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ORIGINAL ARTICLE

Exopolysaccharide production from glycerol by *Bacillus sonorensis* NTV10 under thermophilic condition

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Abstract

Exopolysaccharides or extracellular polymeric substances (EPS) has become an important resource and is being increasingly used in the biotechnology and biopharmaceutical industries. However, its production from glycerol under thermophilic conditions has not been reported. This study is aimed at isolating high-performance, EPS-producing bacteria under thermophilic conditions using glycerol as the substrate. Among the isolated microorganisms, *Bacillus sonorensis* strain NTV10 exhibited the highest EPS production. The optimum cultivation conditions in the enrichment medium (HS medium) were 1 g/L glycerol, 45 °C, and pH 7, with the highest EPS production of 15.97 mg/mL. We confirmed that NTV10 prefers thermophilic conditions for the highest EPS production. However, the utilization of glycerol was low because of the presence of yeast extracts and peptone in the HS medium. Therefore, the ability of glycerol conversion into EPS by NTV10 was evaluated using minimal medium (medium E*). We found that 15 g/L glycerol exhibited the highest EPS production (8.8 mg/mL). The monosaccharide composition of EPS from both media was similar, containing glucose, mannose, and rhamnose in a relative ratio of 5.1:2.2:1. The results of the IR spectrum showed the presence of mainly carboxyl and hydroxyl groups in the EPS product, which was in accordance with the monosaccharide composition. These properties can be applied in various industries such as food processing, cosmetics, and pharmaceuticals. The experimental knowledge derived from this study can be used to promote the use of glycerol as a renewable substrate for bioconversion into highly valuable products, such as EPS production.

Keywords Exopolysaccharide · Glycerol · *Bacillus sonorensis* · Thermophilic condition · Microbial isolation

1 Introduction

One of the major environmental problems is the consumption of fossil fuels, especially in the transportation sector, which alone accounted for 24% of global carbon dioxide (CO_2) emissions in 2019. Approximately 75% of total CO_2 emissions in this sector are from commercial vehicles [1]. Biodiesel, which is primarily used in busses and commercial vehicles, has emerged as a promising alternative to fossil fuel resources. However, the increase in biodiesel production can become troublesome due to the extreme surplus of waste glycerol, about 10% (w/w) of which is generated from the transesterification reaction during the biodiesel production process. This problem directly affects the refined glycerol market and causes a significant reduction in the price of glycerol [2, 3]. Improper disposal of crude glycerol can have adverse environmental effects; further, the expensive nature of the glycerol refining process can be challenging, especially for small and medium-sized biodiesel plants [4]. Consequently, several efforts have been made to convert glycerol into a more valuable product, to reduce the cost of biodiesel production and the environmental problems associated with glycerol disposal [5, 6]. Moreover, most commercially available biotechnological products currently use sugars, starch, or molasses as feedstocks. In recent years, research has focused on the use of renewable and non-edible feedstocks as alternative raw materials. Therefore, glycerol can

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be considered a potential candidate for use as a renewable and low-cost substrate in biological production platforms.

Exopolysaccharides or extracellular polymeric substances (EPS) from prokaryotes are widely used as polysaccharide materials in many industries, such as food, pharmaceuticals, petrochemicals, and cosmetics. EPS can be homo- or heteropolysaccharides, each having different structures and specific properties depending on the type of bacteria and substrate used in its production. EPS has become an important resource and is being increasingly used in the biotechnology and biopharmaceutical industries [7]. The global market for hydrocolloids, which includes numerous polysaccharides, is still dominated by plant and algal polysaccharides, such as starch, galactomannans, and pectin. Considering the growth rate of the plants, eco-anxiety, and high extraction costs, EPS from microorganisms have the opportunity of becoming the dominant polysaccharide in the industry. Currently, some brands in the global market tend to use bacterial EPS such as xanthan, used in chromatographic media by Sigma-Aldrich Co. LLC.; alginate, used in Silvercel® antimicrobial alginate dressing by Johnson & Johnson; and dextran, used for the replacement of blood loss, plasma substitution, and volume expansion by Pharmacosmos [8].

The production of bacterial EPS normally uses different monosaccharides as substrates, such as glucose, fructose, and galactose, which is one of the reasons for their high production cost. Therefore, investigating the use of a low-cost substrate, such as glycerol, for EPS production is of interest. Some bacterial strains have been previously reported, which have the potential to produce EPS from glycerol under mesophilic conditions, such as *Lactobacillus helveticus* ATCC 15807 [9], *Enterobacter* A47 [10], *Gluconacetobacter xylinus* [11], and *Acetobacter xylinum* [12].

EPS production is commonly studied under mesophilic conditions (30–37 °C). However, some researchers have also investigated EPS production under thermophilic conditions (41–65 °C) [13-15]. The advantages of thermophilic conditions—extraordinarily high biochemical reaction rate, limit of bacterial contamination, and requirement of a smaller reactor volume—are attractive to investigate in the bio-production process [16]. These advantages are useful in industrial applications. However, EPS production from glycerol under thermophilic conditions has not been reported, because obtaining a remarkable isolated thermophile with high efficiency of EPS production from glycerol remains challenging. For a mixed culture source for microbial isolation in this study, a compost sample of synthetic food waste after 5 days of composting is considered as a potential resource. The composting temperature of synthetic food waste could reach 60 °C after 5 days of the process, and a high level of acetic acid accumulation occurred during this period [17]. Ua-Arak [18] et al. reported that acetic acid-producing bacteria have the ability to produce a high molecular weight EPS. Consequently, there is a possibility that EPS-producing thermophiles exist in the compost samples after day 5.

This study focused on EPS production from glycerol using an isolated thermophile. The challenge of this research was to identify bacteria that naturally prefer to produce EPS from glycerol under thermophilic conditions. After isolation and identification, the optimum conditions for maximizing EPS production, such as temperature, initial glycerol concentration, and pH, were evaluated. Finally, the EPS product was characterized by its monosaccharide content, structure, and properties, to determine its functions and usefulness in various applications.

2 Materials and methods

2.1 Isolation of EPS-producing bacteria using glycerol substrate, under thermophilic condition

A synthetic food waste sample, which was collected after 5 days of composting and potentially contained EPS-producing bacteria, was used as the source of a mixed culture. The Hestrin–Schramm (HS) medium [19], which contains 20.0 g/L glucose, 5.0 g/L yeast extract, 5.0 g/L peptone, 2.7 g/L Na_2HPO_4, 1.15 g/L citric acid, and 16 g/L bacto-agar, was modified by replacing the main carbon source from glucose with glycerol (20 g/L) and used for the isolation of EPS-producing bacteria. The culture conditions were 50 °C and shaking at 150 rpm. All microbial colonies with different morphologies were isolated, purified, and inoculated in liquid medium to evaluate their EPS production potential. After 48 h of incubation, EPS was extracted from the culture medium and measured using the phenol–sulfuric acid method [20]. The isolated bacterium with the maximum EPS yield was classified by 16S rRNA gene sequence analysis, and the optimal conditions for this reaction were determined.

2.2 Microbial identification by 16S rRNA sequence technique

Total genomic DNA of the isolated bacteria was extracted using a DNA extraction kit (ISOIL for Beads Beating, Nippon Gene Co. Ltd., Toyama, Japan), from the granule sample. The extracted DNA was used as template for the amplification of full-length 16S rRNA, using a universal primer, with TaKaRa PCR Thermal Cycler Dice™ (TP600, Takara Bio Inc., Shiga, Japan). The PCR product was purified using Wizard® SV Gel and PCR Clean-Up System (Promega, USA). Four primer sets (9F-GAGTTTGATCCT GGCTCAG, 515F-GTGCCAGCAGCCGCGGT, 785F-GGA TTAGATACCCTGGTAGTC, and 1099F-GCAACGAGC

GCAACCC) were used to read the sequences and derive high-accuracy sequencing results. Sequences were initially compared to the available databases using BLAST network services to determine their approximate phylogeny.

2.3 Optimization of the culture conditions and culture medium

Glycerol was used as the main carbon source in the HS medium. The suitable culture conditions—temperature (30–60 °C), glycerol concentration (0.5–20 g/L), and pH (5–8)—were investigated and optimized for EPS production.

Medium E* [21], a minimal medium, was modified and used to test EPS production from glycerol, by isolated EPS-producing bacteria. Modified medium E* was composed of (g/L): 1.0 glycerol, 1.0 nitrogen source, 5.8 K_2HPO_4, 3.7 KH_2PO_4, 10 mL $MgSO_4$ solution (100 mM), and 1 mL microelement solution. This microelement solution contains (g/L of 1N HCl): 2.78 $FeSO_4{\cdot}7H_2O$, 1.98 $MnCl_4{\cdot}H_2O$, 2.81 $CoSO_4{\cdot}7H_2O$, 1.67 $CaCl_2{\cdot}2H_2O$, 0.17 $CuCl_2{\cdot}2H_2O$, and 0.29 $ZnSO_4{\cdot}7H_2O$. The effect of various nitrogen sources—yeast extract, peptone, urea, ammonium phosphate dibasic ($(NH_4)_2HPO_4$), ammonium sulfate, ammonium chloride, and potassium nitrate—on EPS production was also investigated. Furthermore, glycerol concentration (1–20 g/L) was examined in the modified medium E*, to determine the optimum concentration, using $(NH_4)_2HPO_4$ as the nitrogen source. Finally, the growth kinetics, glycerol utilization, and EPS production by the isolated bacteria, in the modified medium E*, were evaluated.

2.4 Characterization of the monosaccharides and functional groups in the EPS products

Exopolysaccharide (EPS) products were extracted from the culture medium after 48 h of incubation. The sample was heated at 80 °C for 10 min to inactivate the hydrolysis enzyme and then centrifuged at 9800 × *g* for 20 min at 4 °C. EPS was precipitated out from the cell-free supernatant by mixing it with cold ethanol (3:1) at 4 °C for 48 h. The sample was then centrifuged at 10,000 × *g* for 30 min at 4 °C; the obtained pellet was rinsed and dried by evaporating the ethanol. It was then dissolved in water, dialyzed using a Thermo Scientific™ SnakeSkin™ Dialysis Tubing (10 K MWCO, 22 mm) against distilled water at 4 °C for 48 h, and freeze-dried overnight. Protein in EPS was removed by dissolving the powder in 10% trichloroacetic acid, then dialyzed against distilled water at 4 °C for 120 h, and freeze-dried again.

The monosaccharide content of the purified EPS product was characterized after hydrolysis with 2 M trifluoroacetic acid at 120 °C for 1 h, using reverse-phase high-performance liquid chromatography (RP-HPLC) equipped with Shodex Asahipak NH2P-50 4E; 250 mM H_3PO_4 (aq.)/CH_3CN (20:80 v/v) was used as the eluent. The major structural and functional groups were determined using Fourier transform infrared (FTIR) spectroscopy. The analyzed data was compared with standard substances and previous reports.

2.5 Fourier transform infrared (FTIR) spectroscopic analysis

FTIR spectroscopy can determine the major structural and functional groups present in the purified EPS products. The purified EPS was ground and mixed with spectroscopy-grade potassium bromide (KBr) and pressed to form pellets using the KBr disk technique. FTIR spectral data were recorded from 64 scans in the region 4,000–400 cm^{-1}, at room temperature, using an FT/IR-610 JASCO spectrometer.

3 Results and discussion

3.1 Isolation and identification of EPS-producing bacteria

After the initial isolation, 18 colonies (NTV1–NTV18) with different morphologies were observed, selected, and purified (Supplement 1). All isolated bacteria were cultured in the modified HS medium to test their EPS-producing potential. Of the 18 isolated bacteria, NTV10 was selected as the representative of the isolated bacterium that produced the highest sugar content (Supplement 2). The results of 16S rRNA sequencing method (approximately 1500 bp) showed that NTV10 is closely related to *Bacillus sonorensis* (99.86% identity) (Accession number: MZ310519).

Bacillus sonorensis is a facultative anaerobic, gram-positive bacterium. Its growth temperature range is 15–55 °C (optimum, 30 °C). The growth can be inhibited by 5% NaCl and 0.001% lysozyme solutions. *B. sonorensis* can utilize citrate and propionate and hydrolyze casein and starch. Furthermore, this bacterium can reduce nitrate to nitrite and facilitate acid fermentation without gas production using glucose and other carbohydrates as substrates [22]. Previously, *Bacillus sonorensis* MJM60135, which was isolated from ganjang (fermented soy sauce), was reported to produce EPS from tryptic soy broth (TSB) medium containing glucose, under mesophilic conditions (37 °C) [23]. In contrast, the EPS in this study was obtained from a modified HS medium containing glycerol, by *B. sonorensis* strain NTV10, under thermophilic conditions. After optimizing the culture conditions, EPS was extracted, purified, and freeze-dried (Fig. 1). The purified EPS product was further identified with respect to its monosaccharide content and functional groups.

Fig. 1 Purified EPS product obtained from glycerol substrate, using *Bacillus sonorensis* NTV10, under thermophilic condition

3.2 Optimization of culture conditions for EPS production in modified HS medium

First, the optimal temperature for EPS production was investigated in the modified HS medium, using 20 g/L of glycerol concentration and pH 6.27. Figure 2A shows that the highest EPS concentration (12.08 mg/mL) was obtained at 45 °C. This result confirmed that the *B. sonorensis* NTV10 strain preferred thermophilic over mesophilic conditions, for EPS production. However, microbial growth and EPS production declined gradually when the temperature was above 45 °C. Previously, *Pediococcus pentosaceus* and *Lactobacillus amylovorus* were isolated from tropical fruits of Thailand and studied for EPS production under thermophilic condition. These two bacteria preferred high temperatures (45 °C) for growth and EPS production [13]. However, some bacteria, such as *Pseudomonas oleovorans* NRRL B-14682, preferred mesophilic conditions and were studied to evaluate the influence of temperature (20–40 °C) on EPS production. The results showed that a temperature of 30 °C provided maximum cell growth and EPS production from glycerol [24]. The optimum temperature of 45 °C for the NTV10 strain offers several advantages, such as reducing the risk of contamination from mesophilic bacteria and increasing the biochemical reaction rate of fermentation [16].

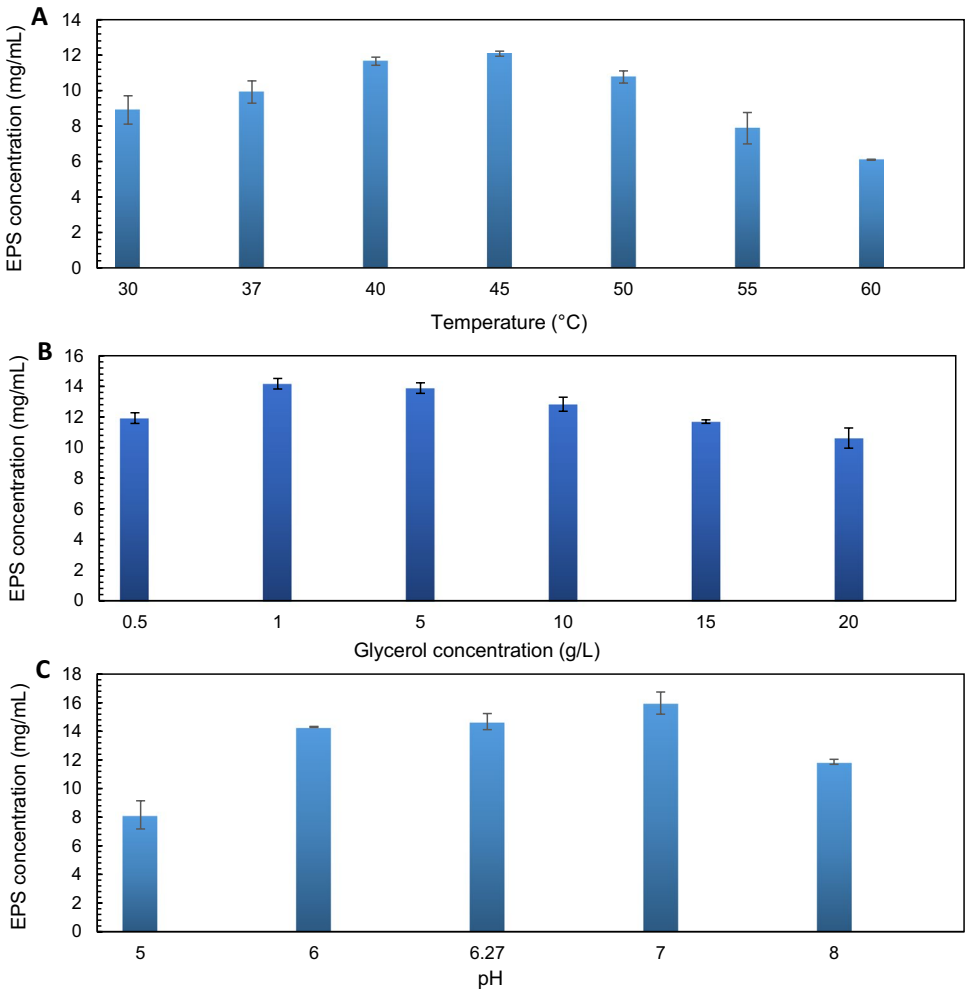

Fig. 2 Effect of physical parameters on EPS production from glycerol, in modified HS medium, by *B. sonorensis* NTV10 strain. (**A**) The effect of temperature (glycerol concentration 20 g/L, pH 6.27); (**B**) the effect of initial glycerol concentrations (temperature 45 °C, pH 6.27); and (**C**) the impact of pH (temperature 45 °C, glycerol concentration 1 g/L)

Moreover, the reaction at 45 °C is energy-efficient as it reduces the cooling cost after the fermentation process.

Second, the optimal glycerol concentration was evaluated at temperature 45 °C and pH 6.27 (Fig. 2B). The highest EPS concentration (14.17 mg/mL) was obtained with an initial glycerol concentration of 1 g/L. EPS production decreased with increasing glycerol concentrations. However, the EPS concentration at 0.5 g/L glycerol concentration was lower than that at 1 g/L. Enrichment substances such as yeast extract and peptone were preferred over glycerol. Therefore, to confirm the ability of *B. sonorensis* NTV10 strain to convert glycerol into EPS, the modified medium E* was used for further experiments. Moreover, the inhibition at high initial glycerol concentrations might have occurred due to the high osmotic pressure conditions. Torino et al. [9] reported that the EPS production, by *Lactobacillus helveticus* ATCC 15807, from glucose, was reduced because of the osmotic stress by adding 5% and 10% of glycerol concentration into the medium. The yield of EPS decreased 10-folds.

Finally, the optimal pH, which is an important factor for biological production processes, was evaluated (Fig. 2C). The highest EPS concentration (15.97 mg/mL) was obtained at an optimum pH of 7, for *B. sonorensis*. However, the optimal pH for EPS production depends on the specific bacterial strain. *Lactobacillus helveticus* ATCC 15807 could produce EPS in acidic conditions (pH 4.5), two-fold higher than that at pH 6.5 [9], whereas *Bacillus licheniformis* T14 prefers alkaline conditions (pH 8) for EPS production [25].

3.3 Optimization of EPS production in medium E*

Medium E*, a minimal medium, was used to evaluate the ability of *B. sonorensis* strain NTV10 to convert glycerol into EPS, and to assess the impact of nitrogen sources on EPS production. Figure 3A shows that organic nitrogen sources (except urea) provide superior EPS production compared to inorganic ones. EPS production did not vary significantly among the inorganic nitrogen sources. This result is similar to that of Moghannem et al. [26], who reported that yeast extract was the best nitrogen source for EPS production from sucrose, compared to peptone, urea, $(NH_4)_2SO_4$, NH_4Cl, and KNO_3, by *Bacillus velezensis* KY471306. Gorret et al. [27] reported that yeast extract not only promoted microbial growth but also directly increased the biosynthesis of EPS.

To investigate the use of glycerol as the sole carbon source for EPS production by NTV10, ammonium phosphate dibasic, which was originally present in medium E*, was used for this experiment. We found that the highest EPS production (8.88 mg/mL) was observed at an initial

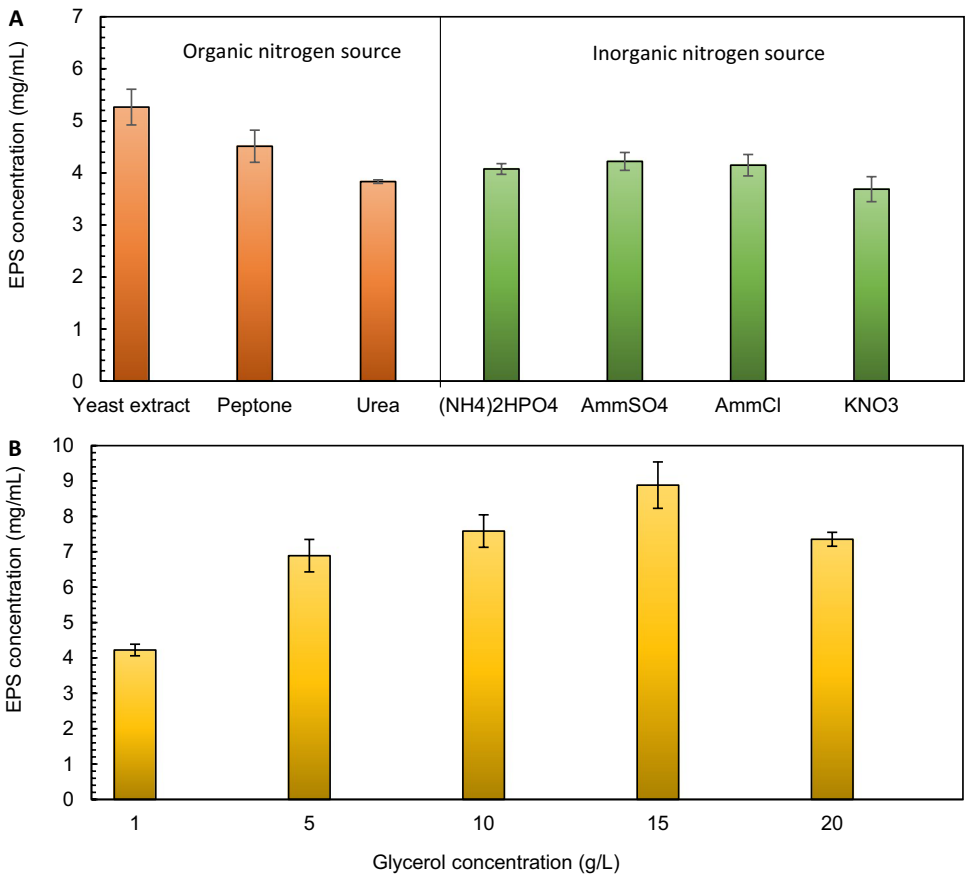

Fig. 3 Comparison of EPS production from glycerol in the medium E* (**A**) using different nitrogen sources and (**B**) at varying initial glycerol concentrations

glycerol concentration of 15 g/L (Fig. 3B). A higher glycerol concentration might cause high osmotic pressure, as previously described. NTV10 showed higher activity for glycerol conversion into EPS in medium E* than in modified HS medium, which contained yeast extract and peptone.

The dynamic relationship between microbial growth and EPS production in medium E* indicated that EPS production corresponded with growth, especially during the logarithmic phase (Fig. 4). This phenomenon was similar to the growth and EPS production of *Klebsiella oxytoca* [28], *Chelatococcus daeguensis* TAD1 [29], and *Lactobacillus delbrueckii* subsp. *bulgaricus* RR [30]. Thus, the enhancement of microbial growth may contribute in increasing the EPS production.

3.4 Characterization of the monosaccharides and determination of the structure of EPS products

The monosaccharide content in the purified EPS products obtained from the modified HS medium and medium E*, by the NTV10 strain, was identified. We found that the monosaccharide contents in the EPSs from both media were similar, containing glucose, mannose, and rhamnose in the ratio 5.1:2.2:1.0. This result indicated that the use of different media (enrichment and minimal media) had no effect on the monosaccharide composition of the EPS. A previous study on EPS production by *B. sonorensis* MJM60135 strain used tryptic soy broth (TSB) as a substrate, which contains glucose as a carbon source and tryptone and soytone as nitrogen sources. The results showed that only glucose and mannose were detected [23]. Although NTV10 and MJM60135 were similar species, their monosaccharide contents in the EPS were slightly different. Similarly, many previous studies used the same bacterial species and carbon sources, but the monosaccharide contents in EPS varied, as shown in Table 1. *Enterobacter* A47 was investigated for EPS production using medium E* containing glycerol, which was similar to the present study, but the monosaccharide content of the EPS was different. Therefore, the type of medium was not the determining factor for the monosaccharide content in EPS. In addition, *Bacillus licheniformis* strains T14 and KS-17 provide different monosaccharide compositions in EPS even when used with the same carbon source (sucrose) and nitrogen source (yeast extract) [25, 31]. Therefore, the monosaccharides in EPS might depend on the microbial strains, but the types of carbon and nitrogen sources might not affect the monosaccharide content.

Figure 5 shows the results of the IR spectrum in the 4000–400 cm^{-1} regions from FTIR spectroscopy that provided information on the major structural and functional groups of the EPS products of *B. sonorensis* NTV10 strain, in modified HS medium and medium E*. We found that there was no significant difference in FTIR results between

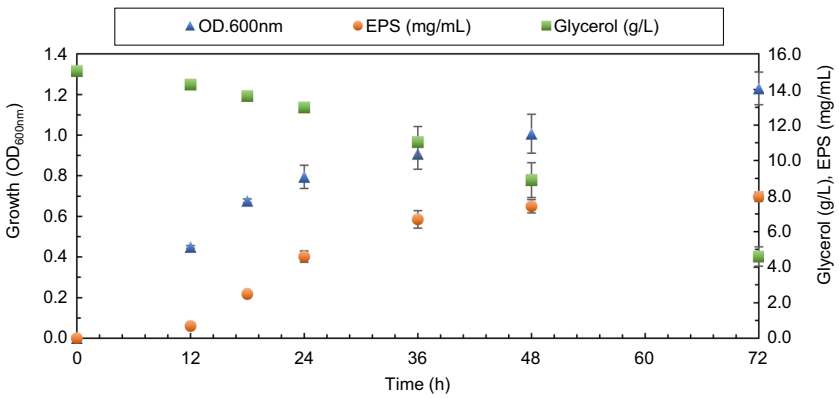

Fig. 4 Time course of growth, EPS production, and glycerol utilization of *B. sonorensis* NTV10 strain in medium E*

Table 1 Monosaccharide composition in EPS of various microbial strains and substrates

Microbial strain	Substrate	Monosaccharide composition	References
Bacillus sonorensis NTV10	Glycerol (modified HS medium)	Glucose, mannose, rhamnose	Present study
Bacillus sonorensis NTV10	Glycerol (modified medium E*)	Glucose, mannose, rhamnose	Present study
Bacillus sonorensis MJM60135	Glucose (tryptic soy broth)	Glucose, mannose	[23]
Bacillus velezensis KY498625	Molasses (yeast extract)	Glucose, galactose, mannose	[26]
Enterobacter A47	Glycerol (medium E*)	Fucose, galactose, glucose, glucuronic acid	[10]
Bacillus licheniformis T14	Sucrose (seawater with yeast extract)	Fructose, fucose, galactose, galactosamine, mannose	[25]
Bacillus licheniformis KS-17	Sucrose (tryptone and yeast extract)	Glucose	[31]

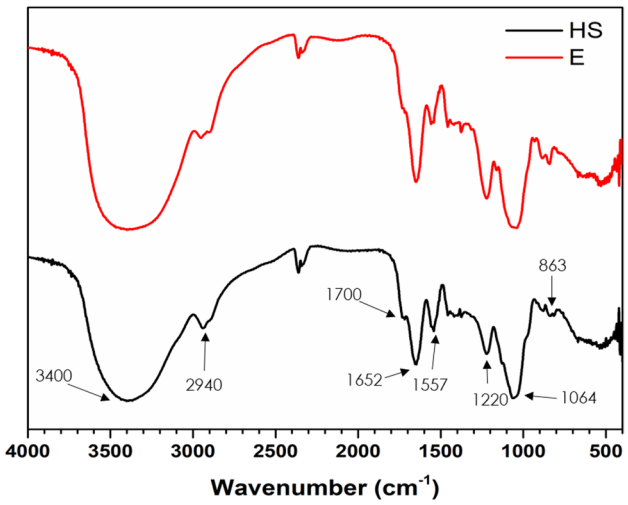

Fig. 5 FTIR spectroscopic analysis of EPS products of *Bacillus sonorensis* NTV10 strain, from HS medium (— HS) and medium E* (— E) in the range 4000–400 cm.$^{-1}$

EPS products of both media, which corresponded to the results of the monosaccharide composition. The characteristics of each band were assigned and are summarized in Table 2. The results of the FTIR spectrum of EPS in this study were similar to those assigned to the major bands of the EPS product from *B. sonorensis* MJM60135. In particular, the presence of carboxyl and hydroxyl groups is essential for binding divalent cations, which aid in enhancing flocculation [23, 32].

Together with the results of monosaccharide composition, the EPS product from NTV10 strain has the major monosaccharides—glucose, mannose, and rhamnose—mostly related to EPS (GalactoPol) from *Pseudomonas oleovorans*, which contains galactose, glucose, mannose, and rhamnose in the ratio 1:0.3:0.06:0.04, respectively [33]. The properties of EPS include flocculating, film-forming, and emulsifying capacities, which can be applied to food processes, cosmetics, pharmaceuticals, oil recovery, packaging, etc. [34]. The results of this study report the optimized culture conditions and efficiency of novel *B. sonorensis* NTV10 strain, for EPS production, from glycerol, under optimal thermophilic conditions. This will be beneficial for industrial EPS production and will facilitate the efficient utilization of glycerol, which, in turn, will have a positive effect on the biodiesel and glycerol industries.

4 Conclusions

Bacillus sonorensis strain NTV10 was isolated and selected as a representative of the EPS-producing bacterium, which can produce EPS from a glycerol-containing medium, under thermophilic conditions. The optimum culture conditions for EPS production by NTV10, using a modified HS medium, were initial glycerol concentration 1 g/L, temperature 45 °C, and pH 7. The highest EPS concentration obtained using the HS medium was 15.97 mg/mL. Glycerol utilization in the HS medium was low because of the presence of yeast extracts and peptones, which were preferred over glycerol. We found that yeast extract was the best nitrogen source for EPS production in minimal medium (medium E*) compared to other types of organic and inorganic nitrogen sources. The ability of glycerol conversion into EPS of NTV10 was evaluated in medium E* using ammonium phosphate dibasic as a nitrogen source. The most suitable glycerol concentration for EPS production in medium E* was 15 g/L, and the highest EPS concentration was 8.8 mg/mL. The kinetic data for EPS production showed that the growth of bacteria corresponded to the production of EPS. The monosaccharide compositions of EPS from both modified HS medium and medium E* were similar and contained glucose, mannose, and rhamnose. The IR spectra corresponded to the monosaccharide composition. The properties of EPS products obtained from NTV10 are applicable in many industries such as food, cosmetics, and pharmaceuticals.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s13399-023-04402-7.

Table 2 Characteristic IR absorptions of the EPS product from glycerol by *B. sonorensis* NTV10 strain

Frequency (cm^{-1})	Bond and functional group
3400	O–H stretching vibration of hydroxyls
2940	C–H stretching vibration of CH_2 and CH_3 groups
1700	C=O stretching vibration of carbonyls in acyl groups
1652	C=O stretching vibration and C–N (amide I)
1557	C–N stretching vibration and N–H deformation vibration (amide II)
1220	C–O–C, C–O stretching vibration between 1000 and 1200 cm^{-1} corresponds to carbohydrates
1064	O–H stretching vibration of polysaccharides
863	α-configuration simultaneously present in EPS
1000–650	=C–H bending vibration of alkenes

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Author contribution All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Nunthaphan Vikromvarasiri. The first draft of the manuscript was written by Nunthaphan Vikromvarasiri. Supervision was done by Kiyohiko Nakasaki. All authors read, revised, and approved the final manuscript.

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Data availability All data generated or analyzed during this study are included in this published article (and its supplementary information files).

Declarations

Ethical approval Not applicable.

Competing interests The authors declare no competing interests.

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