR inoculants is crucial for the efficacy and commercial success of microbial fertilizers. Formulation aims to optimize the survival of microbial strains under specific environmental conditions and enhance their capacity to promote plant growth. This process ensures protection of bacterial cells against harsh conditions such as high temperatures, desiccation, and storage, thereby extending product shelf life. Proper formulation of PGPR inoculants is a critical component for sustainable agricultural practices, playing a significant role in improving both plant health and productivity.

Among PGPR strains, *Bacillus* species are particularly produced and utilized as microbial fertilizers commercially due to their high efficacy potential and long shelf life. However, for large-scale production, strain-specific PGPR formulations need to be developed and optimized to produce PGPR inoculants with high efficacy potential and extended shelf life.

In this study, acidic liquid formulations were prepared using acetic acid for *B. subtilis* EGE-B-36.5 strain, and alkaline liquid formulations were prepared using calcium acetate-calcium hydroxide for *B. subtilis* EGE-B-1.19 strain. The viable cell count in the liquid formulations was statistically compared with the control. In the acidic liquid formulation, statistically significant changes in viable cell count were observed for *B. subtilis* EGE-B-36.5 strain at pH 4.0 after 12 months and for *B. subtilis* EGE-B-1.19 strain at pH 4.0 after 12 months ($p<0.05$). In the alkaline liquid formulation at pH 9.5 there had been a statistically significant ($p<0.05$) difference between control group of the *B. subtilis* EGE-B-1.19.

Keywords

Bacillus subtilis,
PGPRs,
Microbial fertilizer,
Liquid formulation,
pH

Time Scale of Article

Received :02 January 2024
Accepted : 30 July 2024
Online date :29 January 2025

*Corresponding Author: sevgi.islek95@gmail.com

1. INTRODUCTION

The significant increase in the global population is driving up the demand for food. Therefore, farmers use large amounts of chemical fertilizers and their derivatives to obtain maximum crop yield due to limited land resources. However, the continuous and excessive use of these chemical fertilizers and derivatives negatively affects the natural microflora, such as bacteria, fungi, cyanobacteria, and protozoa, present in the rhizosphere or applied area, causing an imbalance in the natural ecosystem [1]. This damage, which initially provides short-term benefits, ultimately leads to inefficiency in production and poor-quality products [2].

Globally, to mitigate the larger problems that the current negative agricultural practices may cause in the future and to ensure the continuity of the most basic human need nutrition the most innovative solution in agricultural production is microbial fertilizers. These fertilizers are environmentally friendly, harmless to human health, and provide essential elements required by plants, competing with chemical fertilizers. Bacteria that establish a positive relationship with plant roots and positively affect plant development and growth are defined as Plant Growth Promoting Rhizobacteria (PGPRs). PGPRs play an active role in reducing the damage caused by plant pathogenic microorganisms, directly or indirectly facilitating plant growth, promoting plant growth by activating insoluble nutrients in the soil, and minimizing abiotic stress [3].

PGPRs have been documented in the literature as beneficial rhizobacteria for the soil ecosystem due to their high adaptation capabilities to various environments, rapid growth rates, and ability to metabolize a wide range of natural and xenobiotic compounds [4]. Although PGPRs encompass many different types of bacteria, many PGPRs developed for commercial applications are predominantly *Bacillus* species. These products are used in the form of endospores, which provide population stability throughout formulation and shelf life [5].

Among *Bacillus* species, *B. subtilis* strains are the most commonly used PGPRs due to their capacity to produce antibiotics and numerous other beneficial properties, which reduce disease incidence in plants [6]. When aerobic, endospore-forming *Bacillus* species are used in agricultural fields, they contribute to crop productivity directly or indirectly. *Bacillus* species possess many physiological characteristics, such as having a Gram-positive cell wall, forming stress-resistant endospores, secreting peptide antibiotics, and producing peptide signal molecules and extracellular enzymes. Particularly, *Bacillus* species can survive for extended periods under adverse environmental conditions due to their endospore formation mechanism. It is known that most *Bacillus* species promote plant growth. The primary mechanisms of growth promotion include the production of growth-stimulating phytohormones, phosphate solubilization, siderophore production, antibiotic production, inhibition of ethylene synthesis in plants, and induction of systemic resistance against pathogens. Numerous studies have shown that *Bacillus* and *Paenibacillus* species exhibit antagonistic activities that suppress pathogens under both in vitro and in vivo conditions [7].

PGPR inoculants are defined as formulations containing one or more beneficial bacterial strains prepared with an easy-to-use and economical carrier material. The key points in PGPR inoculant technology are the selection of an appropriate carrier for the inoculants and the preparation of a suitable formulation [8]. Biomass production, formulation, and determining shelf life are important steps to consider during the development of PGPR inoculants. It must be ensured that a properly produced, formulated, and applied bioinoculant product will deliver all the benefits it is intended to provide. Generally, many private companies globally offer a variety of efficient and effective bioinoculants for diverse soils in response to increasing demand in the international market. However, these inoculants often tend to be of low quality. In some products used in developing or developed countries, rhizospheric microorganisms may be absent or contaminated with other strains. This inconsistency in the beneficial

effect of bioinoculants in the field creates a negative impression in the market [9]. The heterogeneity of soils poses a significant challenge for bioinoculants. Bacteria inoculated into the soil must compete with the better-adapted native microbiota and survive in the soil microbiome. Therefore, a more suitable microenvironment should be provided for bioinoculants with physicochemical protection. This approach will help prevent rapid declines in the number of live bacterial cells [9].

As a result, the goal of inoculant formulations should be to allow PGPRs to survive better in suitable and available forms during storage and application [9]. An ideal bioinoculant formulation should not have phytotoxic effects on the plants where it is applied, demonstrate high tolerance to adverse environmental conditions, have a cost lower than other products in the market, and be reliable in controlling plant diseases [10].

In this study, indigenous *Bacillus subtilis* EGE-B-1.19 and *Bacillus subtilis* EGE-B-36.5 strains with PGPR properties were used. Following production, the pH values of the culture medium were adjusted to acidic and alkaline levels. The shelf life of liquid formulations at different pH values was monitored over 12 months to determine the optimal pH for these strains and to aim for the production of a viable product with extended shelf life.

2. MATERIALS AND METHODS

2.1. Microorganisms, Culture Conditions and Preformulation

Bacillus subtilis EGE-B-1.19 and *Bacillus subtilis* EGE-B-36.5, which have PGPR properties, were used from microbial culture collection, Department of Bioengineering, Ege University, Izmir, Turkey [11, 12]. Initially, growth of two strains on nutrient agar plates at pH 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, and 8.5 was determined. Plates were inoculated and incubated at 30 °C. After incubation, the growth of *Bacillus* strains on petri plates with different pH values was visually graded.

Then, *B. subtilis* EGE-B-1.19 and *B. subtilis* EGE-B-36.5 were grown on *Bacillus* endospore production medium (EPM). This medium contained glucose, 5.0 g; dry yeast, 3.31 g; soy flour, 23.56 g; $(NH_4)_2SO_4$, 0.60 g; glycerol 2.0%; stock salt solution 10 ml; antifoam, 0.1% (w/v); distilled water, 1000 ml; pH 7.0. The stock salt solution was composed of 20.30 g, $MgCl_2.6H_2O$; 10.20 g, $CaCl_2.2H_2O$; 1.00 g, $MnCl_2.4H_2O$; 1000 ml distilled water. Sterilized *Bacillus* EPM was inoculated with each *Bacillus* strain and incubated at 30±2°C. The viable cell count of the culture broth was determined in colony-forming units per milliliter (cfu/ml) using the pour plate method [13]. After production, consecutive serial dilutions ranging from 10^{-1} to 10^{-8} were prepared from the culture liquid, and the viable cell count was determined in colony forming units per milliliter (CFU/ml) using the pour plate method. For this purpose, 1 mL from each dilution ranging from 10^{-4} to 10^{-8} was transferred to sterile glass petri dishes under aseptic conditions. Each dilution was performed in duplicate. Subsequently, the dishes were cooled to 45°-50°C, and approximately 20 mL of nutrient agar medium was poured onto them. After gently swirling the plates by hand until the agar solidified, they were incubated at 28°C for 24-48 hours [14].

Acidic liquid preformulation experiments were carried out with *B. subtilis* EGE-B-36.5 using acetic acid [15], lactic acid [16], propionic acid [16], citric acid [16], and boric acid [17]. *B. subtilis* EGE-B-36.5 culture broth's ($1x10^8$ cfu/ml, pH 8.0) in sterile amber bottles was adjusted to pH 3.0 and 4.0 with lactic acid, citric acid, and propionic acid, pH 3.0, 4.0, and 5.0 with acetic acid, and pH 5.0 with boric acid. Acidic liquid preformulations and culture broth at pH 8.0 as control were stored at room temperature for three months. The viable cell count (cfu/ml) in acidic liquid preformulations and control was analyzed monthly using the pour plate method for three months. In addition, the pH measurements of the preformulations were monitored monthly to determine the relationship between the viable cell count

and the pH change. According to the preformulation results, it was decided which acid should be used to prepare the acidic liquid formulations.

2.2 Experimental Setup for the PGPR Liquid Formulations

The pH of the culture broths ($1x10^8$ cfu/ml, pH 8.0) in sterile amber bottles with *B. subtilis* EGE-B-1.19 and *B. subtilis* EGE-B-36.5 were adjusted to pH 2.0, 3.0, 4.0, and 5.0 with acetic acid. In addition, alkaline liquid formulations were prepared from the culture broth ($1x10^8$ cfu/ml) of only *B. subtilis* EGE-B-1.19 at pH 9.0, 9.5, and 10.0 using calcium acetate-calcium hydroxide. The acidic and alkaline liquid formulations were stored at a room temperature of 25°C. The viable cell count (cfu/ml) of the liquid formulations was monitored monthly using the pour plate method for 12 months. Additionally, the pH values of the liquid formulations were monitored monthly for 12 months using a Milwaukee Mi 150 pH meter.

2.3 Statistical analysis

The experiments were performed in triplicate. The means were statistically analyzed using Tukey's ANOVA ($p<0.05$).

3. RESULTS AND DISCUSSION

3.1 Investigation of the Growth of *Bacillus* strains at Different pH Levels on Petri Plates

In this study, it was observed that *B. subtilis* EGE-B-1.19 and *B. subtilis* EGE-B-36.5 strains grew well between pH 5.5 – pH 7.5 on NA plates after 48 h of incubation. Although *B. subtilis* EGE-B-1.19 grew well at pH 8.0 and 8.5, it showed weak growth after two weeks of incubation at pH 4.5. *B. subtilis* EGE-B-36.5 showed weak growth at pH 8.0 and 8.5, but it did not grow at pH 4.5, as shown in Table 1. Therefore, acidic and alkaline liquid formulations were designed to be below pH 5.5 and above pH 8.5, where *B. subtilis* strains could not grow well. The purpose of designing acidic and alkaline liquid formulations below pH 5.5 and above pH 8.5 is to allow *Bacillus* strains, which produce endospores, to remain in the endospore form for an extended period without transitioning to the vegetative form.

Table 1. Determination of pH tolerance of *Bacillus subtilis* strains on Nutrient Agar plates

Bacillus subtilis strains	pH								
	pH 4.5	pH 5.0	pH 5.5	pH 6.0	pH 6.5	pH 7.0	pH 7.5	pH 8.0	pH 8.5
B. subtilis EGE-B-1.19	+++	+++	+++++	+++++	+++++	+++++	+++++	+++++	+++++
B. subtilis EGE-B-36.5	-	+++	++++	+++++	+++++	+++++	+++++	+++	+++

The pH tolerance of different strains of *B. subtilis* is different, as shown in Table 1. *B. subtilis* can grow at low as pH 4.0 and high as pH 9.0, but 7.8 is where it grows best. Alkaliphile and alkali-tolerant microorganisms grow well in alkali environments above pH 9; however, alkaliphile microorganisms do not show optimal growth below pH 9. It has been shown in studies that *B. subtilis* maintains a cytoplasm pH between 7.3 and 7.6 with its cytoplasmic pH homeostasis ability [18, 19]. Gauvry et al. reported that *the B. subtilis* BSB1 strain grew between pH 4.8 and 9.1 [20]. In our studies, *B. subtilis* EGE-B-1.19 grew at pH 4.5-8.5 and *B. subtilis* EGE-B-36.5 grew at 5.0-8.5. These strains were found to have alkali and acid tolerance but showed different growth rates at different pH values. Accordingly, strain-specific studies should be conducted from formulation development studies even if they belong to the same species.

3.2 Preformulation of *Bacillus subtilis* EGE-B-36.5

The incubation of *Bacillus subtilis* EGE-B-1.19 and *Bacillus subtilis* EGE-B-36.5 in EPM resulted in obtaining spores at concentrations of 5.6 x 10^9 CFU/ml and 1.2 x 10^9 CFU/ml, respectively. Both bacteria were diluted to a concentration of 10^8 CFU/ml through the necessary calculations.

The pH of the culture broth was adjusted to pH 3.0, pH 4.0, and pH 5.0 with lactic acid, propionic acid, citric acid, and acetic acid to prepare the preformulation with *B. subtilis* EGE-B-36.5. In addition, the pH of the culture broth containing *B. subtilis* EGE-B-36.5 was adjusted to pH 5.0 using only boric acid. It has been determined that boric acid must be used in large quantities to adjust the pH of the culture broth to acidic levels such as pH 3.0 and pH 4.0 because it is a weak acid. For this reason, it was decided that using boric acid excessively is not cost-effective (Table 2) As stated in Table 3, preformulations created using five different acids were subjected to statistical analysis using the variance analysis (ANOVA) function in the SPSS package program in the third month to assess the total viable cell count. A significant variation in the viable cell count was observed in the formulation adjusted to pH 3.0 using citric acid ($p < 0.05$). According to the outcomes generated by this software, a second significant change in viable cell count ($p < 0.05$) was identified in the culture medium adjusted to pH 5.0 using acetic acid. In formulations to be conducted on a commercial scale, the unit cost of citric acid is considerably higher than that of acetic acid. Hence, because of the cost-effectiveness of acetic acid, the study proceeded with acetic acid to establish acidic liquid formulations.

Table 2. Monthly viable cell counts (cfu/ml) and pH values of *B. subtilis* EGE-B-36.5 in preformulations

		Preformulation of *B. subtilis* EGE-B-36.5							
Acids	**Storage (Month)**	**0**		**1st month**		**2nd month**		**3rd month**	
		x10^8 CFU/ml	**pH**	**x10^8 CFU/ml**	**pH**	**x10^8 CFU/ml**	**pH**	**x10^8 CFU/ml**	**pH**
Lactic Acid	pH 3.0	3.3 ± 0.03	3.0 ±0.05	2.3 ±0.9	2.9 ±0.03	2.1 ±0.5	3.6 ±0.04	3.2 ±2.0	3.1 ±0.01
	pH 4.0	3.8 ±0.3	4.1 ±0.05	1.7 ±0.8	4.1 ±0.1	2.2 ±0.05	4.6 ±0.1	2.5 ±0.3	6.4 ±0.14
	pH 5.0	-	-	-	-	-	-	-	-
Propionic Acid	pH 3.0	2.8 ±0.4	3.0 ±0.05	1.2 ±0.2	3.0 ±0.05	1.6 ±0.60	3.0 ±0.05	1.4 ±0.14	3.0 ±0.03
	pH 4.0	2.3 ±0.4	4.0 ±0.04	1.9 ±0.9	3.9 ±0.02	3.0 ±0.47	4.0 ±0.04	1.8 ±0.5	4.0 ±0.02
	pH 5.0	-	-	-	-	-	-	-	-
Citric Acid	pH 3.0	3.9 ±0.089	3.0 ±0.07	1.3 ±0.13	2.9 ±0.05	2.2 ±0.6	3.0 ±0.07	4.1 ±1.25	3.1 ±0.01
	pH 4.0	3.1 ±0.40	4.0 ±0.06	1.5 ±0.3	4.1 ±0.16	1.6 ±0.05	4.0 ±0.06	1.6 ±1.1	6.5 ±0.3
	pH 5.0	-	-	-	-	-	-	-	-
Acetic Acid	pH 3.0	3.2 ±1.1	3.1 ±0.05	1.74 ±0.17	2.9 ±0.04	2.0 ±0.53	3.1 ±0.05	1.9 ±0.5	3.0 ±0.04
Boric Acid	pH 3.0	-	-	-	-	-	-	-	-
	pH 4.0	-	-	-	-	-	-	-	-
	pH 5.0	1.5 ±0.2	4.7 ±0.3	1.4 ±0.25	4.7 ±0.3	1.8 ±0.7	4.7 ±0.25	1.6 ±0.4	4.7 ± 0.3
Control		2.5 ±0.07	8.6 ±0.05	1.7 ±0.2	6.9 ±0.03	3.4 ±1.2	7.5 ±0.06	2.0 ±0.8	7.5 ±0.18

-: Acidic liquid formulation was not prepared.

Table 3. Comparison of viable cell counts (CFU/ml) of *B. subtilis* EGE-B-36.5 in the third month of acidic liquid formulations prepared with different acids

	Population density (x10^8 cfu/ml)		
Acids	pH 3.0	pH 4.0	pH 5.0
Citric Acid	4.1 ± 1.25 ^{*a}	1.6 ± 1.1 ^{d}	-
Propionic Acid	1.4 ± 0.14 ^{cd}	1.8 ± 0.5 ^{bcd}	-
Boric Acid	-	-	1.6 ± 0.4 ^{bcd}
Lactic Acid	3.2 ± 2.0 ^{abc}	2.5 ± 0.3 ^{abcd}	-
Acetic Acid	1.9 ± 0.5 ^{bcd}	3.2 ± 0.15 ^{ab}	3.4± 0.9 ^{ab}
Control	2.0 ± 0.8 ^{abcd}		

*In a column, means that are followed by the same letter are statistically similar. NA: not applicable

3.3. Shelf Life of the Acidic Liquid Formulations

Acetic acid formulations of the *B. subtilis* strains were prepared at pH 2.0, 3.0, 4.0, and 5.0, as shown in Tables 4a and 4b. The viable cell count in the formulation at pH 2.0 for both *Bacillus* strains declined in the second month, and no viable cells were observed in the third month. *Bacillus* strains typically thrive in environments with high pH values. Low pH values, such as pH 2.0, represent highly acidic conditions that typically have a negative impact on the growth of most *Bacillus* strains. However, some *Bacillus* species are acidophiles, which means that they can tolerate lower pH values. Extremophilic *Bacillus* species can survive in low pH environments because they have adapted to extreme conditions [21]. As a result, neither of the strains can survive at pH 2.

When viable cell counts were compared with the control group by performing the ANOVA test in the SPSS package program, it was determined that there had been a statistically significant ($p<0.05$) difference between the control and the acetic acid formulation of the *B. subtilis* EGE-B-36.5 strain at pH 4.0 and the acetic acid formulation of the *B. subtilis* EGE-B-1.19 at pH 4.0 (Table 4a and 4b).

Table 4a. Monthly viable cell counts (cfu/ml) and pH values of *B. subtilis* EGE-B-36.5 in acidic liquid formulations

	Acidic liquid formulation of *B. subtilis* EGE-B-36.5									
Storage (Month)	**pH 2.0**		**pH 3.0**		**pH 4.0**		**pH 5.0**		**Control**	
	Population density (x10^8 CFU/ml)	**pH**	**Population density (x10^8 CFU/ml)**	**pH**	**Population density (x10^8 CFU/ml)**	**pH**	**Population density (x10^8 CFU/ml)**	**pH**	**Population density (x10^8 CFU/ml)**	**pH**
0	1.8 ± 1.4	2.0 ± 0	3.2 ± 1.1	3.1 ± 0.05	3.4 ± 0.1	4.0 ± 0.01	4.1 ± 0.05	5.1 ± 0.1	2.5 ± 0.07	8.6 ± 0.05
1	0.8 ± 0.5	2.28 ± 0.01	1.74 ± 0.17	2.9 ± 0.04	2.5 ± 1.42	3.9 ± 0.02	3.1 ± 0.4	8.2 ± 0.2	1.7 ± 0.2	6.9 ± 0,03
2	0.04 ± 0.2	2.03 ± 0.7	2.0 ± 0.53	3.1 ± 0.05	2.2 ± 0.05	4.0 ± 0.01	2.0 ± 1.2	5.1 ± 0.1	3.4 ± 1.2	7.5 ± 0.06
3	-	-	1.9 ± 0.5	3.0 ± 0.04	3.2 ± 0.15	4.0 ± 0.02	3.4 ± 0.9	7.5 ± 0.3	2.0 ± 0.8	7.5 ± 0.18
4	-	-	1.6 ± 0.3	3.0 ± 0.02	2.5 ± 0.5	4.1 ± 0.03	1.9 ± 0.25	7.6 ± 0.3	2.0 ± 0.7	7.5 ± 0.07
5	-	-	1.8 ± 0.1	3.0 ± 0.01	2.2 ± 0.2	4.0 ± 0.01	1.9 ± 0.12	7.6 ± 0.2	1.9 ± 0.15	7.5 ± 0.06
6	-	-	1.5 ± 0.06	3.0 ± 0.02	2.1 ± 0.16	4.0 ± 0.01	1.8 ± 0.2	7.6 ± 0.25	1.8 ± 0.45	7.5 ± 0.03
7	-	-	1.5 ± 0.2	3.0 ± 0.015	2.1 ± 0.1	4.0 ± 0.01	1.5 ± 0.27	7.7 ± 0.16	1.9 ± 0.1	7.6 ± 0.03
8	-	-	1.0 ± 0.1	3.1 ± 0.015	1.8 ± 0.2	4.0 ± 0.015	1.1 ± 0.1	7.8 ± 0.18	1.6 ± 0.2	7.6 ± 0.04
9	-	-	0.9 ± 0.2	3.1 ± 0.01	1.7 ± 0.2	4.0 ± 0.02	0.8 ± 0.1	7.8 ± 0.15	1.4 ± 0.17	7.7 ± 0.08
10	-	-	0,8 ± 0,2	3,1 ± 0,03	1.5 ± 0.14	4.0 ± 0.02	0.5 ± 0.17	7.8 ± 0.4	0.9 ± 0.2	7.8 ± 0.15
11	-	-	0.8 ± 0.25	3.2 ± 0.03	1.4 ± 0.1	4.0 ± 0.02	0.3 ± 0.25	7.8 ± 0.4	0.5 ± 0.14	7.9 ± 0.13
12	-	-	0.6 ± 0.1	3.2 ± 0.04	1.4 ± 0.1	4.0 ± 0.03	0.2 ± 0.1	7.9 ± 0.4	0.5 ± 0.15	7.9 ± 0.29

-: No growth

Table 4b. Monthly viable cell counts (cfu/ml) and pH values of *B. subtilis* EGE-B-1.19 in acidic liquid formulations

Storage (Month)	Acidic liquid formulation of *B. subtilis* EGE-B-1.19									
	pH 2.0		pH 3.0		pH 4.0		pH 5.0		Control	
	Population density (x10^8 CFU/ml)	pH	Population density (x10^8 CFU/ml)	pH	Population density (x10^8 CFU/ml)	pH	Population density (x10^8 CFU/ml)	pH	Population density (x10^8 CFU/ml)	pH
0	2.9 ± 0.8	2.0 ± 0	3.0 ± 1.3	3.0 ± 0	3.2 ± 0.7	4.0 ± 0	3.5 ± 0.5	5.0 ± 0	3.5 ± 1.14	8.0 ± 0
1	1.3 ± 0.1	2.3 ± 0.01	1.2 ± 0.70	2.9 ± 0.01	2.2 ± 0.5	5.1 ± 0.02	4.1 ± 0.05	7.2 ± 0.3	3.3 ± 0.7	7.9 ± 0.2
2	0.03 ± 0.2	2.2 ± 0.05	0.5 ± 0.2	2.8 ± 0.03	4.0 ± 1.45	5.1 ± 0.07	4.8 ± 0.9	7.0 ± 0.2	3.6 ± 0.15	7.9 ± 0.4
3	-	-	1.1 ± 0.5	2.8 ± 0.01	4.8 ± 1	5.2 ± 0.1	6.5 ± 0.5	7.5 ± 0.6	2.8 ± 0.06	7.8 ± 0.8
4	-	-	0.4 ± 0.24	2.8 ± 0.02	4.6 ± 2.6	5.9 ± 0.7	4.7 ± 1.1	8.3 ± 1.05	4.2 ± 0.4	8.4 ± 0.9
5	-	-	0.1 ± 0.02	3.0 ± 0.02	4.5 ± 0.7	6.2 ± 1.3	3.5 ± 1	8.1 ± 1.03	7.8 ± 0.4	8.7 ± 0.28
6	-	-	0.1 ± 0.04	3.0 ± 0.01	2.8 ± 0.45	6.6 ± 1.7	2.35 ± 0.55	7.8 ± 0.9	1.9 ± 0.75	8.2 ± 0.64
7	-	-	0.1 ± 0.01	3.0 ± 0.01	3.1 ± 1	6.7 ± 1.7	4 ± 1.6	8.1 ± 1.2	3.9 ± 0.2	8.3 ± 0.77
8	-	-	0.4 ± 0.12	2.9 ± 0.03	4.25 ± 0.25	6.8 ± 1.4	2.8 ± 0.95	7.8 ± 1.04	1.75 ± 0.15	8.1 ± 0.53
9	-	-	0.1 ± 0.01	2.9 ± 0.03	3.7 ± 1.6	6.8 ± 1.4	3.7 ± 1.35	7.8 ± 1.1	2.45 ± 0.45	8.2 ± 0.2
10	-	-	0.1 ± 0.08	2.9 ± 0.05	2.9 ± 0.7	6.9 ± 1.3	2.6 ± 0.6	7.7 ± 1.05	1.5 ± 0.3	8.2 ± 0.27
11	-	-	0.15 ± 0.01	2.9 ± 0.01	2.85 ± 0.95	7.0 ± 1.2	1.3 ± 0.6	7.7 ± 1.1	3.2 ± 0.95	8.1 ± 0.4
12	-	-	0.1 ± 0.01	2.8 ± 0.03	3.35 ± 0.55	7.1 ± 1.2	2.4 ± 0.5	7.8 ± 1.1	1.5 ± 0.95	8.2 ± 0.5

-:No growth

In the study conducted by Muis, it was determined that *B. subtilis* grows easily between pH 5.0 and pH 8.0, and the optimum pH is pH 6.0 [22]. Because formulations formed in these ranges will support the growth of bacteria, lower acidic values were used for acetic formulations in our study. In addition, it has been noted that the growth of contamination is inhibited when the culture media is provided at a low pH level [15]. Issahary et al. investigated the activation of *B. cereus* endospores under low pH at different temperatures (50 ℃, 60 ℃ and 70 ℃)[23]. Because of the treatment of endospores at pH 1.0 and all temperatures, they showed that endospores were activated much faster than the control group (water only), but endospores entered the death phase very early. As the temperature increased, the endospores were activated more quickly and entered the death phase more quickly. In addition, in our study, it was determined that the acidic liquid formulation at pH 2.0 did not have viable cells at the end of the 3rd month. In the study, it was observed that the endospores of both *Bacillus* strains were lysed at pH 2.0. Wilks et al. determined the resistance of *B. subtilis* AG174 to extreme acidic and alkaline culture broth *B. subtilis* AG174 was cultured at pH 6.0 and pH 7.0, and the culture broth was adjusted to pH 4.5 [18]. After 2 h, the viability of the strain was determined as 60-100% and 5%−15%, respectively. In addition, the *B. subtilis* strain was cultured at pH 7.0 and pH 9.0, and the culture broth was adjusted to pH 10.0. After 2 h, *B. subtilis* strain viability was determined as 1-5% and 40%−100%, respectively. In this study, they showed the importance of the pH of the growth medium for viability. In our study, *Bacillus* strains in the endospore form were produced under optimum production conditions (pH 7.0, 30 ºC). Therefore, the survival time of the bacteria increased and reached 12 months under similar pH conditions.

Vehapi and Özçimen adjusted the pH of the Luria –Bertani broth (LB) medium to pH 3.0, pH 5.0, and pH 7.0 in their study to investigate the growth of *the B. subtilis* strain [24]. They found that the specific growth rate of the *B. subtilis* strain was seven times higher in the culture medium adjusted to pH 7.0 than in the culture medium adjusted to pH 3 and almost equal to that in the culture medium adjusted to pH 5. The formulations are designed to maintain a steady viable cell count in the strain that will be used. If the pH range of the formulation is suitable for the growth of the strain, the strain may die after a while because of factors such as insufficient nutrients in the environment. Therefore, studies should be conducted on formulations that maintain a steady viable cell count in the strain over time. Furthermore, based on the results obtained in the study, it was concluded that different acidic formulations at various pH values should be developed for both *B. subtilis* strains.

3.4 Shelf Life of the Alkaline Liquid *Bacillus Subtilis* Ege-B-1.19 Formulations

Calcium acetate and calcium hydroxide, which are suitable for use as food additives, are pH regulating substances that enhance bioavailability [25]. In our study for 12 months, it was determined that the alkaline formulations used for *the B. subtilis* EGE-B-1.19 strain provided suitable conditions (Table 5). When the viable cell count was compared with the control group using the ANOVA test in the SPSS package program, it was determined that there had been a statistically significant ($p<0.05$) difference between the control and the alkaline formulation of the *B. subtilis* EGE-B-1.19 strain at pH 9.5 (Table 6)

Table 5. Monthly total number of viable cells (cfu/ml) and pH values of *B. subtilis* EGE-B-1.19 in alkaline liquid formulations

Storage (Month)	Alkaline liquid formulation of *B. subtilis* EGE-B-1.19							
	pH 9.0		pH 9.5		pH 10.0		Control	
	Population density ($x10^8$ CFU/ml)	pH	Population density ($x10^8$ CFU/ml)	pH	Population density ($x10^8$ CFU/ml)	pH	Population density ($x10^8$ CFU/ml)	pH
0	3.5 ± 0.45	9.0 ± 0	3.7 ± 0.45	9.5 ± 0	3.8 ± 1.45	10.0 ± 0	3.5 ± 1.14	8.0 ± 0
1	3.6 ± 1.05	7.6 ± 0.1	3.8 ± 1,1	9.1 ± 0.15	3.8 ± 0.8	9.7 ± 0.02	3.3 ± 0.7	7.9 ± 0.2
2	4.3 ± 0.7	7.1 ± 0.16	2.7 ± 0.7	8.6 ± 0.04	3.8 ± 0.5	9.4 ± 0.16	3.6 ± 0.15	7.9 ± 0.4
3	4.3 ± 1.1	7.3 ± 0.5	4.8 ± 2.15	7.8 ± 0.65	2.8 ± 0.6	9.2 ± 0.4	2.8 ± 0.06	7.8 ± 0.8
4	5.8 ± 1.55	8.1 ± 0.9	2.0 ± 0.1	7.9 ± 0.4	3.55 ± 0.05	8.9 ± 0.8	4.2 ± 0.4	8.4 ± 0.9
5	3.2 ± 0.1	8.0 ± 0.45	4.7 ± 1.2	7.2 ± 0.1	2.05 ± 0.45	8.1 ± 0.9	7.8 ± 0.4	8.7 ± 0.28
6	3.0 ± 1	7.9 ± 0.7	2.6 ± 0.2	7.3 ± 0.6	2.1 ± 0.8	7.7 ± 0.3	1.9 ± 0.75	8.2 ± 0.64
7	3.5 ± 0.6	7.9 ± 0.8	3.6 ± 0.9	7.2 ± 0.3	3.2 ± 1.45	7.8 ± 0.2	3.9 ± 0.2	8.3 ± 0.77
8	1.85 ± 0.75	7.9 ± 0.9	2.15 ± 0.25	7.1 ± 0.25	0.9 ± 0.2	7.9 ± 0.4	1.75 ± 0.15	8.1 ± 0.53
9	1.9 ± 0	8.1 ± 0.3	1.4 ± 0.2	7.1 ± 0.1	1.55 ± 0.55	8.0 ± 0.15	2.45 ± 0.45	8.2 ± 0.2
10	4.7 ± 0.2	7.9 ± 0.5	3.8 ± 0.1	7.3 ± 0.3	1.3 ± 0.5	8.2 ± 0.1	1.5 ± 0.3	8.2 ± 0.27
11	3.4 ± 1.2	7.8 ± 0.7	1.25 ± 0.25	7.2 ± 0.6	1.1 ± 0.2	8.2 ± 0.1	3.2 ± 0.95	8.1 ± 0.4
12	2.0 ± 0.25	7.9 ± 0.7	3.7 ± 0.3	7.2 ± 0.5	1.7 ± 0.6	8.3 ± 0.2	1.5 ± 0.95	8.2 ± 0.5

Table 6. Statistical values of liquid formulation of *B. subtilis* EGE-B-1.19 and *B. subtilis* EGE-B-36.5

***Bacillus subtilis* EGE-B.1.19**				***Bacillus subtilis* EGE-B.36.5**	
Acidic pH	**Population density ($x10^8$ cfu/ml)**	**Alkaline pH**	**Population density ($x10^8$ cfu/ml)**	**Acidic pH**	**Population density ($x10^8$ cfu/ml)**
Control	1.5 ± 0.95 *[b]	**Control**	1.5 ± 0.95 [b]	**Control**	0.5 ± 0.15 [b]
pH 3.0	0.1 ± 0.01 [c]	**pH 9.0**	2.0 ± 0.25 [ab]	**pH 3.0**	0.6 ± 0.1 [ab]
pH 4.0	3.35 ± 0.55 [a]	**pH 9.5**	3.7 ± 0.3 [a]	**pH 4.0**	1.4 ± 0.1 [a]
pH 5.0	2.4 ± 0.5 [ab]	**pH 10.0**	1.7 ± 0.6 [b]	**pH 5.0**	0.2 ± 0.1 [c]

*In a column, means that are followed by the same letter are statistically similar.

When the literature is examined, it is difficult to suppress growth in neutral and weakly alkaline pH conditions. Chung et al. showed that the strong alkaline structure of the zeolite NaA they used prevented the transformation of endospores into a vegetative form [26].The liquid formulations produced from *Bacillus* strains were adjusted to pH 5.0, pH 9.0, and pH 11.0 and the viable cells count. In the formulations created with pH 5.0 and pH 9.0, there was a decrease in the viable cell count on the 20th day, and no viable cells were found in the formulations after the 60th day. In the formulation created at pH 11.0, they reported that endospores were preserved without growth for up to 60 days.

4. CONCLUSION

These results showed that acidic liquid formulations using acetic acid stabilized the viable cell count of both *B. subtilis*EGE-B-36.5 and *B. subtilis* EGE-B-1.19 strains. The optimum pH value for the shelf life of *B. subtilis* EGE-B-36.5 and *B. subtilis* EGE-B-1.19 was found to be pH 4.0. In addition, the optimum pH for the alkaline formulation of *B. subtilis* EGE-B-1.19 was determined to be pH 9.5. According to this study, the optimum pH value should be specifically determined for each strain when preparing acidic and alkaline liquid formulations.

ACKNOWLEDGEMENTS

This work was supported by Ege University Scientific Research Projects Coordination Unit. Project Number FYL-2020-21642.

CONFLICT OF INTEREST

The authors stated that there are no conflicts of interest regarding the publication of this article.

CRediT AUTHOR STATEMENT

Sevgi İşlek: Conceptualization, Methodology, Validation, Investigation, Writing – Original Draft, Writing – Review & Editing. **Kemal Karaca:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – Original Draft, Writing – Review & Editing. **Rengin Eltem:.** Conceptualization, Methodology, Investigation, Writing – Review & Editing, Supervision, Project administration, Funding acquisition

REFERENCES

[1] Singh M, Singh D, Gupta A, Pandey KD, Singh PK, Kumar A. Plant Growth Promoting Rhizobacteria. PGPR Amelioration in Sustainable Agriculture 2019; 41–66.

[2] Chandini, Kumar R, Kumar R, Prakash O. The impact of chemical fertilizers on our environment and ecosystem. Research trends in environmental sciences 2019; 69-86.

[3] Tabassum B, Khan A, Tariq M, Ramzan M, Iqbal Khan MS, Shahid N, Aaliya K. Bottlenecks in commercialisation and future prospects of PGPR. Applied Soil Ecology 2017; 121: 102–117.

[4] Bhattacharyya PN, Jha DK. Plant growth-promoting rhizobacteria (PGPR): Emergence in agriculture. In World Journal of Microbiology and Biotechnology 2012; 28: 1327–1350.

[5] Beneduzi A, Passaglia LM. Genetic and phenotypic diversity of plant growth promoting bacilli. In Bacteria in agrobiology: plant growth responses, Springer, Berlin, Heidelberg 2011; 1-20.

[6] Akinrinlola RJ, Yuen GY, Drijber RA, Adesemoye AO. Evaluation of *Bacillus* Strains for Plant Growth Promotion and Predictability of Efficacy by In Vitro Physiological Traits. International Journal of Microbiology 2018; 1: 5686874.

[7] Kumar A, Prakash A, Johri BN. *Bacillus* as PGPR in Crop Ecosystem, In Bacteria in Agrobiology: Crop Ecosystems, Springer, Berlin, Heidelberg 2011; 37–59.

[8] Malusá E, Sas-Paszt L, Ciesielska J. Technologies for beneficial microorganisms inocula used as biofertilizers, In The Scientific World Journal 2012; 1: 491206

[9] Lobo CB, Juárez Tomás MS, Viruel E, Ferrero MA, Lucca ME. Development of low-cost formulations of plant growth-promoting bacteria to be used as inoculants in beneficial agricultural Technologies. In Microbiological Research 2019: 219: 12–25.

[10] Nakkeeran S, Fernando WD, Siddiqui ZA. Plant growth promoting rhizobacteria formulations and its scope in commercialization for the management of pests and diseases. PGPR: Biocontrol and biofertilization 2005; 257-296.

[11] Bahadir PS, Liaqat F, Eltem R. Plant growth promoting properties of phosphate solubilizing *Bacillus* species isolated from the Aegean Region of Turkey. Turkish Journal of Botany 2018; 42(2): 183-196.

[12] Oztopuz O, Sarigul N, Liaqat F, Park RD, Eltem R. Chitinolytic *Bacillus subtilis* Ege-B-1.19 as a biocontrol agent against mycotoxigenic and phytopathogenic fungi. Turkish Journal of Biochemistry 2019; 44(3): 323-331.

[13] Liaqat F, Bahadır PS, Elibol M, Eltem R. Optimization of chitosanase production by *Bacillus mojavensis* EGE-B-5.2 i. Journal of basic microbiology 2018; 58(10): 836-847.

[14] Kumar AP, Janardhan A, Radha S, Viswanath B, Narasimha G. Statistical Approach to Optimize Production of Biosurfactant by *Pseudomonas aeruginosa* 2297. 3 Biotech 2015; 5: 71-79.

[15] Reuter CJ. Compositions for stabilizing *Bacillus* spores and methods of use thereof. 2011; U.S. Patent No. 20110200572

[16] Daniels RS. Corn Steep Liquor as a biostimulant composition 2012; U.S. Patent No. 2012015454

[17] Turan M. Mısır maserasyon sıvısı, çinko oksit, borik asit ile bakteri karışımından meydana gelen bir organik gübre. 2017; TR Patent No. 201613931

[18] Wilks JC, Kitko RD, Cleeton SH, Lee GE, Ugwu CS, Jones BD, BonDurant SS, Slonczewski JL. Acid and base stress and transcriptomic responses in *Bacillus subtilis*. Applied and environmental microbiology 2009; 75(4): 981-99.

[19] Bayram S, Aydogan MN. Searching for Versatile Polysaccharide-Degrading Alkali-tolerant or Alkaliphilic *Bacillus* Strains. Journal of the Institute of Science and Technology 2022; 12(1): 133-141.

[20] Gauvry E, Mathot AG, Couvert O, Leguérinel I, Coroller L. Effects of temperature, pH and water activity on the growth and the sporulation abilities of *Bacillus subtilis* BSB1. International Journal of Food Microbiology 2021; 337:108915.

[21] To HTA, Chhetri V, Settachaimongkon S, Prakitchaiwattana C. Stress tolerance-*Bacillus* with a wide spectrum bacteriocin as an alternative approach for food bio-protective culture production. Food Control 2022; 133: 108598.

[22] Muis A. Biomass production and formulation of *Bacillus subtilis* for biological control. Indonesian journal of agricultural science 2006; 7(2): 51-56.

[23] Issahary G, Evenchik Z, Keynan A. Low-p H Activation of *Bacillus cereus* Spores. Journal of Bacteriology 1970; 101(2): 418-422.

[24] Vehapi M, Özçimen D. Investigation of *B. subtilis* viability at different pH ranges for use in microbial cleaner formulation. Bulletin of Biotechnology 2020; 1(1): 1-7.

[25] Jha S, Singh R, Pandey A, Bhardwaj M, Tripathi SK, Mishra RK, Dikshit A. Bacterial toxicological assay of calcium oxide nanoparticles against some plant growth-promoting rhizobacteria. Int. J. Res. Appl. Sci. Eng. Technol 2018; 6(11): 460-466.

[26] Chung S, Lim JH, Kim SD. Powder formulation using heat resistant endospores of two multi-functional plant growth promoting rhizobacteria *Bacillus* strains having phytophtora blight suppression and growth promoting functions. Journal of the Korean Society for Applied Biological Chemistry 2010; 53(4): 485-492.