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Research Article

The effect of environmental factors on surfactin production of *Bacillus subtilis*

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Abstract: Surfactin is one of the most efficient biosurfactants excreted by *Bacillus subtilis* which displays the highest potential as induced systemic resistance elicitor among all metabolites produced by *B. subtilis*. Environmental factors have considerable effect on surfactin production. In this study surfactin production of two *Bacillus subtilis* strains were analyzed using high performance liquid chromatography (HPLC). C14 and C15 surfactins were detected in the ethanol extract from acid-precipitated supernatant. HPLC analyses of different media including Nutrient Broth (NB) medium, NB plus 40g/l glucose, NB plus 10% soil extract and NB plus 10% plant extract medium, clearly showed that these bacteria produced different amounts of surfactins C14 and C15 in these media. Surfactin production in NB/plant medium was relatively the highest in quantity. Microelements analysis of media containing plant and soil extract with atomic absorption spectrometry showed high amounts of Fe, Mn and Zn in medium containing plant extract compared with that of soil extract. Since these elements play an important role in surfactin production, high amounts of Fe, Mn and Zn in NB/plant extract medium compared to the NB/soil extract medium could be the possible reason for relatively higher amounts of surfactins C14 and C15 produced in NB/plant medium. So adding these important elements to soil may boost biocontrol effect of *B. subtilis* against plant pathogens.

Keywords: Surfactin, HPLC, *Bacillus* spp., biocontrol

Introduction

Surfactin, one of the most efficient biosurfactants, belongs to the lipopeptide family and is produced by *Bacillus subtilis* (Copper *et al*., 1981). It is a cyclic lipopeptide composed of a heptapeptide and a β-hydroxy fatty acid with variable chain lengths of 13-15 carbon atoms (Baumgart, *et al*., 1991; Oka, *et al*., 1993). Usually, for each variant, Bacillus strains may co-produce several homologues of different length of the fatty acid chain (Ongena and Jacques, 2008). The cyclic lipopeptide (C14 and C15) surfactin might have an efficient role at triggering systemic resistance (Henry *et al*., 2011). The increasing interest in surfactin is because of its amphiphilic character, which is responsible for its excellent surface-active properties as it reduces the surface tension of water from 72 to 27mN/m at a concentration as low as 0.005% (Arima *et al*., 1968). These compounds have potential applications in both medical and biotechnological fields (Asad *et al*.,

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2010). This biosurfactant possesses antifungal and antibacterial activity, inhibits fibrin clot formation, induces formation of ion channels in lipid bilayer membranes, inhibits cyclic adenosine monophosphate (cAMP), platelet and spleen cytosolic phospholipase A2 (PLA2) and also exhibits antitumor activities (Kim *et al*., 1998; Singh and Cameotra, 2004).

Surfactin does not have a specific affinity for plant sterols in contrast to other Bacillus lipopeptides such as iturin (Maget-dana and Peypoux, 1994) and fengycin (Eeman *et al*., 2009). This may partly explain the differential bioactivities of these structurally closely-related lipopeptide families on various cell types. In Bacillus, surfactin plays an important role in surface attachment and biofilm formation (Bais *et al*., 2004; Raaijmakers *et al*., 2010). It was demonstrated that the biocontrol ability of a wild-type *B. subtilis* strain 6051 against *Psedomonas syringa* depended on the amount of surfactin production (Bais *et al*., 2004). Bais *et al*. (2004) mentioned that formation of biofilms by *B. subtilis* was a complex process that included secretion of surfactin. To determine the role of surfactin in biocontrol by *B. subtilis*, a mutant strain, M1, with a deletion in a surfactin synthase gene was tested. It was found that *B. subtilis* M1 was ineffective as a biocontrol agent against *P. syringae* infectivity in Arabidopsis and also failed to form robust biofilms on either roots or inert surfaces. Crude extracellular lipopeptide surfactin was found to be also significantly effective in promoting the growth of soybean seedlings under greenhouse conditions, compared with the non-treated control (Preecha *et al*., 2010). Guerra-Santos *et al*. (1986) reported the effect of environmental factors such as, pH, temperature, and aeration on the bacterial cell growth and biosurfactant production. The influence of metal ions on biosurfactant production has been reported by others (Chayabutra *et al*., 2001; Copper *et al*., 1981). Lang *et al*. (1999) and Wei *et al*. (2005) reported the pronounced effect of carbon source used in bacterial culture on biosurfactant production. Nitschke *et al*. (2005) also reported the effect and the role of nitrogen sources on the production of surface-active compounds by microorganisms.

The objective of this study was to investigate the effect of environmental factors on surfactin production of *Bacillus subtilis*. Hence, we used two strains of *B. subtilis* including ATCC 21332 and *B. subtilis* OKB105 strain FKR3 (with different amounts of surfactin secretion ability), and different media including glucose, soil extract and plant extract. In addition, microelements analysis of plant and soil extracts was carried out with atomic absorption spectrometry. In this way we could find out the effect of glucose, soil and plant extracts (three main environmental factors) on surfactin production.

Materials and Methods

Bacterial strains and growth conditions

Bacterial strains used in this study were *B. subtilis* ATCC 21332 (wild type, American Type Culture Collection) and *B. subtilis* OKB105 strain FKR3 provided kindly by M. A. Marahiel (Department of Chemistry, Biochemistry, Philipps-University Marburg, Germany). The strains were inoculated in 50 ml of Nutrient Broth medium, Nutrient Broth plus 40 gr/lit Glucose (Yeh *et al*., 2005), Nutrient Broth plus 10% soil extract and Nutrient Broth plus 10% plant (*Vitis* sp.) extract, then incubated in a shaker-incubator at 180 rpm/28 °C for 24 h.

Preparation of soil extract

To prepare soil extract, 1 kg of garden soil was added to 1 liter of water and autoclaved at 121 °C for 20 min then filtered and restored volume to 1 liter (Lochhead and Burton, 1957).

Preparation of plant extract

According to the modified protocol of Leila (1977), the dried leaves of *Vitis* sp. were added to sterile distilled water in a ratio of 1: 20 (50g in 1L water), and mixture was stirred for1 hour at room temperature. The extract was then filtered and autoclaved at 121 °C for 20 min.

Purification of Surfactin

Liquid medium containing bacteria was subjected to centrifugation at 8000 rpm for 5 min to remove

the bacterial cells. The supernatant was then subjected to an acid precipitation with 6 M HCl by adjusting the pH to 2.0 and refrigerated (4 °C) overnight to facilitate precipitation of lipoproteins. The precipitant was collected by centrifugation at 8000 rpm for 5 min. Then it was dissolved in distilled water and the pH adjusted to 7.0 with 10 M NaOH. The suspension was extracted with equal volume of dichloromethane (Merck) for 30 min at room temperature. The dichloromethane phase was resolved by centrifugation and evaporation. The white precipitate was then dissolved in 0.5 ml of ethanol and filtered using a 0.2 mm filter, and then used for chromatography (Cooper *et al*, 1981, Jacques *et al*., 1999; Araujo *et al*., 2002).

Quantification of Surfactin

The concentration of Surfactin was determined using an HPLC-UV/vis quantification method. The analysis was performed with a standard HPLC device (Waters Series 600, Meriland, USA) equipped with a reverse-phase column (C18150 × 3.9 mm, 60Å, Cartridge column, Ireland) at 30 °C. Mobile phase consisted of a mixture of acetonitrile (ACN) and 3.8 mM trifluoroacetic acid (TFA) solution in water at the ratio of 80:20 and were pumped with an isocratic mode at a flow rate of 0.7 ml/min for30 min. The sample injection was set at 50 µl and the peptide bonds of Surfactin were detected at 210 nm. Standard surfactin (98% purity) was purchased from Sigma-Aldrich.

Surfactin calibration curve

The obtained chromatograms were analyzed using Empower login software. The surfactin calibration curve was constructed by plotting graphs of the total peak area (TPA) against various concentrations (50ppm, 125ppm, 250ppm, 500ppm and 1000ppm) of standard surfactin.

Soil and plant analysis

Elemental analyses of soils and plants were conducted after bringing the sample into solution using acid digestion. Trace elements (Zn, Mn and Fe) in soil samples were extracted with Diethylene triamine-penta acetic acid (DTPA) solution (Lindsey and Norvell, 1978) and in leaf samples were determined after decomposition by acid mixture of HNO3 (65%) + HClO4 (70%) (4:1 ratio), and HF (40%) in an open system at 200 °C (Manouchehri, *et al*., 2006). A blank digestion solution was made for comparison.

Instrumentation

The atomic absorption spectrometry (AAS) (AA-650, Shimadzu, and Kyoto, Japan), technique was used for the determination of metal elements including Zn, Mn and Fe in soil and leaf samples. This technique features a high accuracy and precision of the micro and trace determinations of elements on the condition that the analyzed sample is adequately prepared (Moralejo and Acebal, 2014). The results were analyzed with SPSS ver 16.0 (SPSS, Chicago, IL, USA). P value ≤ 0.05 was considered significant. Data are shown as mean ± standard deviation (SD).

Results and Discussion

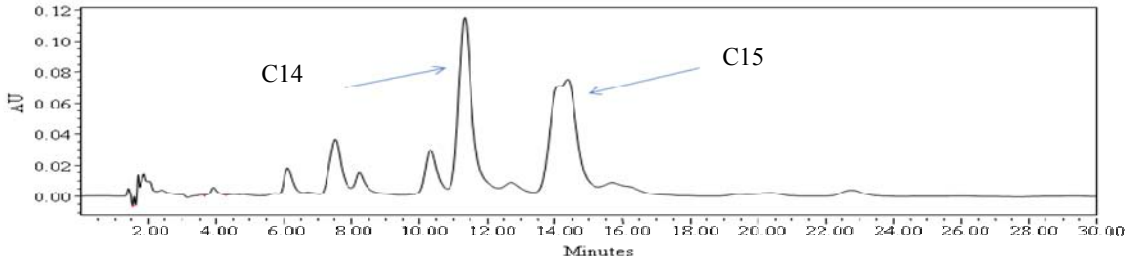
Peptide production

In order to compare lipopeptide biosynthesis of *B. subtilis* ATCC 21332 and FKR3 strains, lipopeptides were extracted from the culture supernatant of both strains with ethanol after 24 h of growth in the four different broth media, then they were analyzed by HPLC. As expected, the strain *B. subtilis* ATCC 21332 produced the lipoheptapeptide surfactins C14 and C15, peaks of which were recognized in retention times of 10.98 and 13.62 min, respectively (Fig. 1).

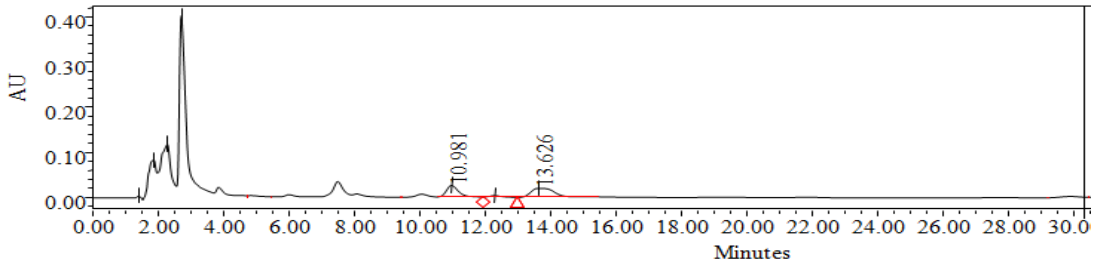
Considering calibration curve (Fig. 2) the production rates of surfactins C14 and C15 in the strain *B. subtilis* ATCC 21332 and FKR3 strains were estimated according to table 1. We used only two isomers of surfactin, C14 and C15, in our study, but it is clear that our strain may also produce other isomers as well (Akpa *et al*., 2001; Ongena and Jacques, 2008). This study showed that bacterial growth in different media such as NB, NB/ Glucose, NB/soil extract and NB/plant extract, had different effects on the amount of surfactin (C14 and C15) production in the two different strains of *B. subtilis*, ATCC 21332 and FKR3 when measured with HPLC. The amount of

surfactin production was significantly higher in NB/plant medium than in other media. Strain ATCC21332 produced surfactin in all media but surfactin production was not detected in the two media NB and NB/Glucose by the strain FKR3. In the latter strain application of soil and plant extract to the NB medium lead to surfactin production indicating that these extracts have inducible effects on FKR3 for surfactin production. The effect of the plant extract was much higher than the soil extract on surfactin production (15.9 fold). This increase in Surfactin production was also observed when plant extract was applied in NB for the strain ATCC21332 (5.6 fold). In agreement with our findings in this study, many authors have reported that environmental factors such as pH, temperature, and aeration have considerable effects on the bacterial cell growth and biosurfactant production (Nitschke *et al*., 2005; Guerra-Santos *et al*., 1986). Nitschke *et al*. (2005) reported the effect and role of nitrogen sources on the production of surface-active compounds by microorganisms. HPLC analyses of surfactin production in four different studied media by ATCC21332 and FKR3 strains, showed that these bacteria produced different amounts of surfactin C14 and C15 depending on their different environmental conditions similar to those found by other scientists (Nitschke *et al*., 2005; Guerra-Santos *et al*., 1986).

A) Standard Surfactin.



B) Bacillus (ATCC21332).



C) Bacillus (FKR3).

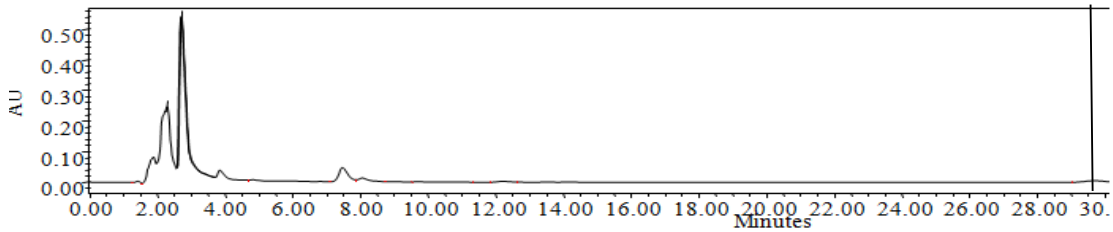


Figure 1. HPLC chromatograms of Surfactin compounds in different strains of *Bacillus subtilis*. A) Chromatogram of standard surfactin. B) Surfactin chromatogram of *Bacillus subtilis* strain ATCC21332. C) Surfactin chromatogram of *B. subtilis* OKB105 strain FKR3.

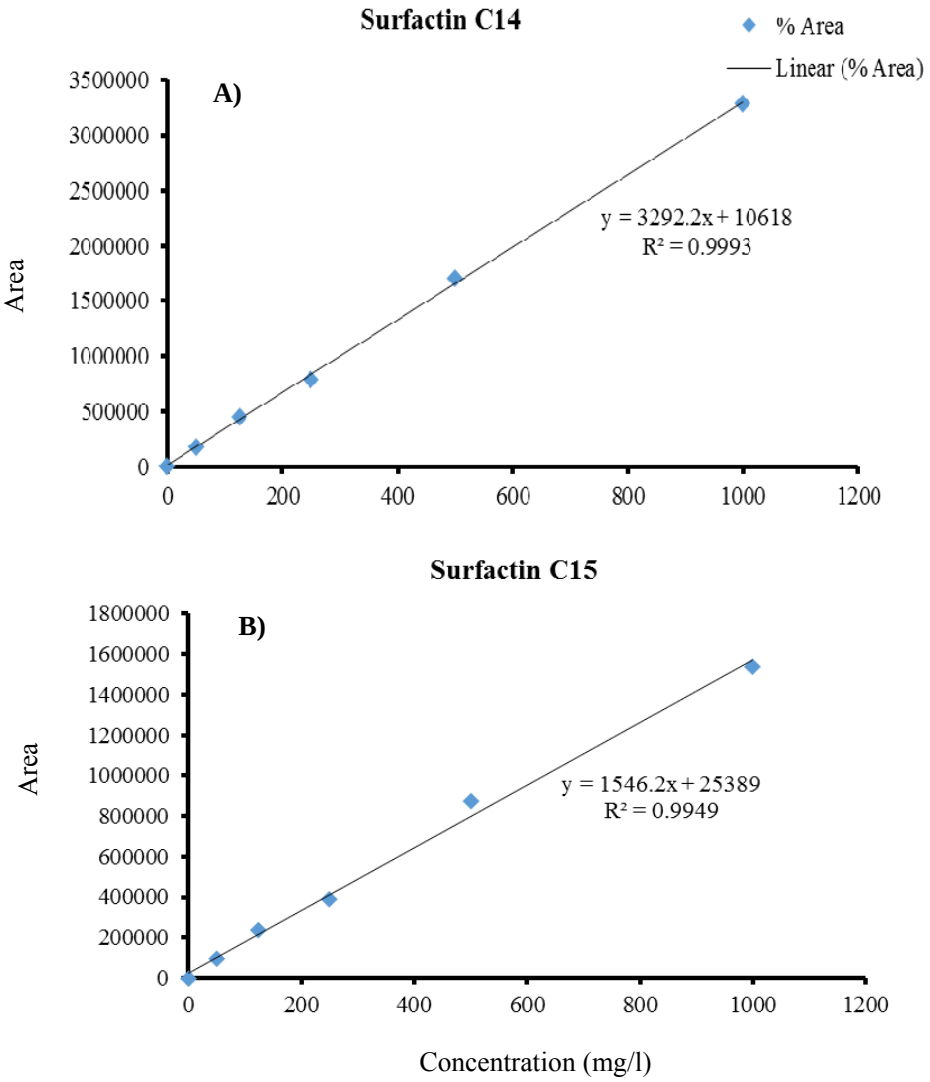


Figure 2. The surfactin calibration curve. A) The calibration curve of surfactin C14. B) The calibration curve of surfactin C15.

Table 1. Surfactin production by two strains of *Bacillus subtilis* in different media.

Media	Surfactin content (C14 + C15) (µg per 8 x 10^8cells)	
	Strain ATCC21332	Strain FKR3
Nutrient Broth	45.60 ± 2.21^{d}	0
Nutrient Broth plus40 g/l glucose	22.13 ± 1.00^{de}	0
Nutrient Broth plus10% soil extracted	400.89 ± 26.19^{b}	11.87 ± 0.34^{de}
Nutrient Broth plus10% plant extracted (*Vitis* sp.)	2249.62 ± 17.90^{a}	189.20 ± 9.57^{c}

Means (± SE) followed by the same letters are not significantly different at P ≤ 0.05 level.

Both bacterial strains grown in NB/plant-extract showed highly significant increase in the surfactin production amounts compared with the other treatments including NB medium, NB/Glucose medium and NB/soil extract (Table 1).

Total content of elements in soil and plant

In order to determine the total content of micronutrients in the soil and plant (*Vitis* sp.) extracts, Fe, Mn and Zn elements were measured using the atomic absorption spectrometry (Moralejo and Acebal, 2014). The levels of Zn, Mn and Fe in plant and soil samples are given in Table 2. The analysis showed that the plant samples contained significantly higher levels of micronutrients compared to the soil samples.

Table 2. Concentration of trace elements in soil and plant extracts.

Element	Mean ± SE (mg/kg)	
	Plant extraction	Soil extraction
Fe	1.64 ± 0.03^{c}	0.53 ± 0.02^{d}
Mn	3.01 ± 0.02^{b}	0.31 ± 0.01^{e}
Zn	3.22 ± 0.02^{a}	0.23 ± 0.01^{f}

Means followed by the same letters are not significantly different at $P \leq 0.05$.

Microelements analysis of plant and soil extracts with atomic absorption spectrometry showed high amount of Fe, Mn and Zn in plant extract compared with the soil extract. Since these elements play an important role in the surfactin production (Copper *et al*., 1981), high amounts of Fe, Mn and Zn in NB/plant - extract medium compared to the NB/soil-extract medium could be the possible reason for relatively high amount of surfactin C14 and C15 production in NB/plant medium. Growing the ATCC21332 strain in iron-enriched minimal salt (MSI) medium (Wei *et al*., 2004) consisting of glucose 40g/L resulted in optimal surfactin production (Yeh *et al*., 2005). On the contrary, our investigations showed that Glucose 40g/L in NB medium did not show a positive significant effect on increasing surfactin C14 and C15 production compared to the NB medium as a control (Table 1). This contrasting result may be due to measuring only two isomers of surfactin in the present study instated of the whole surfactins and also using the NB medium which is not optimal.

According to our findings significantly high amounts of Fe, Mn and Zn elements in NB/plant medium were detected compared to the NB/soil medium that could be the possible reason for relatively higher amount of surfactin C14 and C15 production in NB/plant medium than NB/soil medium. These results are in agreement with findings of Copper *et al*. (1981) and Chayabutra *et al*. (2001). Copper *et al*. (1981) reported among large number of metal salts including MnSO4, FeSO4, and $Fe_2(SO4)_3$, $MgSO_4$, $CaCl_2$, Na_2HPO_4, KH2PO4, $NaNO_3$, $ZrOCl_2$, $UO_2(C2H3O_2)_2$, $VOSO_4$, $CuSO_4$, $NiSO_4$, $CoSO_4$, and $A1_2(SO4)_3$ that was added to culture media, only iron and manganese salts caused significant enhancement of surfactin production. The surfactin could be improved by the addition of either iron or manganese salts (Chayabutra *et al*., 2001; Copper *et al*., 1981). In consistence with findings of Copper *et al*. (1981), analyzing metal elements in plant and soil extract in the present study showed high amount of Fe, Mn and Zn in plant extract compared with soil extract (Table 2). According to Copper *et al*. (1981), a small amount of $MnSO_4$ (about 10^{-6} M) and soluble iron (0.3 mg/liter) are needed for the maximum effect on surfactin production by *B. subtilis*. So adding these important elements to soil can be useful in biocontrol effect of *B. subtilis* against plant pathogens by formation of biofilms and secretion of surfactin that acts as a lipopeptide antimicrobial agent (Bais *et al*., 2004; Copper *et al*., 1981). Surfactin is the main elicitor secreted by *B. subtilis* and *B. amyloliquefaciens* S499 displaying consistent induced systemic resistance activity in plants (Henry *et al*., 2011; Jourdan *et al*., 2009; Ongena *et al*., 2007; Ongena and Jacques, 2008;).

This study has provided a field to improve the positive biological control effects of *B. subtilis*

that could be used as a promising agent against plant pathogens in sustainable agriculture.

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بررسی تأثیر عوامل محیطی بر میزان سورفکتین تولیدی در باکتری *Bacillus subtilis*

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چکیده: سورفکتین یکی از مؤثرترین سورفکتانت‌های زیستی است که توسط باکتری *Bacillus subtilis* ترشح می‌شود و بیش‌ترین نقش را به‌عنوان تحریک‌کننده واکنش ISR در میان کلیه متابولیت‌های تولید شده این باکتری دارد. عوامل محیطی اثرات چشم‌گیری روی تولید سورفکتین دارند. در این تحقیق، با کمک دستگاه کروماتوگرافی با کارایی بالا (HPLC) تولید سورفکتین توسط دو استرین *B. subtilis* مورد بررسی قرار گرفت. سورفکتین‌های ۱۴ و ۱۵ کربنه طی استخراج اتانولی از محلول رویی ته‌نشین شده با اسید به‌دست آمدند. بررسی‌های HPLC محیط کشت‌های مختلف شامل نوترینت براث (NB)، NB حاوی ۴۰ گرم در لیتر گلوکز، NB حاوی ۱۰٪ عصاره خاک و NB حاوی ۱۰٪ عصاره گیاه به‌وضوح نشان داد که این باکتری‌ها مقادیر متفاوتی از سورفکتین‌های ۱۴ و ۱۵ کربنه را در این محیط‌های کشت تولید کردند. تولید سورفکتین در محیط کشت NB حاوی عصاره گیاه بالاترین میزان بود. آنالیز میکروالمان‌های حاصل در محیط‌های حاوی عصاره گیاه و خاک با طیف‌سنجی جذب اتمی نشان داد که مقادیر عناصر Fe، Mn و Zn در محیط کشت حاوی عصاره گیاه در مقایسه با محیط کشت حاوی عصاره خاک بیش‌تر بود. از آنجایی‌که این عناصر نقش مهمی در تولید سورفکتین دارند، لذا مقادیر بیش‌تر آنها در محیط حاوی عصاره گیاهی نسبت به محیط حاوی عصاره خاک می‌تواند دلیل احتمالی تولید بیش‌تر سورفکتین ۱۴ و ۱۵ کربنه در محیط حاوی عصاره گیاهی باشد. بنابراین افزودن این عناصر مهم به خاک می‌تواند در فرایند کنترل بیولوژیکی *B. subtilis* بر علیه بیمارگرهای گیاهی مفید باشد.

واژگان کلیدی: سورفکتین، کروماتوگرافی مایع با کارایی بالا، باسیلوس، بیوکنترل