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# Mg-Fe Layered Double Hydroxides Enhance Surfactin Production in Bacterial Cells

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**Abstract:** In this study, four additives—montmorillonite, activated carbon, and the layered double hydroxides (LDHs), Mg<sub>2</sub>Fe-LDH and Mg<sub>2</sub>Al-LDH—were tested for their ability to promote surfactin production in a *Bacillus subtilis* ATCC 21332 culture. Among these tested materials, the addition of 4 g/L of the Mg-Fe LDH, which featured an Mg/Fe molar ratio of 2:1, produced the highest surfactin yield of 5280 mg/L. During the time course of *B. subtilis* cultivation with the added LDH, two phases of cell growth were evident: Growth and decay. In the growth phase, the cells grew slowly and secreted a high amount of surfactin; in the decay phase, the cells degraded rapidly. The production in the presence of the Mg<sub>2</sub>Fe-LDH had three characteristics: (i) High surfactin production at low biomass, indicating a high specific surfactin yield of 3.19 g/g DCW; (ii) rapid surfactin production within 24 h, inferring remarkably high productivity (4660 mg/L/d); and (iii) a lower carbon source flux to biomass, suggesting an efficient carbon flux to surfactin, giving a high carbon yield of 52.8%. The addition of Mg<sub>2</sub>Fe-LDH is an effective means of enhancing surfactin production, with many potential applications and future industrial scale-up.

**Keywords:** *Bacillus subtilis*; surfactin; quantitative analysis; fermentation; growth phase; layered double hydroxides

## 1. Introduction

Biosurfactants are amphipathic molecules produced by microorganisms [1–3] with the capability of decreasing surface and interfacial tension [4]. Depending on their chemical composition and their producing organism, biosurfactants can possess high biodegradability, low toxicity, ecological acceptability, and high efficiency. Accordingly, they have been investigated as possible alternatives to chemical surfactants [5,6]. *Bacillus* spp., bacterial strains of complicated physiological diversity, can be used to produce many bioactive peptides with potential biotechnological and biopharmaceutical applications. Among these peptides, the lipopeptides that feature an alkyl group and a circular peptide group are the most popular biosurfactants [7]; these materials include surfactins [8–10], iturins [11,12], and fengycins [13].

The surfactin produced by *B. subtilis* is one of the strongest biosurfactants available [7]. Its chemical composition is that of a cyclic lipopeptide (comprising seven amino acids) with a 12 to 19-carbon atom hydrophobic fatty acid chain [14]. Surfactin can lower the surface tension of water to 27 mN/m even when its concentration is as low as 0.005% [7,10,15,16], suggesting its great potential applicability. Nevertheless, the high expense and low yield of surfactin production have limited its commercial use. Yeh et al. found that limiting the concentration of the carbon source (glucose) affected the surfactin production mediated by *B. subtilis* [17]. Davis et al. observed the highest production of surfactin when

ammonium nitrate was the nitrogen source during *B. subtilis* cultivation in a defined medium [18]. Sen et al. noted that the ratio of Mn and Fe mineral salts in the medium was a factor affecting the production of surfactin [19]. Wei and Chu found that the yield of surfactin increased dramatically, over those obtained using genetic strains, when employing 0.01 mM  $Mn^{2+}$  [20]. Furthermore, Wei et al. employed an iron-enriched (4 mM  $Fe^{2+}$ ) minimal salt medium to produce 3000 mg/L of surfactin [21]. Moreover, some of these studies revealed that the addition of solid additives (e.g., activated carbon (AC) or expanded clay) could increase surfactin production significantly. For example, Yeh et al. added AC and increased the yield of surfactin to 3600 mg/L [17].

Layered double hydroxides (LDHs)—also known as anionic clays—comprise cationic brucite-like layers with exchangeable interlayer anions [22]. Because a positive ionic charge appears on the surface layer, many types of molecules can be intercalated into LDHs [23–27]. Several methods have been developed to widen the layered gallery, with globular macromolecules as intercalating agents [28,29]. Conterosito et al. intercalated various pharmaceuticals drugs and cosmetic sunscreen into Mg-Al\_LDH and Zn-Al\_LDH. They revealed that different bioactive molecules could interact with inorganic LDH and demonstrated the relationship between the molecular length and an enlarged interlayer spacing [30]. Toson et al. showed the intercalation of organic molecules into the LDH interlayer by the liquid-assisted grinding method. The intercalation mechanism for layer widening with intercalated organic molecules was investigated [31]. Choy et al. employed supramolecular inorganic species (e.g., nanoscale Mg-Al LDH) as biomolecule reservoirs that could be used for gene and drug delivery [23,32]. Nevertheless, few studies have focused on using LDHs as additives for microbial cultivation. Kan et al. prepared  $Mg_2Al$ -LDH and investigated its effect as an additive on surfactin production and surfactin intercalation [33]. When considering the application of the surfactin-intercalated LDH as a slow release bio-pesticide, however, this aluminum salt was prohibited from field tests [34]. For agricultural applications, iron salts are generally considered less toxic. Therefore, in this present study, we prepared several Mg-Fe LDH derivatives with potentially greater practicality. We tested the effects of their addition on the production of surfactin from a *B. subtilis* culture. To our surprise, replacing the additive to  $Mg_2Fe$ -LDH had an extraordinary effect on the surfactin production. Accordingly, we examined various  $Mg_nFe$ -LDH ( $n = 1, 2, 3$ ) compositions and concentrations to determine the optimal conditions for surfactin production. In addition, we examined the time course of the production in the optimal culture. An extra low biomass of cells yielded the highest surfactin production. This result was quite different from that obtained after the addition of  $Mg_2Al$ -LDH. Furthermore, we compared the effects of the LDHs with those of other additives (e.g., montmorillonite (MMT), AC), and determined the conditions for the highest production of surfactin through quantitative analysis. Herein, we also suggest possible reasons for the enhancement of surfactin production mediated by LDHs.

## 2. Materials and Methods

### 2.1. Chemicals and Reagents

$Al(NO_3)_3 \cdot 9H_2O$ ,  $Fe(NO_3)_3 \cdot 9H_2O$ , and  $Mg(NO_3)_2 \cdot 6H_2O$  were purchased from SHOWA, USA. MMT was obtained from Alfa Aesar, USA. The AC was obtained from China Activated Carbon (Taipei, Taiwan); it had a diameter of 3 to 4 mm, a height of 9 mm, and a specific surface area of 1200  $m^2/g$ , and was prepared from bituminous coal with an iodine number of 1150 mg/g. Surfactin ( $\geq 98\%$ , Sigma-Aldrich, Missouri, MO, USA) was used as the standard. All solvents and other chemicals were of analytical grade.

### 2.2. Microorganisms and Culture Conditions

The strain, *B. subtilis* ATCC 21332, was obtained from Professor Wei Yu-Hong of Yuan Ze University. This strain was kept on a nutrient-agar plate at 30 °C. For cultivation, its seed medium comprised 1% glucose, 0.5% yeast extract, 1% peptone, and 1% NaCl. The seed culture was performed in Erlenmeyer flasks (500 mL) containing the seed medium (100 mL) inoculated with two loops of cells.

The cultivation was conducted at 200 rpm and 30 °C for 12 h. The main shake-flask culture was conducted in an Erlenmeyer flask (500 mL) containing the main medium (100 mL) comprising 10 g/L sucrose, 5 g/L (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 5.67 g/L Na<sub>2</sub>HPO<sub>4</sub>, 4.08 g/L KH<sub>2</sub>PO<sub>4</sub>, 0.2 g/L MgSO<sub>4</sub>·7H<sub>2</sub>O, and 0.57 g/L FeSO<sub>4</sub>·7H<sub>2</sub>O. The media were sterilized (121 °C, 20 min); the carbon source was autoclaved separately. The medium (90 mL) was inoculated with the seed broth (10 mL). The flasks were incubated on a rotary shaker (200 rpm, 30 °C, 5 days). When testing additives, MMT, AC, and the prepared LDHs were added (2 g/L) to the culture medium at the beginning of the culture process.

### 2.3. Mg<sub>2</sub>Al-LDH and Mg<sub>2</sub>Fe-LDH

Mg<sub>2</sub>Al-NO<sub>3</sub>-LDH and Mg<sub>2</sub>Fe-NO<sub>3</sub>-LDH were prepared through co-precipitation, as described previously [27]. To prepare the Mg<sub>2</sub>Al-LDH sample, Mg(NO<sub>3</sub>)<sub>2</sub>·6H<sub>2</sub>O (120 g) and Al(NO<sub>3</sub>)<sub>3</sub>·9H<sub>2</sub>O (90 g) were dissolved in deionized H<sub>2</sub>O (1 L). To prepare the Mg<sub>2</sub>Fe-LDH sample, Mg(NO<sub>3</sub>)<sub>2</sub>·6H<sub>2</sub>O (169 g) and Fe(NO<sub>3</sub>)<sub>3</sub>·9H<sub>2</sub>O (134 g) were mixed in deionized H<sub>2</sub>O (1 L). To prepare samples with Mg/Fe molar ratios of 1.0 and 3.0, appropriate amounts of Mg(NO<sub>3</sub>)<sub>2</sub>·6H<sub>2</sub>O and Fe(NO<sub>3</sub>)<sub>3</sub>·9H<sub>2</sub>O were used. Each aqueous solution was stirred vigorously at 80 °C while purging with nitrogen gas. When preparing the Mg<sub>2</sub>Al-LDH sample, the pH was maintained at 10 ± 0.2 by adding 4 N NaOH in portions. For the Mg<sub>2</sub>Fe-NO<sub>3</sub>-LDH sample, the pH was adjusted to 9.5 ± 0.2 by using a mixture of NaOH and K<sub>3</sub>[Fe(CN)<sub>6</sub>], prepared based on the following compositions: [OH<sup>-</sup>]/([Mg<sup>2+</sup>] + [Fe<sup>3+</sup>]) = 1.6 and [[Fe(CN)<sub>6</sub>]<sup>3-</sup>]/[Fe<sup>3+</sup>] = 3. The suspension that formed was stirred at 80 °C for 24 h. The obtained precipitates—white Mg<sub>2</sub>Al-LDH and dark-red Mg<sub>2</sub>Fe-LDH—were filtered off and washed (deionized H<sub>2</sub>O). The filtered cakes were lyophilized (freeze-drying). The dried LDHs were characterized using x-ray diffraction (XRD; PANalytical, X'Pert PRO MRD, Almelo, Netherlands) and attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy (Thermo Scientific, Nicolet iS50 FTIR, Madison, WI, USA).

### 2.4. Quantitative Analysis

To study the effects of LDH addition, the following quantitative terms are defined. The surfactin yield (mg/L) is expressed by the volumetric concentration. The carbon source yield is defined as:

$$Y_{\frac{P}{S}} = \frac{\Delta P}{\Delta S} \quad (1)$$

The productivity is defined as:

$$\text{Productivity} = \frac{\Delta P}{\Delta t} \quad (2)$$

The specific yield is defined as:

$$Y_{\frac{P}{X}} = \frac{\Delta P}{\Delta X} \quad (3)$$

where  $P$  represents the surfactin concentration,  $S$  is the carbon source concentration,  $X$  is the concentration of biomass dried cells, and  $t$  is the duration of cultivation. All concentrations are expressed herein on a volumetric basis.

### 2.5. Assays

The surfactin concentration was measured using a modified approach called salt-assisted homogeneous liquid-liquid extraction via high-performance liquid chromatography (HPLC) [33,35]. The culture broth (1 mL) was subjected to a centrifugation (3200 g, 3 min, 4 °C) to remove the solid pellets. The supernatant was mixed with MeCN (0.5 mL) and ammonium sulfate (0.8 g) and subjected to vigorous stirring for 1 min, and then centrifuged (3200 g, 3 min). The supernatant was filtered (0.22 μm) to obtain the sample for injection. HPLC analysis was performed under the following conditions: A reversed-phase C-18A column (5 mm, 18 mm × 100 mm BDS-Hypersil, Thermo Fisher Scientific, Waltham, MA, USA); a mobile phase comprising CF<sub>3</sub>CO<sub>2</sub>H, MeCN, and deionized H<sub>2</sub>O

(0.1:400:100); a flow rate of 1.0 mL/min; an injection sample volume of 20  $\mu$ L; and a UV–Vis detector (JASCO, Tokyo, Japan) operated at 220 nm. A standard curve was constructed using a freshly prepared solution of surfactin (Sigma). The chromatogram of the standard (supplemental Figure S1) revealed various ratios of the surfactin isoforms A–F. The surfactin produced using *B. subtilis* ATCC 21332 featured the same surfactin isoforms A–F at various ratios. In the surfactin assay, the whole isoforms were measured and added up for quantitative calculation. To analyze the cells' dried weight (CDW), 5 mL of the broth sample was subjected to centrifugation (12,000 g, 10 min) to obtain a pellet. Distilled H<sub>2</sub>O (5 mL) was added to the pellet; after adjusting to pH 2.0, the sample was vigorously stirred (1 min). The mixture was centrifuged (12,000 g, 5 min). The pellet obtained was dissolved in distilled H<sub>2</sub>O (5 mL); the pH was adjusted to 7.0 and the mixture was again subjected to centrifugation. The obtained pellet was washed with distilled H<sub>2</sub>O (2  $\times$  5 mL), dried (80  $^{\circ}$ C, 12 h), and then weighed. The basal spacing of the LDH was determined using a Shimadzu SD-D1 X-ray diffractometer with a Cu target (scanning rate: 1 $^{\circ}$ /min). The basal spacing was estimated using the Bragg equation ( $n\lambda = 2d\sin\theta$ ).

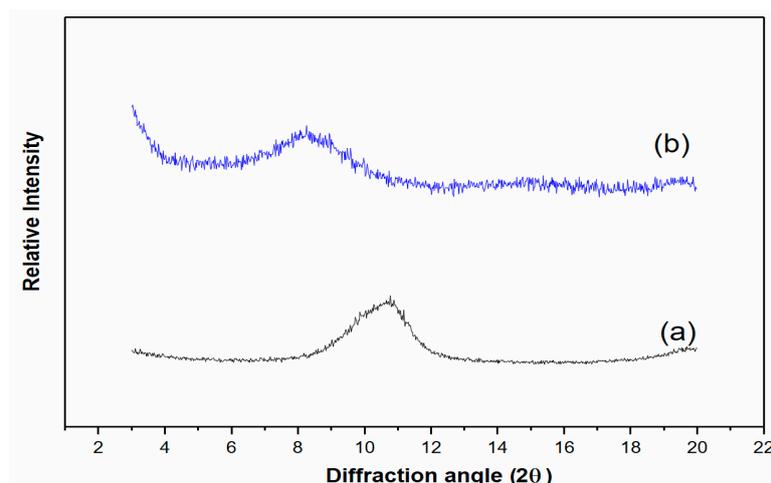
### 2.6. Statistical Analysis

Multiple flasks were run concurrently. Three flasks were employed each time for daily sampling. Each data point is expressed as a mean plus standard deviation. The Tukey test was applied for the comparison of results ( $p \leq 0.05$ ).

## 3. Results and Discussion

### 3.1. Preparation of MgFe–LDH

The addition of a small quantity of a solid carrier (AC or expanded clay) has been claimed as an effective approach toward increasing surfactin production [17]. LDHs are layered anionic exchange substances that have been intercalated with various macromolecules for the purpose of their slow release [36,37]. The addition of Mg<sub>2</sub>Al–LDH to a surfactin production fermentation system involving *B. subtilis* incubation revealed that surfactin could indeed intercalate into the LDH layer gallery to form a surfactin–LDH complex; this phenomenon occurred with a significant increase in the production of surfactin [33]. In consideration of a slow-release composite for agricultural use, Mg<sub>2</sub>Al–LDH would be inappropriate for field trials. For this study, therefore, we prepared Mg<sub>2</sub>Fe–LDH instead. We examined the effect of adding this iron salt LDH to *B. subtilis* cultivation to study whether it, too, would promote surfactin production. The prepared Mg<sub>2</sub>Fe–LDH was subjected to XRD and ATR-FTIR spectroscopic analysis. These analyses revealed an Mg<sub>2</sub>Fe–LDH layer spacing of 7.8  $\text{\AA}$  at a value of  $2\theta$  of 11.3 $^{\circ}$ , derived from the calculation of Bragg's equation (Figure 1a), and a typical adsorption peak (1381  $\text{cm}^{-1}$ ) for NO<sub>3</sub><sup>−</sup> anions within the prepared LDH (data not shown). In addition, to confirm the interaction between LDH and bacterial cells, the LDH after the cultivation was collected and subjected to XRD analysis. The result in Figure 1b shows that the collected LDH did vary its  $2\theta$  from the original 11.3 $^{\circ}$  to 8.3 $^{\circ}$ , indicating a d-spacing of 10.8  $\text{\AA}$ . The original XRD peak with a d-spacing of 7.8  $\text{\AA}$  completely disappeared. The enlarged spacing was likely due to the LDH interaction with surfactin molecules. The isoelectric point (IEP) of surfactin is around pH 5, and the fermentation process while applying LDH to the cultivation was around pH 7.4. The pH higher than the IEP would allow the surfactin to possess a negative charge, giving the chance of anion exchange for LDH intercalation. Besides, the interlayer spacing expansion of LDH might be ascribed not only to the surface interaction of surfactin intercalation but also the combination of water and other anion molecules in the culture medium into the Mg<sub>2</sub>Fe–LDH interlayer.



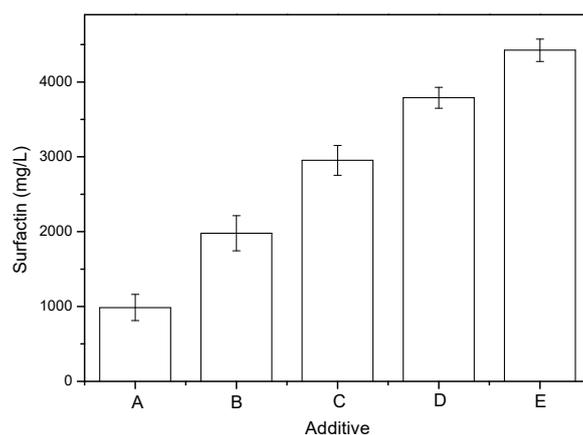
**Figure 1.** XRD pattern of (a) pristine  $Mg_2Fe$ -LDH and (b)  $Mg_2Fe$ -LDH collected after fermentation.

### 3.2. Effect of Solid Additives on Surfactin Production

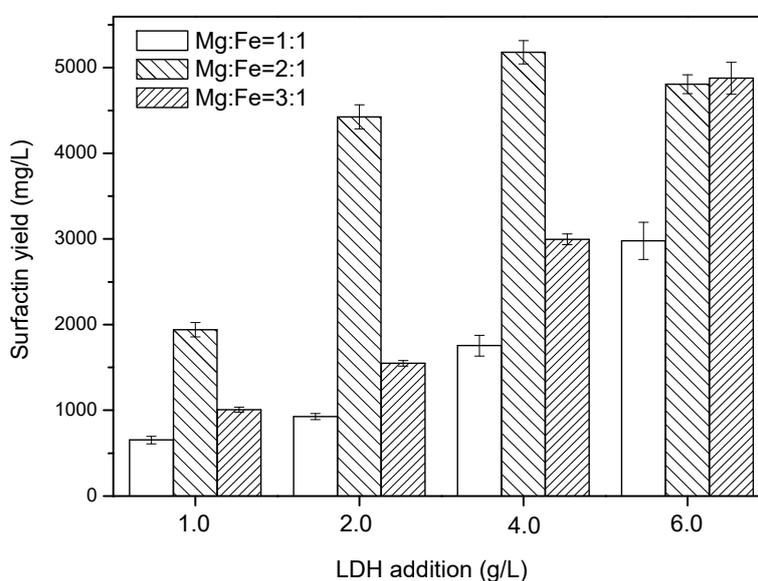
As reported previously, the addition of some solid additives can enhance surfactin production [17]. For this present study, four solid additives—MMT, AC, and two LDHs—were prepared and added respectively to the *B. subtilis* culture medium; the medium prepared without any additives was used as the control during the five-day fermentation. The surfactin production increased when the culture medium contained each of these solid additives, relative to the control. The addition of MMT, AC, and the two LDHs (2 g/L) resulted in surfactin yields that had increased by 2.0-, 3.0-, 3.8-, and 4.5-fold, respectively, when compared with the control (Figure 2). It is noteworthy that the AC with the alkaline characterization might lead to surfactin linearization and surfactin binding on the AC surface, which may be an underestimation of the actual production. Thus, the LDHs were the most effective carriers for enhanced surfactin production in a culture of *B. subtilis* ATCC 21332. Furthermore, the amount of surfactin produced in the presence of  $Mg_2Fe$ -LDH was more than that produced in the presence of  $Mg_2Al$ -LDH. Indeed,  $Mg_2Fe$ -LDH had an extraordinary stimulatory effect on promoting surfactin production.

### 3.3. Effect of $MgFe$ -LDH Composition on Surfactin Production

To study the effect of the Mg/Fe molar ratio on surfactin production, LDHs were prepared with Mg:Fe molar ratios of 1:1, 2:1, and 3:1 and added into *B. subtilis* cultivation. The concentrations of the additive ranged from 1 to 6 g/L in the fermentation medium. The cultivation was performed for 5 days. Figure 3 reveals that the LDH prepared with a Mg:Fe molar ratio of 2:1 had the greatest effect at promoting surfactin production. In general, LDHs possessing different ratios of divalent and trivalent metal ions possess different types of positively charged sheets and different layer dimensions in their resulting layered structures [38–41]. In the brucite-like layers of an LDH, a fraction of the divalent metal ions is replaced by trivalent metal ions, with the molar ratio of  $M^{3+}:(M^{3+} + M^{2+})$  ( $x$ ) normally positioned between 0.2 and 0.4 [24,42]. In this present study, an Mg:Fe ratio of 2:1 ( $x = 0.33$ ) had the best effect on improving surfactin production. Thus, it appears that the layer size associated with the positively charged sheets of the  $Mg_2Fe$ -LDH structure had the strongest stimulatory effect on the cells.



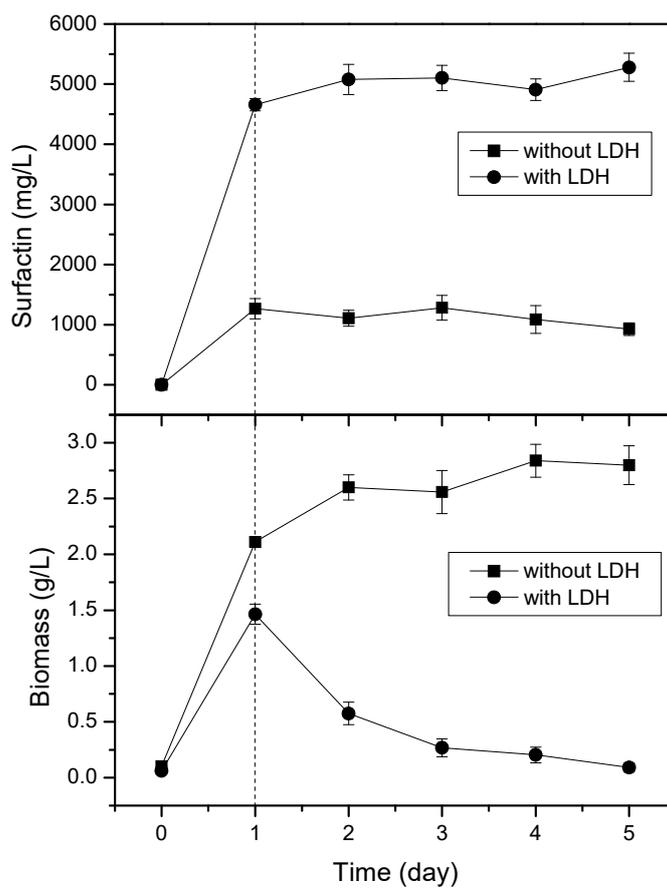
**Figure 2.** Effects of solid additives on surfactin yield in *B. subtilis* ATCC 21332 cultivation in a 5-day fermentation: (A) none; (B) MMT; (C) AC; (D) Mg<sub>2</sub>Al-LDH; (E) Mg<sub>2</sub>Fe-LDH. The surfactin level in the supernatant of the broth was determined. Error bars indicate the standard deviations from three tests.



**Figure 3.** Effect of Mg<sub>2</sub>Fe-LDH concentration (1, 2, 4, and 6 g/L) on the specific surfactin yield, where bars with different patterns represent various Mg<sup>2+</sup>:Fe<sup>3+</sup> ratios: blank, 1:1; back slash, 2:1; slash, 3:1. Error bars indicate standard deviations from three tests.

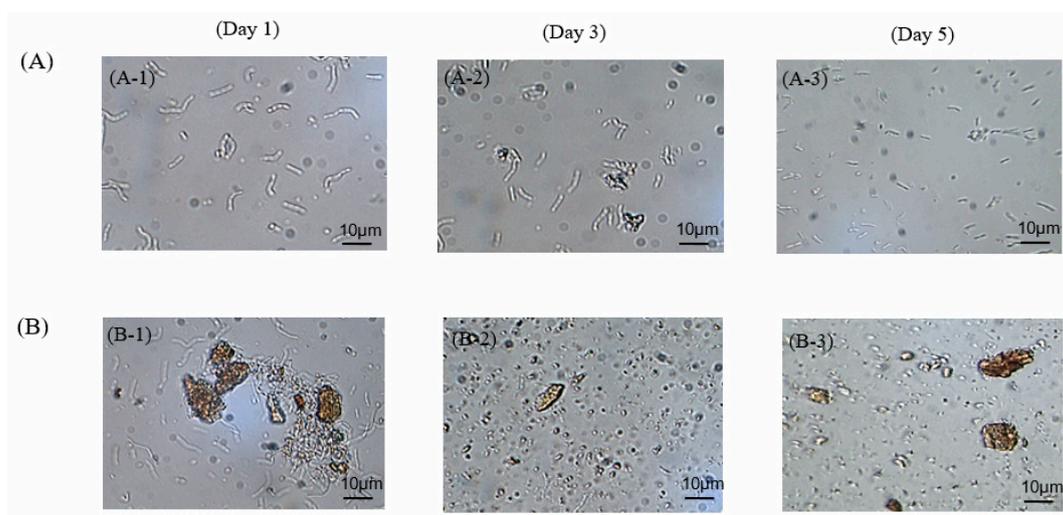
#### 3.4. Time Course of Cultivation with LDH Addition

To study the cell growth after adding LDH, the time courses of the cultivation events performed with and without added LDH were recorded (Figure 4). Although the addition of Mg<sub>2</sub>Fe-LDH promoted surfactin production, relative to that of the control, it was interesting to observe that the cell growth ended on the first day, where the amount of surfactin reached 4.8 g/L. In terms of product formation kinetics, this behavior was a clear growth-associated pattern: The cells grew and surfactin was produced. After day 1, the cells began to degrade in a decay phase, with the surfactin production decelerating. In contrast, the growth of cells was very rapid in the culture medium prepared without LDH, but the level of surfactin production was very low. Thus, a slight inhibition of cell growth appeared to trigger the cells to secrete more surfactin. We suspect that the surfactin secreted by the cells performed a role as a protecting agent that kept the cells from coming into direct contact with the LDH.



**Figure 4.** Time courses of surfactin and biomass production in the presence and absence of  $Mg_2Fe-LDH$  (4 g/L). Error bars indicate the standard deviations from three tests.

Figure 5A,B present microscopy images of the morphologies of the cells grown in the presence of the LDH. In the culture medium lacking the LDH, the cells had a short and rod-like morphology from day 1 to day 3 of culturing. By the fifth day, some cells became slenderer than the original short-rod cells. In contrast, in the culture medium incorporating the LDH, the cells grew in a short-rod shape on the first day, but, by the third day, most of the cells had decayed and shrunk, with many endospores present. By the fifth day, almost none of the cells were evident in the broth, with only some spores remaining in the culture. This observation is consistent with the amounts of cells measured in the study. Therefore, the addition of LDH did inhibit the growth of cells during the cell growth phase, but it also enhanced the production of surfactin. Accordingly, in addition to the high surfactin yield of the culture incorporating the LDH, an extremely high productivity also ensued. Because of the lower number of cells, not only was the specific production elevated, the carbon source conversion to surfactin was also enhanced and provided a high carbon yield.



**Figure 5.** Optical microscopy images of *B. subtilis* ATCC 21332 growth, taken after various numbers of cultivation days; (A) in the absence of any LDH; (B) in the presence of  $Mg_2Fe$ -LDH.

### 3.5. Comparison of Surfactin Production

Table 1 compares the surfactin production in this present study with those reported previously in the literature. Four factors characterize surfactin production in these bioprocesses in terms of their efficiency for fermentation on industrial scale: the surfactin yield, the carbon yield, the productivity, and the specific production. Due to the variation on surfactin quantification, the surfactin assays, such as HPLC, surface tensions, and acid precipitation, were also listed. As evident in Table 1, the addition of  $Mg_2Fe$ -LDH had a unique effect in promoting surfactin production. Historically, surfactin production has improved gradually from an original yield of less than 1000 mg/L two decades ago to approximately 2000 to 3000 mg/L recently. When using this present approach, the yield of surfactin after the addition of  $Mg_2Fe$ -LDH was enhanced significantly, to greater than 5000 mg/L. Furthermore, the addition of  $Mg_2Fe$ -LDH ensured that the carbon source mostly flowed to surfactin production. Indeed, the carbon source yield was approximately 52.8%. This high carbon yield characterizes a surfactin production process with a highly efficient use of the raw material. In addition, the presence of  $Mg_2Fe$ -LDH caused the surfactin yield to reach 4660 mg/L after one day of culturing; that is, the productivity was 4660 mg/L, a remarkably high value as compared in the literature. In addition, because the number of cells decreased in the presence of  $Mg_2Fe$ -LDH, the smaller amount of biomass and the higher surfactin yield led to a specific yield of 3.19 g/g DCW. The addition of  $Mg_2Fe$ -LDH in *B. subtilis* submerged cultivation provided a high carbon yield, high productivity, and high specific production of surfactin; such a high efficiency appears well suited to industrial applications.

At the beginning of our approach, the change of  $Mg_2Al$ -LDH to  $Mg_2Fe$ -LDH was due to the practical need in agricultural applications, where the aluminum salt is prohibited from field tests. However, to our surprise, the replacement of additive to  $Mg_2Fe$ -LDH did give an extraordinarily high surfactin production. Due to this effect, the three critical characteristics affecting surfactin production were evaluated. It was found that a high specific surfactin yield, a high productivity, and a high carbon yield could be obtained in the presence of the  $Mg_2Fe$ -LDH. To explain the difference between  $Mg_2Fe$ -LDH and  $Mg_2Al$ -LDH additions, the effect of the  $Mg_2Fe$ -LDH addition with the leaking iron trace element in the culture was the possible reason for this highly efficient surfactin production. To decipher the cause of the extraordinarily high stimulatory effect of  $Mg_2Fe$ -LDH, the following considerations might be taken into account. In some previous studies, ferric ions have been found to serve as trace element stimulators, with an excellent ability to promote surfactin production [21,43,44]. In addition, the use of pristine  $Mg_2Al$ -LDH has been claimed to enhance surfactin production as a result of its toxicity toward the cells [33]. Accordingly, the presence of  $Mg_2Fe$ -LDH was expected to not only inhibit cell growth and promote surfactin production (similar to the behavior of  $Mg_2Al$ -LDH)

but also to slowly release some iron salts to serve as trace elements in the medium, thereby also improving the surfactin production. The higher production obtained using Mg<sub>2</sub>Fe-LDH, compared with that of Mg<sub>2</sub>Al-LDH, might be due to the synergistic effect of the Mg<sub>2</sub>Fe-LDH crystalline structure and the trace iron salts in the medium, with both combining to promote surfactin production to such a high level.

**Table 1.** Various approaches used for surfactin production.

Approach	Yield <sup>a</sup> (mg/L)	Carbon Yield (g/g carbon source)	Productivity <sup>b</sup> (mg/L/day)	Specific production <sup>c</sup> (g/g DCW)	Surfactin Quantification	Ref.
Iron addition & product in foam	800	0.02	–	0.615	Acid precipitate	[45]
Peat hydrolysate medium	160	0.004	160	0.208	HPLC	[46]
Aqueous two phase	350	0.07	350	–	Surface tensions	[47]
Optimized medium	760	0.021	–	–	Surface tensions	[19]
Optimized nitrogen source	439	0.015	219	0.075	HPLC	[18]
Recombinant strain	350	0.035	350	–	HPLC	[48]
Strain mutation and product in foam	562	0.014	562	0.323	Surface tensions	[16]
Iron-enriched medium	3500	0.088	–	–	HPLC	[43]
Optimized iron supplement	3000	–	–	0.162	HPLC	[21]
Activated carbon addition	3600	0.09	1200	–	HPLC	[17]
Optimized trace element	3340	0.084	–	–	HPLC	[49]
Mn <sup>2+</sup> addition	2600	0.065	–	0.289	HPLC	[20]
Mg <sub>2</sub> Al LDH addition	3789	0.379	–	–	HPLC	This study
Mg <sub>2</sub> Fe LDH addition	5280	0.528	4660	3.19	HPLC	This study

<sup>a</sup> Maximum yield in whole culture. <sup>b</sup> Maximum productivity in whole culture. <sup>c</sup> Specific production when reaching maximum yield.

#### 4. Conclusions

We investigated the effects of LDHs on the production of biomass and surfactin in a *B. subtilis* ATCC 21332 culture. The highest yield of surfactin (5280 mg/L) was obtained after 5 days of cultivation in the presence of 4 g/L Mg<sub>2</sub>Fe-LDH. This study demonstrated that LDHs have potential for use as additives to enhance the production of surfactin in *B. subtilis* ATCC 21332. Furthermore, microscopy revealed the inhibition of cell growth in the presence of the LDH, suggesting an efficient process for the production of surfactin through greater conversion of the carbon source.

**Supplementary Materials:** The following are available online at <http://www.mdpi.com/2073-4352/9/7/355/s1>, Figure S1: Typical surfactin standard chromatogram in HPLC showing surfactin isoform A–F.

**Author Contributions:** Data curation and methodology, P.-H.C. and S.-Y.L., conceptualization, writing—original draft preparation; writing—review and editing, project administration, T.-Y.J. and Y.-C.L.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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