Journal of the Taiwan Institute of Chemical Engineers 000 (2017) 1–6

Contents lists available at ScienceDirect

Journal of the Taiwan Institute of Chemical Engineers

journal homepage: www.elsevier.com/locate/jtice

# Enhanced surfactin production via the addition of layered double hydroxides

Shu-Chen Kan[a,1], Chih-Chen Lee[a,1], Yu-Che Hsu[a], Yu-Hsiang Peng[b], Cheng-Chun Chen[a], Jia-Jun Huang[a], Jenn-Wen Huang[b], Chwen-Jen Shieh[c], Tzong-Yuan Juang[d,*], Yung-Chuan Liu[a,*]

[a] Department of Chemical Engineering, National Chung Hsing University, 250 Kuo Kuang Road, Taichung 40227, Taiwan
[b] Department of Plant Pathology, National Chung Hsing University, 250 Kuo Kuang Road, Taichung 40227, Taiwan
[c] Biotechnology Center, National Chung Hsing University, 250 Kuo Kuang Road, Taichung 40227, Taiwan
[d] Department of Cosmeceutics, China Medical University, Taichung 40402, Taiwan

ARTICLE INFO

*Article history:*
Received 12 April 2017
Accepted 9 June 2017
Available online xxx

*Keywords:*
*Bacillus subtilis* CWS1
Surfactin
Solid additives
LDH
Fermentation

ABSTRACT

In this study, Mg–Al layered double hydride (LDH) was prepared and the impact of LDH addition on the biomass and surfactin yield for cultures of *Bacillus subtilis* CWS1 was examined. Various solid additives, including montmorillonite (MMT), activated carbon (AC) and LDH, were tested. Among these, LDH triggered the highest surfactin yield at 3789 mg/L. The optimal conditions for surfactin production were 2 g/L LDH supplied on the beginning of a 5-day cultivation, resulting in a 3.8-fold increase in yield. To reveal the mechanism of surfactin enhancement, observation was conducted via the optical microscope. The resulted images showed that when LDH was added, the cells became segmented, short and narrow compared to cells without any addition. It is apparent that LDH exhibits a slightly toxic effect, limiting cell growth. The surviving cells were forced to secrete more surfactin to protect themselves from the harsh environment. Since the cells could no longer grow, their only function was to secrete surfactin into the broth, leading to an extraordinarily high surfactin yield with a low biomass production. This suggests that an alternative means for efficient surfactin production is through the addition of LDH.


## 1. Introduction

Biosurfactants are surfactants produced by a variety of microorganisms [1,2]. They are amphipathic molecules with both hydrophobic and hydrophilic moieties [3], and are capable of reducing surface and interfacial tension [4]. Based on the chemical composition and organism, biosurfactants fall into one of five groups: glycolipids, phospholipids, lipopeptides, fatty acids or polymeric biosurfactants [5]. Because of their high biodegradability, ecological acceptability, low toxicity and high efficiency, biosurfactants are being investigated as possible alternatives to chemical surfactants [6].

*Bacillus* spp. strains prossess complicated physiological diversity and could be used to produce numerous bioactive peptides with potential biotechnological and biopharmaceutical applications [7]. Compounds such as polypeptides, lipopeptides and antibiotics with potent antibacterial activities have been reported [8,9]. Among these compounds, the lipopeptides, which consist of a lipid connected to a peptide, are the most popular biosurfactants [10]. Some of the known classes of this group include surfactins [11–13], iturins [14,15] and fengycins [16].

Surfactin produced by *Bacillus subtilis* is recognized as one of the strongest biosurfactants available [10]. Its chemical composition includes a cyclic lipopeptide consisting of seven amino acids and a 12-19-carbon hydrophobic fatty acid chain [17]. Reports have shown that surfactin can reduce surface tension to 27 mN/m even when the concentration is as low as 0.005% [10,13,18,19], suggesting great potential in commercial applications. However, the high cost and low yield involved in surfactin's production has limited its commercial use thus far, prompting further research. Yeh et al. proposed that limiting carbon source (glucose) concentration affected surfactin production by *B. subtilis* [20]. Davis et al. found that the highest surfactin production was achieved when using ammonium nitrate as the nitrogen source for *B. subtilis* cultivation in a defined medium [21]. Sen et al. reported that the mineral salts ratio, Mn/Fe, in the medium was a critical factor in the enhanced production of surfactin [22]. Wei and Chu not only found that

* Corresponding authors.
*E-mail addresses:* tyjuang@mail.cmu.edu.tw (T.-Y. Juang), ycliu@dragon.nchu.edu.tw (Y.-C. Liu).
[1] Both authors equally contributed to this work.

http://dx.doi.org/10.1016/j.jtice.2017.06.017
1876-1070/

surfactin production increased from 0.33 g/L to 2.6 g/L when 0.01 mM $Mn^{2+}$ was added to a defined glucose medium, but the final yield exceeded that of the genetically-improved strains [23]. Wei et al. further found that when using an iron-enriched (4 mM $Fe^{2+}$) minimal salt medium, 3000 mg/L of surfactin was produced [24]. Moreover, some of the studies mentioned that the addition of a small quantity of solid additives, such as activated carbon or expanded clay, could significantly increase surfactin production. In fact, the addition of activated carbon increased the yield to 3600 mg/L [20], suggesting that solid additives may strongly stimulate surfactin production.

Layered double hydroxides (LDH), the so-called anionic clays, consist of cationic brucite-like layers and exchangeable interlayer anions [25]. Mg–Al LDH is a family of anionic clays. Due to the presence of positive ion charges on the surface layer, they can be intercalated with various molecules [26–30]. Recently, the use of globular macromolecules as intercalating agents to widen the layered gallery was revealed [31,32]. Choy et al. demonstrated that inorganic supramolecules, such as nanoscale Mg–Al LDH, can act as biomolecule reservoirs as well as gene and drugs carriers [26]. However, none of the studies focused on the use of this kind of layered material as an additive in microbial cultivation. In our study, the effect of (hereafter referred to as LDH) on the biomass and surfactin production of the *B. subtilis* CWS1 culture was examined. A comparison of LDH with others additives, including montmorillonite (MMT) and activated carbon (AC), was performed and the conditions for the highest surfactin production were also determined. The possible mechanism for surfactin enhancement was revealed and discussed.

## 2. Materials and methods

### 2.1. Chemicals and reagents

All of the solvents and chemicals used were of analytical grade. The compounds $Al(NO_3)_3{\cdot}9H_2O$ and $Mg(NO_3)_2{\cdot}6H_2O$ were obtained from SHOWA, USA. MMT was purchased from Alfa Aesar, USA. AC was purchased from China Activated Carbon Co. Ltd. (Taipei, Taiwan). Surfactin (≧98%), used as the standard, was purchased from Sigma-Aldrich, USA.

### 2.2. Microorganisms and culture conditions

The strain *Bacillus subtilis* CWS1 was a gift from Prof. Wei Yu-Hong in Yuan Ze University. It was maintained on a nutrient-agar plate at 30 °C. The seed media consisted of the following components: 1% glucose, 0.5% yeast extract, 1% peptone and 1% NaCl. Cultivation was conducted in 500 ml Erlenmeyer flasks containing 100 ml of seed media and inoculated with two loops of bacteria on a rotary shaker. The cultivation conditions were set at 200 rpm and 30 °C for 12 h. Various additives (2 g/L), including LDH, MMT and AC were then added to the culture medium The cultivation conditions were set at 200 rpm and 30 °C for 5 days.

### 2.3. Flask cultivation

Shake-flask culture was performed in a 500 ml Erlenmeyer flask containing 100 ml of the main medium. The main medium consisted of the following components: 10 g/L sucrose, 5 g/L $(NH4)_2SO_4$, 5.67 g/L $Na_2HPO_4$, 4.08 g/L $KH_2PO_4$, 0.2 g/L $MgSO_4{\cdot}7H_2O$ and 0.57 g/L $FeSO_4{\cdot}7H_2O$. 90 mL of medium in a 500 mL shake flask was inoculated with 10 mL inoculum. Media were sterilized at 121 °C for 20 min and the carbon source was autoclaved separately. The flasks were incubated on a rotary shaker at 200 rpm and 30 °C for 3–5days.

### 2.4. Preparation of $Mg_2Al$–$NO_3$–LDH

$Mg_2Al$–$NO_3$–LDH was prepared by co-precipitation, according to previously reported procedures [30]. In brief, a mixture of $Mg(NO_3)_2{\cdot}6H_2O$ (120 g, 0.48 mol) and $Al(NO_3)_3{\cdot}9H_2O$ (90 g, 0.24 mol) was dissolved in 1 L of deionized water (Mg/Al molar ratio of 2.0). The aqueous solution was vigorously stirred at 60 °C under nitrogen purge in order to minimize contamination with atmospheric $CO_2$. The pH was maintained at $10 \pm 0.2$ by portion-wise addition of 4 N NaOH. The resulting suspension was stirred at 60 °C for 16 h. The white precipitates were isolated by filtration and washed thoroughly with deionized water. The washed white precipitates were then lyophilized by freeze dryer. The dried LDH was submitted for X-ray diffraction analysis (PANalytical, X'Pert PRO MRD, Netherlands) and attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectra measurement in the wave number 400–4000 $cm^{-1}$ (Thermo Scientific, Nicolet iS50 FTIR, Madison, WI, USA).

### 2.5 Disintegration of LDH containing surfactin in pellet

To disintegrate LDH to release surfactin for analysis, 500 µL of 6N hydrochloric acid was added to the broth pellet. The mixture was stirred vigorously until the LDH was completely dissolved. The mixture was then placed in a refrigerator at 4 °C for 24 h, followed by centrifugation at 12,000 g for 5 min. The precipitate was collected and redissolved by adding 20 µL of ammonia solution (28%, Showa, Japan) and 1 mL of DI water. The supernatant was collected and sent for analysis of surfactin content.

### 2.6. Assays

The surfactin concentration was measured according to a modified HPLC method [33]. In brief, 1 mL of the surfactin sample was mixed with 0.5 mL of acetonitrile and 0.8 g of ammonium sulfate. The mixture was vigorously mixed, followed by centrifugation at 3200 g for 3 min; 20 µl of the filtered supernatant was used as the injection sample. The HPLC system (JASCO, Japan) was equipped with a reverse phase C-18A column (5 mm, 18 mm × 100 mm BDS-Hypersil, Thermo Fisher Scientific Inc., USA). The column temperature was set at 30 °C. The mobile phase was composed of a mixture of trifluoroacetic acid, acetonitrile and deionized water (0.1:400:100) with the flow rate set at 1.0 mL/min. A UV–vis detector (JASCO, Japan) with a wavelength of 220 nm was used. A standard curve was built with the freshly-prepared surfactin (Sigma) solution.

### 2.7. Statistical assay

Multiple flasks were run concurrently and three flasks were used each time for daily sampling. Each data point was expressed as a mean with a standard deviation. The Tukey test was used to compare the results ($P \leqq 0.05$).

## 3. Results and discussion

### 3.1. Preparation of $Mg_2Al$–$NO_3$–LDH

It was reported that cell growth and metabolites production by microorganisms could be affected by the supplement of different additives, such as metal ions and salts [23,34]. Some studies mentioned that the addition of a small quantity of solid carriers (activated carbon or expanded clay) could significantly increase

Please cite this article as: S.-C. Kan et al., Enhanced surfactin production via the addition of layered double hydroxides, Journal of the Taiwan Institute of Chemical Engineers (2017), http://dx.doi.org/10.1016/j.jtice.2017.06.017