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# Characterization of A Lipopeptide Surfactant From Lipase-Producing *Bacillus amyloliquefaciens* E1PA and Its Role in Lipase-Catalyzed Oil Hydrolysis

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**Abstract**

The lipase-catalyzed hydrolysis of fat and oil is well recognized and considered to be an important reaction in fatty acid production. Generally, lipase activity is poor in oil-water biphasic systems. In this study, surfactins were purified, identified, and molecularly characterized from 2 biosurfactant-producing strains (*Bacillus amyloliquefaciens* E1PA and *B. subtilis* TISTR 1248). A high surfactin yield was achieved from *B. amyloliquefaciens* E1PA culture when 4 % (v/v) glycerol was used as a carbon source. Moreover, the addition of 2 % palm oil to the fermentation broth significantly increased biosurfactant production 2-fold during culture in Mineral Salt Medium (MSM). The biosurfactant synthesized by both strains was partially categorized as a lipopeptide via Fourier transform infrared spectroscopy (FTIR) analysis. The E1PA biosurfactant was subjected to analysis of its interfacial activation in the hydrolysis reaction. The poor hydrolytic activity (12.4 %) of lipase was significantly increased (97.3 %) when surfactin (1 % v/v) was used to generate the emulsion. In addition, the gene involved in *B. amyloliquefaciens* E1PA surfactin biosynthesis was characterized. The surfactin (*sfp*) gene was PCR amplified, and then cloning and expression of recombinant *sfp* was performed in *Escherichia coli*. Thus, heterogeneous expression of *sfp* gene in *E. coli* may be used for the efficient production of active recombinant E1PA surfactin. These results suggest that the lipopeptide biosurfactant produced by *B. amyloliquefaciens* E1PA could be useful as a biosurfactant in the oleochemical industry to produce fatty acids and/or fatty acid ethyl esters (biodiesel).

**Keywords:** *Bacillus amyloliquefaciens*, biosurfactant, emulsification, surfactin, hydrolysis, vegetable oils

## Introduction

Surface-active substances, or surfactants, are amphiphilic molecules, exhibiting both hydrophilic (oil lover) and hydrophobic (water lover) properties that allow them to accumulate between fluid phases and reduce surface tension [1]. Synthetic surfactants are extensively used in many industrial processes, including in foaming detergents, dispersion, wetting, and emulsification. Surfactants can be derived through chemical synthesis; however, these surfactants are non-biodegradable, toxic xenobiotic compounds [2]. Recently, naturally-occurring surface active substances isolated from microbes have attracted attention, due to their lower toxicity, higher biodegradability, biocompatibility, and environmental friendliness. Biosurfactants produced from many bacteria have been isolated and

identified, especially from Bacillus species, which produce lipopeptides such as *Bacillus* sp. I-15 [3] and *B. subtilis* SPB1 [4]. Lipopeptides are primarily synthesized by microorganisms belonging to the *Bacillus* genus. The cyclic lipopeptide surfactin is one of the most effective biosurfactants. Surfactin comprises a heptapeptide head group with a β-hydroxy fatty acid with a 12- to 16-carbon atom chain length that forms a cyclic lactone ring structure [5].

Increasing interest has focused on the isolation of novel enzymes and novel enzyme-producing bacteria for application in industrial conversions [6]. Lipases are enzymes that catalyze the hydrolysis reaction at oil-water interfaces by converting triglycerides (fat) to fatty acids and glycerol, a by-product. Lipases can also catalyze the reverse trans-esterification and ester production reactions in non-aqueous systems [7]. Thus, these enzymes are good candidates for fatty acid production in the oleochemical industry. Additionally, lipases exhibit hydrolytic activity in the presence of various substrates, and retain the ability to alter substrates with C4-C18 chain lengths, including the alkaline-purified recombinant *B. amyloliquefaciens* E1PA lipase [8] and the *B. subtilis* lipase [9]. Some lipase-producing bacteria can potently destroy or degrade the biological activity of both animal- and vegetable-derived edible fats and oils comprising long-chain fatty acids [8]. However, these lipases are lipolytic enzymes that function at the oil-water interface, and their substrates are poorly soluble in water. This phenomenon generates fewer unwanted by-products from the hydrolysis of fats and oils [10]. Because interfacial activation is necessary for the catalytic activity of lipases [11], a biosurfactant is needed to emulsify an oil in the aqueous phase and enhance the hydrolytic activity of lipases. In this study, we characterized the lipopeptide biosurfactant of *B. amyloliquefaciens* E1PA. This biosurfactant shows desirable properties as either a versatile lipolytic enzyme or a surface active agent. Additionally, the genetic properties of the surfactin (*sfp*) gene were characterized following successful expression and purification from a heterologous *E. coli* host. The potential application of this surfactin for the lipase-catalyzed hydrolysis of vegetable oil is discussed.

## Materials and methods

### Reagents and chemicals

Chemicals used in this study were purchased from Fluka (Switzerland) or Sigma-Aldrich (USA). All chemicals were of analytical or molecular biology grade. $T_4$ DNA ligase, *Pfu* DNA polymerase, restriction enzymes, agarose gel, ampicillin, lysozyme, and isopropyl ß-D-thiogalactoside (IPTG) were obtained from Invitrogen (USA). Chloramphenicol was purchased from Sigma-Aldrich (USA). DNA ladder was purchased from Fermentas. Culture media, including nutrient broth and agar, tryptone, and yeast extract, were acquired from Difco (France). The Ni-NTA His-Bind resin column was purchased from Novagen (USA). The vegetable oils were purchased from a local market in Khon Kaen, Thailand.

### Bacteria and culture media

Two lipase-producing strains [*B. amyloliquefaciens* E1PA, which were screened and isolated from lipid-rich food waste at Khon Kaen University [8], and *B. subtilis* TISTR 1248, which was isolated from water and soil from a shrimp pond located at the Microbiological Resource Centre, Thailand Institute of Scientific and Technological Research (TISTR)], were used in this study. The *E. coli* Tuner (DE3) pLacI strain (Novagen) was employed for protein expression, and *E. coli* NovaBlue was used as the cloning host. The pETBlue-2 vector (Novagen) was used for both gene cloning and protein expression. For biosurfactant activity screening and molecular identification, *B. amyloliquefaciens* E1PA and *B. subtilis* TISTR 1248 were cultured on Luria-Bertani (LB) medium at 30°C with agitation at 150 rpm. *E. coli* was cultured in LB medium at 37 °C and 150 rpm supplemented with chloramphenicol and ampicillin at concentrations of 100 µg/ml and 34 µg/ml, respectively.

### Screening for biosurfactant activity

To examine the bacterial strains for the production of biosurfactant, a single colony of each strain was inoculated in a test tube containing 5 ml of LB broth and cultured for 24 h. The cell-free supernatants were used to screen for biosurfactant activity [12-14].

**Emulsification determination**

To determine the emulsification capacity of the biosurfactant, emulsification activity was evaluated according to the method of Cooper and Goldenberg [12], with a slight alteration. One part biosurfactant and one part mineral oil were mixed by vortexing at max speed for 2 min. The reaction was allowed to stand for 24 h prior to evaluation. Emulsification activity was calculated by dividing the total height of the emulsion layer by the total height of the solution. The result was reported as a percentage.

$$\%E24 = (\text{emulsion layer} / \text{total height}) \times 100$$

**Oil displacement test**

To examine biosurfactant activity, an oil displacement test was performed in a Petri dish filled with 40 ml of distilled water. A 15-µl aliquot of engine oil was dropped on the surface of the water, and 10 µl of biosurfactant extract was gently pipetted into the midpoint of the oil film. After 30 sec, the clear halo zone and diameter were observed under visible light and computed as described by Morikawa et al. [13].

**Drop collapsing test**

Each well of a microtiter plate lid was filled with 2 µl of mineral oil. The microtiter plate was equilibrated at ambient temperature for 1 h, and 5 µl of the biosurfactant was pipetted onto the surface of the oil. The characteristics of the bead on the oil surface were checked after 1 min. Biosurfactant activity that resulted in flat drops was recorded as positive (+). Conversely, curved drops were recorded as negative (-) and indicated an absence of biosurfactant activity [14].

**Hemolysis test**

Strains were singly streaked on blood agar (TSA with sheep blood) medium. The streak plate was incubated at 30 °C for 24 h. Observations were scored for α, β, and γ hemolysis; observation of hemolytic activity indicated biosurfactant production.

**Tilted glass slide (TGS) test**

The biosurfactant extract was gently merged with one drop of normal saline (0.9 % w/v NaCl). The mixture was placed at one end of a glass slide. To confirm biosurfactant activity, the slide was tilted. Movement of the drop downward indicated biosurfactant activity.

**Growth with glycerol and biosurfactant production**

The biosurfactant-producing strains were cultivated in NB medium and incubated overnight at 30 °C with 150 rpm agitation. The production medium was transferred to fresh mineral salt (MS) medium (100 ml) amended with 4 % (v/v) glycerol. Glycerol was used as the carbon source in the biosurfactant production medium. The culture was incubated at 30 °C for 96 h. The MS medium contained (in g/l): $Na_2HPO_4$, 2.2; $KH_2PO_4$, 1.4; $(NH_4)2SO_4$, 3; $MgSO_47H_2O$, 0.6; NaCl, 0.05; yeast extract, 1; $CaCl_2$ $7H_2O$, 0.02; and $FeSO_4$ $7H_2O$, 0.01. Additionally, the MS medium was modified by adding 2 ml of a trace element solution consisting of (in g/l): $ZnSO_4$, 0.29; $CuSO_4$, 0.25; $CaCl_2$, 0.24; and $MnSO_4$, 0.17. Growth and emulsification activity were determined at 24-h intervals up to 96 h. The data were statistically analyzed using ANOVA and Duncan's test considering $p < 0.05$ as the significance threshold.

**Palm oil-induced biosurfactant production**

To evaluate palm oil induction, the bacterial strains were cultured in NB medium and incubated overnight at 30 °C for 18 h with 150 rpm agitation. The production medium was transferred to 100 ml of MS medium containing 2 % (v/v) palm oil as the inducer, followed by incubation at 30 °C for 96 h. Aliquots of the bacterial cultures were taken at 24 h intervals up to 96 h to assess emulsification activity in comparison with uninduced cultures.

**Biosurfactant production**

The biosurfactant-producing bacteria were grown in MS medium with agitation at 150 rpm for 48 h. The extracellular biosurfactants were extracted with chloroform-methanol [15]. The culture broth (200 ml) was harvested and centrifuged at 8000 rpm for 10 min at 4 °C, and 1 M sulfuric acid was added to the cell-free supernatant to adjust the pH to 2. The solution was left in the refrigerator for 12 h prior to extraction with an equal volume of chloroform-methanol (2:1). The mixture was subsequently shaken for 3 h at 30 °C at 200 rpm. The organic phase was then separated using a separating funnel, and the biosurfactant was concentrated using a rotary evaporator at 60 - 70 °C and dehydrated via lyophilization.

**Biochemical and structural characterization of the biosurfactant**

The functional groups of the biosurfactants were identified through Fourier transform infrared (FTIR) spectroscopy (Bruker, TENSOR27). The FTIR spectrum was analyzed in transmittance mode at a 4 $cm^{-1}$ resolution, with the wave number ranging from 400 to 4,000 $cm^{-1}$. The lipopeptide biosurfactant surfactin (Sigma-Aldrich, USA) was used as the biosurfactant standard.

**Effect of biosurfactants on lipase-catalyzed palm oil hydrolysis**

The purified recombinant lipase was immobilized via entrapment in calcium alginate beads. This recombinant lipase was prepared as designated by Saengsanga *et al*. [8]. Sodium alginate was melted in distilled water to obtain a 1.5 % (w/v) concentration. $CaCl_2$ was dissolved in 100 ml of deionized water to obtain a 2 % (w/v) concentration. Then, 10 ml of the sodium alginate slurry was mixed with 1 ml of the lipase solution, and the mixture was dropped into the $CaCl_2$ solution to obtain lipase-calcium alginate gel beads. The calcium alginate beads were incubated at room temperature for 30 min and hardened through re-suspension in 20 ml of fresh $CaCl_2$ solution for 24 h at 4 °C [16]. These beads were washed with 0.1 M Tris-Cl (pH 7.7), followed by distilled water to remove excess calcium ions and unentrapped enzymes.

For the hydrolysis reaction, 8 mL of 0.1 M phosphate buffer (pH 7.2), 1 mL of palm oil, 1 g of immobilized lipase, and 1 % (v/v) surfactin were mixed in a 15 mL screw-cap conical tube [11]. Hydrolysis was performed at 40 °C at 150 rpm for 6 h. To stop the reaction, the immobilized lipase was withdrawn. Liberation of free fatty acid by hydrolysis was observed by titrating the reaction against NaOH solution (0.02 N) using phenolphthalein as the indicator [17]. The data were statistically analyzed using ANOVA and Duncan's test considering $p < 0.05$ as the significance threshold.

**Molecular characterization of the *sfp* gene from *B. amyloliquefaciens* E1PA**

***Isolation of chromosomal DNA***

*B. amyloliquefaciens* E1PA was cultivated in LB broth at 37 °C with agitation at 150 rpm for 24 h. Genomic DNA was prepared using the procedure described by Sambrook *et al*. [18]. The adapted alkaline lysis method described by Ish-Horowicz and Burke [19] was used to prepare plasmid DNA.

***sfp gene cloning and sequencing analysis***

To isolate the *sfp* gene from *B. amyloliquefaciens* E1PA, a new primer was designed and synthesized, based on the known sequence of *B. amyloliquefaciens* subsp. *plantarum* CAU B946 (accession no. HE617159). The primers were Bamy*sfp*-F: 5'-GAAGATTTACGGAATTTATATG-3' and Bamy*sfp*-R: 5'-ATCTCGAGTAAAAGCTCTTCGTACGTT-3'; the reverse primer contained an engineered *Xho*I restriction site. To amplify the *sfp* gene, polymerase chain reaction (PCR) was conducted in a 50-µl volume consisting of 0.25 U of *Pfu* DNA polymerase (Fermentas), 100 ng of template DNA, 1x PCR buffer, 2 mM $MgSO_4$, 0.2 mM each dNTP, and 0.5 µM F and R primers. PCR was performed over 35 cycles of denaturation at 95 °C for 30 sec, primer annealing at 46 °C for 30 sec, and extension at 72 °C for 60 sec. The *sfp* gene fragment was digested with *Xho*I and purified using a PCR purification kit (Qiagen). After purification, the obtained DNA fragment was ligated into pETBlue-2 vector linearized by digestion with *Eco*RV/*Xho*I; the inserted fragment was cut with *Xho*I prior to ligation, resulting in the addition of 2 amino acids (Met-Ala) at the N-terminus. The resultant plasmid was transformed into *E. coli* NovaBlue competent cells, and the cells were spread on LB agar containing 100 µg/ml ampicillin. The recombinant plasmid was sequenced to confirm the correct expression frame.

The sequence of the *sfp* gene was compared with the sequences of other *sfp* genes retrieved from the GenBank database using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The *sfp* gene of *B. amyloliquefaciens* E1PA was submitted to GenBank under accession number AGN92427. The polypeptide sequence and open reading frame (ORF) encoded by the *sfp* nucleotide sequence were predicted by ExPASy (http://www.expasy.org/) and aligned with ClustalX 2.0 [20]. The aligned amino acid sequences were used to construct a phylogenetic tree using the maximum likelihood (ML) method in the MEGA5 program with 1,000 bootstrap replicates [21].

**Expression and purification of recombinant surfactin**

To express the surfactin in a heterogeneous host, recombinant pET*sfp* was expressed under regulation by the $T_7$ promoter in *E. coli* Tuner (DE3) p*Lac*I. The transformants were cultivated in 20 ml of LB medium amended with 100 µg/ml ampicillin and 34 µg/ml chloramphenicol at 37 °C at 150 rpm. For induction, 1 mM ITPG was added when the $OD_{600}$ reached 0.5 - 1, followed by incubation for an additional 7 h. The induced cells were harvested via centrifugation at 8,000×g for 15 min. The crude protein was solubilized with the BugBuster protein reagent. The biosurfactant-soluble proteins were loaded into an Ni-NTA His-Bind resin column to obtain purified surfactin, based on the manufacturer's recommendations. The protein fraction was subjected to 12.5 % (w/v) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS - PAGE), followed by Coomassie Brilliant Blue staining.

## Results and discussion

**Screening for biosurfactant production**

Biosurfactants have been shown to play biological roles in improving the bioavailability of water-insoluble substrates by emulsification. Therefore, they can enhance the hydrolysis of fat and oil. The emulsification of various oils in water is essential, and allows the hydrolysis of the oil via enzymatic reactions. This process enhances the bioavailability of the oil, and thereby increases the hydrolysis rate [11]. In the present study, biosurfactant production by *B. amyloliquefaciens* E1PA and *B. subtilis* TISTR 1248 on different oils was detected using numerous approaches, including the oil displacement test, emulsification index (E24), drop collapse test, blood agar hemolysis, hydrocarbon overlay agar (HOA) assay, and TGS test. The highest emulsification index associated with biosurfactant production was obtained when palm oil was used for the cultivation of *B. amyloliquefaciens* E1PA and *B. subtilis* TISTR 1248 (**Table 1**). However, E1PA showed an emulsification index of 49.63±4.63, which was higher than the emulsification index of *B. subtilis* TISTR 1248 (41.36±3.61). Biosurfactant production was also estimated based on emulsification activity. The biosurfactant produced by *B. amyloliquefaciens* E1PA showed better emulsification activity than the biosurfactant synthesized by *B. subtilis* TISTR 1248. Among the different oils tested, palm oil was best for *B. amyloliquefaciens* E1PA, whereas engine oil was suitable for *B. subtilis* TISTR 1248 (**Table 2**). Additionally, the drop collapse test, oil displacement tests, and HOA were performed; these tests are typically used to indicate surface and wetting activities [22, 23]. In these experiments, the surface activities of the crude biosurfactants from the E1PA and TISTR 1248 strains were consistent with the previous experiments, which indicated high surface activity (**Table 3**).

**Table 1** Emulsification index (E24).

| Bacteria | Emulsification index (E24) | | |
|---|---|---|---|
| | Palm oil | Engine oil | Mineral oil |
| *B. amyloliquefaciens* E1PA | 49.63±4.63 | 5.24±1.37 | 34.90±0.81 |
| *B. subtilis* TISTR 1248 | 41.36±3.61 | 2.27±0.05 | 0.00 |

**Table 2** Emulsification activity.

| Bacteria | Emulsification activity (U $mL^{-1}$) | | |
|---|---|---|---|
| | **Palm oil** | **Engine oil** | **Mineral oil** |
| *B. amyloliquefaciens* E1PA | 396.67±16.07 | 321.67±7.64 | 328.33±49.33 |
| *B. subtilis* TISTR 1248 | 246.67±27.54 | 286.67±2.89 | 243.33±14.43 |

**Table 3** Results of the oil displacement test (ODT), hydrocarbon overlay assay (HOA), drop collapse test, and blood hemolytic activity of selected bacteria.

| Bacteria | ODT | HOA | Drop collapse test | Hemolysis |
|---|---|---|---|---|
| *B. amyloliquefaciens* E1PA | + | + | + | ß-hemolysis |
| *B. subtilis* TISTR 1248 | + | + | + | γ-hemolysis |
| $H_2O$ | - | - | - | - |

Positive (+) indicated a biosurfactant activity, and negative (-) indicated an absence of biosurfactant activity.

In the blood agar assay, *B. amyloliquefaciens* E1PA and *B. subtilis* TISTR 1248 showed β and γ hemolysis (**Figure 1**), respectively. Hemolytic activity is a characteristic of biosurfactant producers [24], and is necessary for all biosurfactant producers [25]. In the present work, only E1PA displayed excellent hemolytic activity. Our data from screening assays unequivocally confirmed that *B. amyloliquefaciens* E1PA and *B. subtilis* TISTR 1248 synthesized biosurfactants.

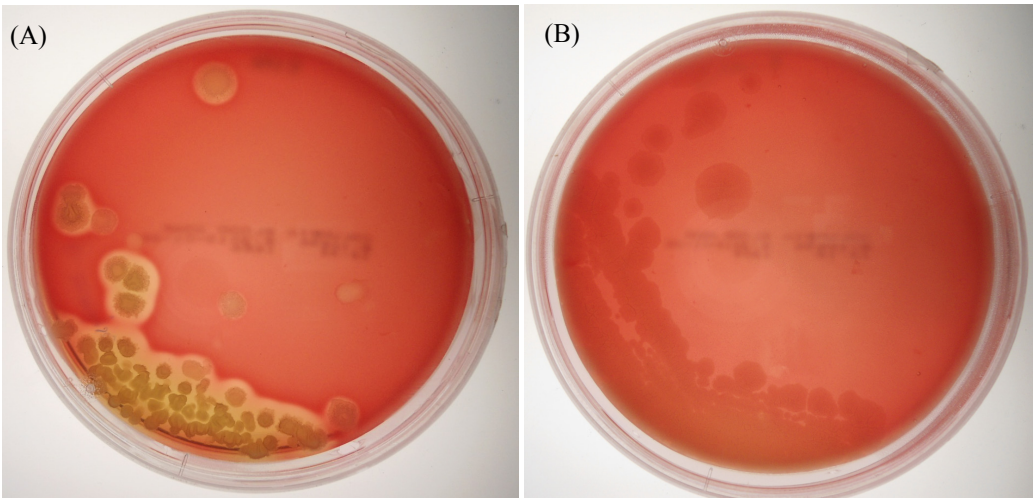



**Figure 1** Hemolytic activity of *B. amyloliquefaciens* E1PA (A) and *B. subtilis* TISTR 1248 (B) on blood agar.

**Growth with glycerol and biosurfactant production by *B. amyloliquefaciens* E1PA and *B. subtilis* TISTR 1248**

To verify biosurfactant production by *B. amyloliquefaciens* E1PA and *B. subtilis* TISTR 1248, biosurfactant production (emulsification activity) and growth curves were monitored during the growth of these strains with glycerol (4 % v/v) in MS medium (**Figure 2**). Based on the observed growth patterns, the cells exhibited exponential-phase growth for 48 h, after which the cells entered the stationary phase of growth. Biosurfactant production was highest at 48 h of incubation in all strains. *B. amyloliquefaciens* E1PA showed emulsification activity of 443 U/ml. At this time point, *B. subtilis* TISTR 1248 displayed emulsification activity of 313 U/ml, which was lower than the emulsification activity of *B. amyloliquefaciens* E1PA. The emulsification activity slightly decreased from 48 to 96 h of incubation. This result unequivocally confirmed the production of biosurfactants by the *B. amyloliquefaciens* E1PA and *B. subtilis* TISTR 1248 cultures when grown on glycerol (a byproduct of biodiesel production) as the sole carbon source. Biosurfactant production requires a carbon source, which plays a crucial role in the type, yield, and activity of the produced biosurfactants [26,27]. The carbon source (glycerol) used in biosurfactant production liberated the biosurfactant, which was initially associated with the cell wall [28].

**Palm oil-induced biosurfactant production**

Next, we investigated the effect of palm oil on biosurfactant production by *B. amyloliquefaciens* E1PA and *B. subtilis* TISTR 1248. Biosurfactant production determined based on emulsification activity revealed that growth with palm oil in MS medium enhanced emulsification activity (**Figure 3**). The biosurfactant produced by *B. amyloliquefaciens* E1PA showed the highest yield (975 U/ml) at 48 h of incubation. *B. subtilis* TISTR 1248 exhibited emulsification activity of 598 U/ml at the same time point. Therefore, the results suggest that palm oil is required, and facilitates, biosurfactant production. Emulsification activity increased 2-fold when palm oil was used as the inducer. The difference in emulsification activity between induced and uninduced cells may be due to the stimulation of biosurfactant production by fatty acids [29].

**Biochemical and structural characterization of the biosurfactant**

The biosurfactants from *B. amyloliquefaciens* E1PA and *B. subtilis* TISTR 1248 were extracted and purified through chloroform-methanol extraction. A viscous, light brown material was obtained and analyzed by FTIR. The IR spectra of the biosurfactants were investigated to obtain insights into their chemical compositions (**Figure 4**). The results were compared with the IR spectrum data from the surfactin standard (Sigma) (**Figure 4A**). The *B. amyloliquefaciens* E1PA and *B. subtilis* TISTR 1248 biosurfactants exhibited strong absorption bands in the range of 3000 - 3500 $cm^{-1}$, with a maximum of approximately 3300 $cm^{-1}$, demonstrating the occurrence of -OH and -NH stretching vibrations (**Figures 4B** and **4C**). This spectrum is typical of carbon-containing compounds with amino groups. Another strong band was detected at 1641 $cm^{-1}$, which implied the stretching mode of a CO-N bond. This band indicates the presence of peptide groups in the biosurfactant [30]. Additionally, the presence of an aliphatic chain was detected based on the modes of C-H stretching at 2942 and 1,431 $cm^{-1}$. The results of the FTIR analysis suggested that the biosurfactants of *B. amyloliquefaciens* E1PA and *B. subtilis* TISTR 1248 are cyclic lipopeptides, because the spectra of the commercially available surfactin (Sigma) and the crude extract were almost identical. Therefore, we can conclude that the biosurfactants produced by *B. amyloliquefaciens* E1PA and *B. subtilis* TISTR 1248 in glycerol culture exhibit a lipopeptide structure, and are of the surfactin biosurfactant type. The lipopeptide biosurfactant surfactin has been widely investigated, and is produced by Bacillus species. Banat *et al*. [31] found that surfactin was formed only by *B. subtilis* and *B. pumilus*. However, according to the FTIR spectra obtained in the present study, strain E1PA of *B. amyloliquefaciens* produces a surfactin biosurfactant similar to that of *B. amyloliquefaciens* KPS46 [32].

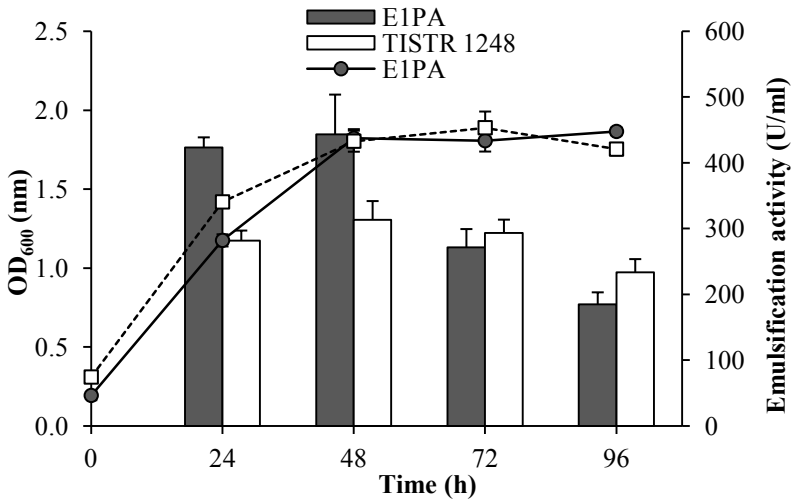



**Figure 2** Growth of *B. amyloliquefaciens* E1PA and *B. subtilis* TISTR 1248 on glycerol (4 %, v/v), accompanied by the emulsification activity of the growth medium. Samples were taken every 24 h during incubation. Growth was determined using spectrophotometry at an optical density of 600 nm. Emulsification activity was determined via mixing with palm oil. An uninoculated control was used as a blank. All values are expressed as the mean with the standard deviation of triplicate measurements.

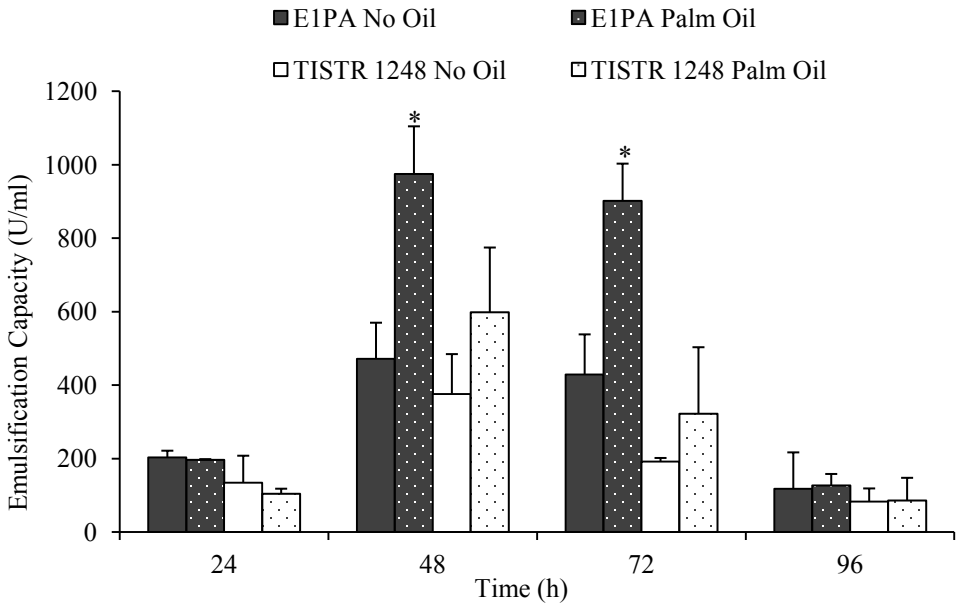



**Figure 3** Emulsification activity of *B. amyloliquefaciens* E1PA and *B. subtilis* TISTR 1248 grown on MSM medium supplemented with and without 2 % (v/v) palm oil. Samples were collected every 24 h during incubation. Emulsification activity was determined via mixing with palm oil. An uninoculated control was used as a blank. All values are expressed as the mean with the standard deviation of triplicate measurements. Asterisk (*) indicates a significant difference at $p < 0.05$.

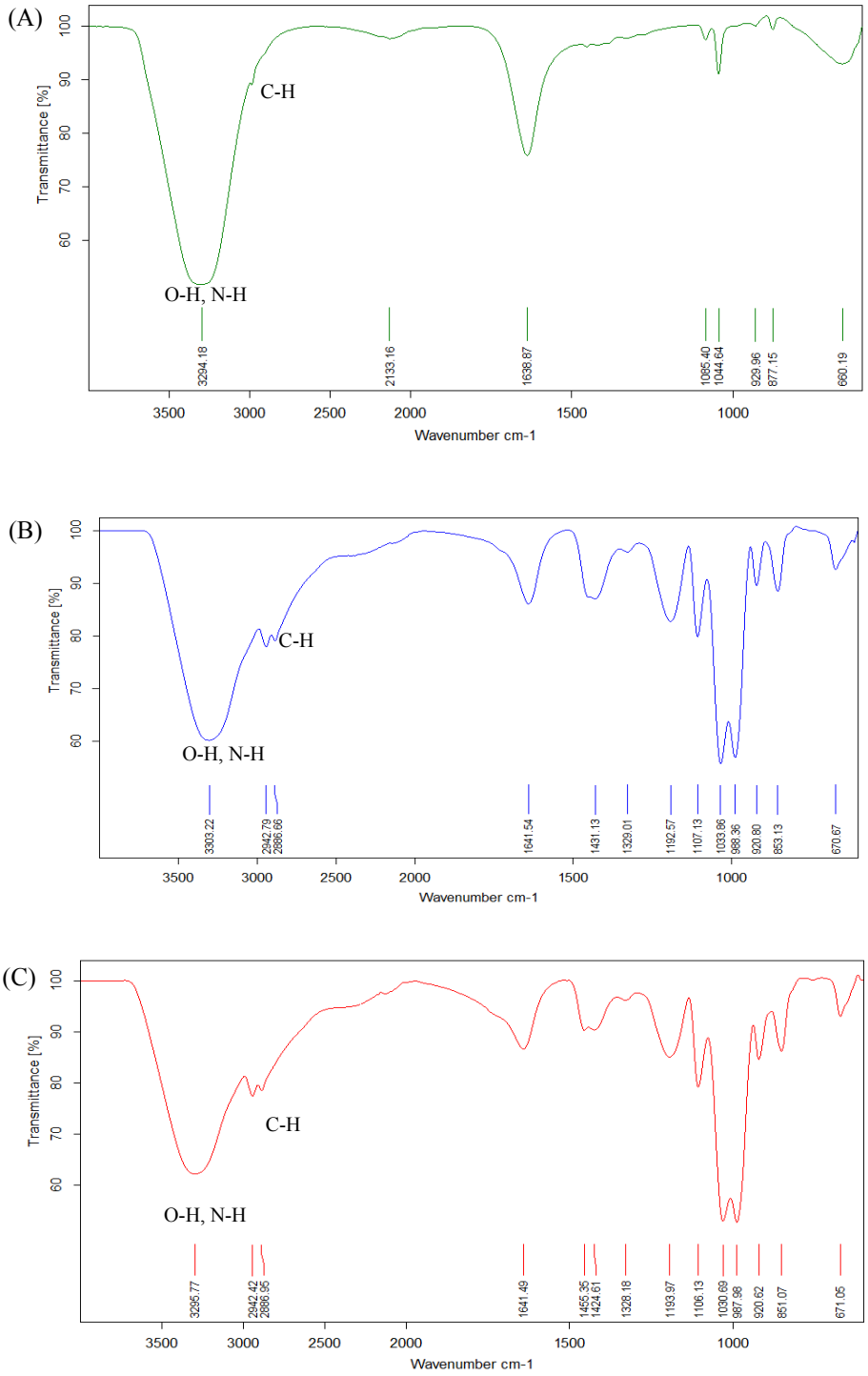



**Figure 4** The FTIR spectra of the surfactin standard (A) and crude surfactin extracted from *B. amyloliquefaciens* E1PA (B) and *B. subtilis* (C).

**Effect of the E1PA surfactin on the ability of immobilized lipase to catalyze palm oil hydrolysis**

The hydrolysis of palm oil in a two-phase system (oil/water) by lipase catalysis was evaluated using the purified recombinant E1PA lipase described in a study by Saengsanga *et al.* [8]. This recombinant lipase (3.5 U/mg) was immobilized in Ca-alginate beads via entrapment. When we assessed the hydrolytic activity of this lipase (**Figure 5**), the highest degree of palm oil hydrolysis was observed following the addition of 1 % (v/v) surfactin. This result indicated that the hydrolytic activity of the immobilized lipase was 12.4 %. The hydrolytic activity increased to 97.3 % when surfactin (1 % v/v) was applied to prepare the emulsion, which was then subjected to the lipase-catalyzed hydrolysis of palm oil. The low hydrolysis activity of the lipase in the absence of surfactin may be attributable to the small surface-to-volume ratio of the oil in the reaction mixture, which resulted in low substrate availability for catalysis and a lack of interfacial lipase activation in this two-phase system [11].

**Molecular characterization of the *sfp* gene involved in E1PA surfactin biosynthesis**

The *sfp* gene of B. *amyloliquefaciens* E1PA was successfully amplified and then cloned into the pETBlue-2 vector. A 615 bp ORF was retrieved, which encoded a mature protein with 204 amino acid residues. The complete *B. amyloliquefaciens* E1PA *sfp* gene was submitted to the GenBank database at NCBI under accession number KC711052. The sequence of the putative lipase gene was 100 % identical to *B. amyloliquefaciens* S20 and *B. subtilis* ZK8 (**Table 4**). The *sfp* gene has been identified in *Bacillus* spp., and is required for the biosynthesis of the lipopeptide surfactin [33,34]. The *sfp* phylogenetic tree is shown in **Figure 6A**. The E1PA *sfp* gene was in the same group as the *sfp* genes from *B. subtilis* ZK8 (KT750873) and *B. amyloliquefaciens* strains S20 (JX414225) and SP-1-13LM (JX684070). The phylogenetic analysis was consistent with the FTIR analysis, which indicated that the biosurfactant was surfactin (**Figure 7**).

Surfactin undergoes non-ribosomal synthesis by a mega-multienzyme peptide synthetase complex that consists of four protein subunits (SrfA, SrfB, SrfC, and SrfD) [31]. The *sfp* gene is vital for surfactin production, and encodes a protein that functions as a primer for non-ribosomal peptide synthesis [35]. This protein acts as an activator of surfactin synthetase during the conversion of the inactive surfactin synthetase apo form to the cofactor-containing holo form through posttranslational modification [36].

**Expression and purification of *sfp* in *E. coli* Tuner**

We attempted to transfer the *sfp* gene from the wild-type *B. amyloliquefaciens* E1PA strain to a surfactin-negative *E. coli* strain. To this end, the recombinant pET*sfp*-4 plasmid was constructed. To express and purify the *sfp* gene, the pET*sfp*-4 plasmid was transformed into the host strain, *E. coli* Tuner. This plasmid was expressed and fused with 6x-His at the C-terminus and was under the regulation of the $T_7$ promoter. For induction, recombinant *E. coli* was inoculated in LB medium containing 100 µg/ml ampicillin and 34 µg/ml chloramphenicol and cultivated at 37 °C with shaking (150 rpm) until the $OD_{600}$ reached 0.4 - 0.5 (5 h). IPTG was added at a final concentration of 1.0 mM, and induction was continued for 3, 6, 9, 12, or 18 h. The cells were subsequently solubilized and analyzed via SDS-PAGE (**Figure 6B**). A target band of approximately 23 kDa in size was observed in the induced recombinant cells harboring the *sfp* gene. No extra protein band was found at the same position in *E. coli*. These results confirmed that the *sfp* gene from *B. amyloliquefaciens* E1PA was expressed in the recombinant strain.

For purification, the crude extract induced with the BugBuster reagent was purified with Ni-NTA resin, and the fraction was analyzed via SDS-PAGE. The purified recombinant *sfp* migrated as a single band in the SDS-PAGE gel with the expected molecular weight of approximately 23 kDa (**Figure 6C**). FTIR analysis of the purified surfactin revealed strong broad absorption bands that were identical (99.84 %) to the surfactin standard (Sigma). Therefore, we concluded that the biosurfactant synthesized by *B. amyloliquefaciens* E1PA had a lipopeptide structure.

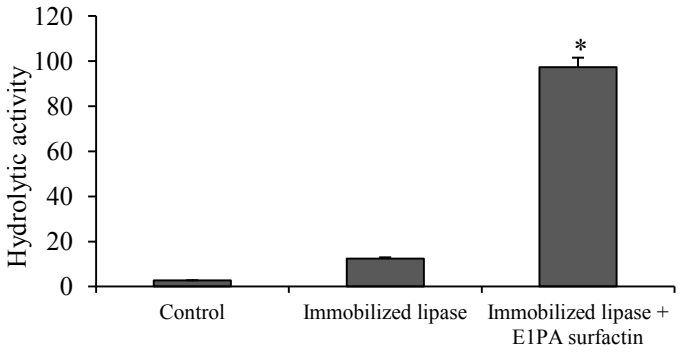
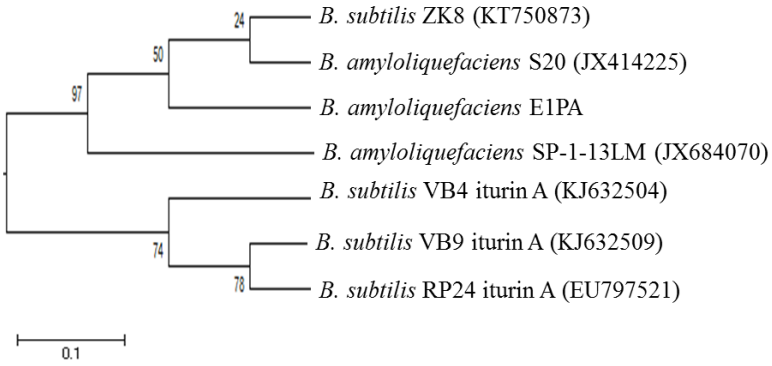
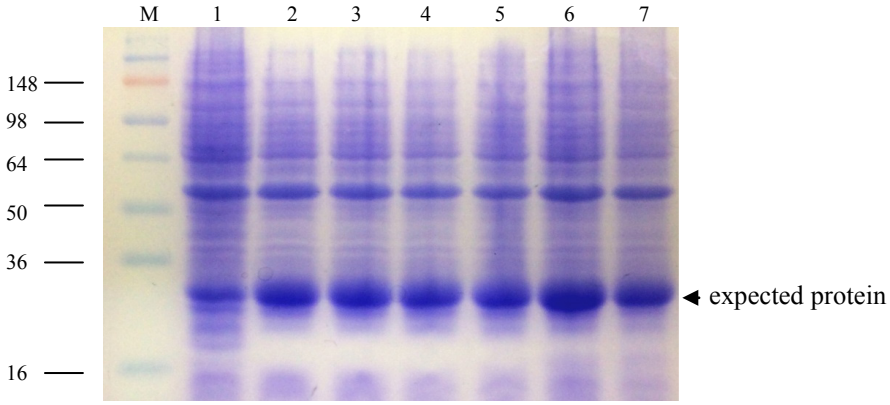



**Figure 5** Hydrolytic activity of the immobilized lipase on palm oil with and without surfactin. Asterisk (*) indicates a significant difference at $p < 0.05$.

(A)




(B)



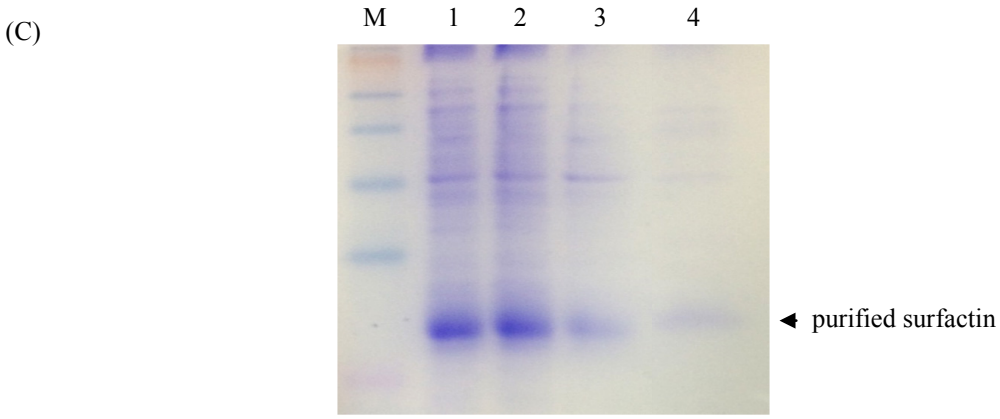



**Figure 6** (A) Phylogenetic tree analysis of the *B. amyloliquefaciens* E1PA *sfp* gene and closely related sequences. (B) SDS-PAGE analysis of recombinant pETP*sfp*-4 expressed in *E. coli* Tuner. The cells were cultured until the $OD_{600}$ reached 0.3 - 0.4 (approximately 5 h); then, IPTG was added at a final concentration of 1 mM. Lane M: Protein marker; Lane 1: *E. coli* Tuner harboring intact pETBlue-2; Lane 2: uninduced *E. coli* Tuner harboring pET*sfp*-4; Lanes 3-7: *E. coli* Tuner harboring pET*sfp*-4 induced with 1 mM IPTG for 3, 6, 9, 12, or 18 h, respectively. (C) SDS-PAGE analysis of the purification step for recombinant pET*sfp*-4. Lane M: standard protein marker; Lane 1: cell lysate; Lane 2: unbound protein, Lane 3: washing step; Lane 4: purified surfactin.

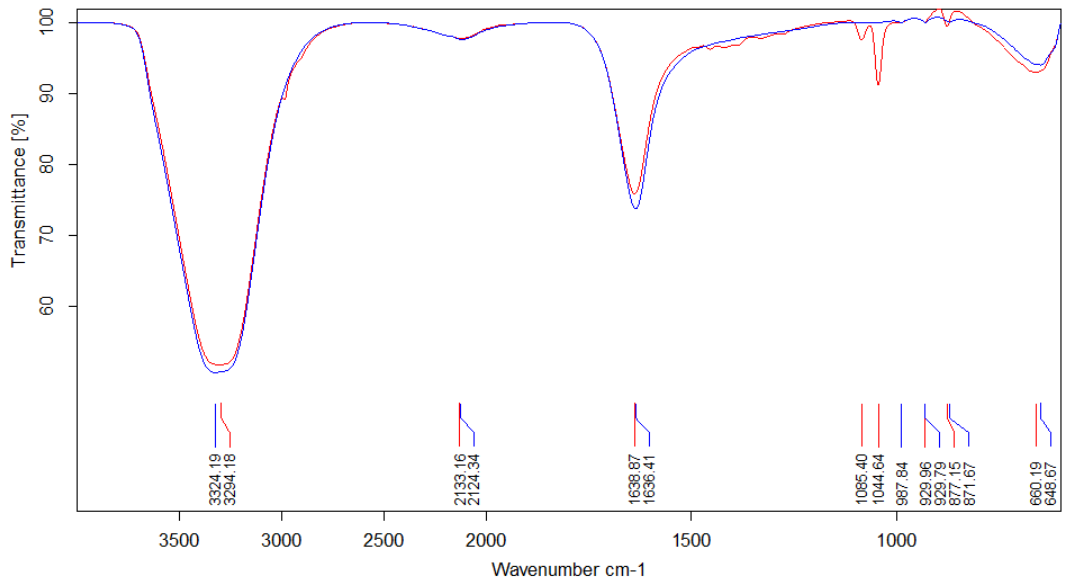



**Figure 7** The FTIR spectra of the surfactin standard (red line) and the purified surfactin from *B. amyloliquefaciens* E1PA (blue line).

## Conclusions

The findings of the present study show that *B. amyloliquefaciens* E1PA produces a biosurfactant using glycerol as a carbon source and exhibits higher emulsification activity when induced with palm oil. The significant increase in interfacial activation observed in the hydrolysis reaction makes the E1PA biosurfactant a good candidate for application in the oleochemical industry. Additionally, we successfully cloned, sequenced, and expressed the *sfp* gene in *E. coli*. Our data suggest that this biosurfactant has a lipopeptide structure and corresponds to surfactin. *E. coli* expressing this *sfp* may have potential applications in the production of recombinant E1PA surfactin.

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