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ORIGINAL ARTICLE

Control of agitation and aeration rates in the production of surfactin in foam overflowing fed-batch culture with industrial fermentation

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KEYWORDS
Surfactin;
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Abstract *Bacillus amyloliquefaciens* fmb50 produces a high yield of surfactin, a lipopeptide-type biosurfactant that has been widely studied and has potential applications in many fields. A foam overflowing culture has been successfully used in the combined production-enrichment fermentation of surfactin. In this study, the agitation and aeration rates were found to have relationships with foam formation and surfactin enrichment. A maximum surfactin concentration of 4.7 g/l of foam was obtained after 21 h of culture with an agitation rate of 150 rpm and an aeration rate of 1 vvm in fed-batch culture. By controlling the foam overflow rate (f_{out}) of a fed-batch culture, surfactin concentration in the foam was continuously maintained above 4 g/l.

PALABRAS CLAVE
Surfactina;
Tasa de agitación;
Tasa de aireación;
Espuma desbordante;
Cultivo *fed-batch*;
Fermentación industrial

Control de las tasas de aireación y agitación en la producción de surfactina en un cultivo alimentado *(fed-batch)* en espuma desbordante con fermentación industrial

Resumen *Bacillus amyloliquefaciens* fmb50 produce gran cantidad de surfactina, un biosurfactante de tipo lipopeptídico que ha sido objeto de estudios pormenorizados y tiene aplicaciones en muchos campos. El cultivo en espuma desbordante se ha utilizado con éxito en la fermentación combinada de producción-enriquecimiento de surfactina. En este estudio, se halló que las tasas de aireación y agitación tienen relación con la formación de espuma y el enriquecimiento de la surfactina. Se obtuvo una concentración máxima de surfactina de 4,7 g/l de espuma después de 21 h de cultivo con una tasa de agitación de 150 rpm y una tasa de

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aireación de 1 vvm en un cultivo alimentado *(fed-batch)*. Al controlar la tasa de espuma desbordante (f_{out}) de un cultivo *fed-batch*, la concentración de surfactina en la espuma se mantuvo continua por encima de 4 g/l.

Introduction

Biosurfactants are amphiphilic molecules widely produced by a variety of microorganisms, which have been considered as an alternative to chemical surfactants. Lipopeptides are one of the major types of biosurfactants[14]. As the most effective biosurfactant that has been found so far, surfactin can lower the surface tension of water from 72 to 27 mN/m[5,12]. Surfactin exhibits antibacterial and antiviral properties and biodegradability, and appears to be promising for applications in areas such as bioremediation and oil recovery[1,2,15]. However, after almost 50 years, surfactin is not yet a viable alternative to chemical surfactants because of the low yield in bioreactors[13], its relatively high medium cost, and severe foaming in aerated and stirred bioreactors[16]. Therefore, the production of surfactin is still limited to laboratory scale[5]. To cope with these problems, renewable substrates and foam overflow fermentation were used in recent years[4,9].

Some studies on fed-batch culture of surfactin have been undertaken in the past few years[4,5,16]. In foam overflowing fed-batch culture (FOFC), the flow rates of the feed and of the overflow foam were equal, and thus the broth volume in the bioreactor was kept constant, and continuous enrichment of surfactin in foam overflow was accomplished[6]. It has been shown that by controlling the agitation and aeration rates, maximum surfactin productivity could be achieved when the oxygen volumetric mass transfer coefficient (k_La) value was 0.0132/s[16]. However, the effect of agitation and aeration rates on surfactin enrichment in the foam has not been reported.

In our previous studies, we identified a strain of *Bacillus amyloliquefaciens* as producer of five surfactin homologues by using high performance liquid chromatography (HPLC) and electrospray ionization mass spectrometry methods[13]. A high yield of surfactin from strain *B. amyloliquefaciens* fmb50 was obtained by genome shuffling, and a method for surfactin determination was established[17].

In this work, a semi-defined medium, which is of low cost and high yield compared with the commonly used Landy medium, was initially combined with foam overflow batch culture. The effect of agitation and aeration rates on the foam overflowing rate (f_{out}) and surfactin enrichment in the batch culture process were studied. By using fed-batch culture, a continuous, high concentration enrichment of surfactin could be achieved. This novel fermentation technology has a potential for the industrial application of surfactin production.

Materials and methods

Microorganism and culture media

B. amyloliquefaciens fmb50 (CGMCC No. 6249), the surfactin producer used in this study, is registered by the China Committee for Culture Collection of Microorganisms.

The seed medium (BPY) consisted of: beef extract 5.0 g/l, peptone 10.0 g/l, yeast extract 5.0 g/l, glucose 10.0 g/l and NaCl 5.0 g/l (pH 7.0).

The semi-defined medium (IBM) used for fermentation was optimized by the Taguchi method in our previous work, and consisted of: corn powder 35 g/l, ammonium nitrate 15 g/l, urea 6 g/l, KCl 1.47 g/l, NaH_2PO_4 20 mmol/l, $MnSO_4$ 0.5 mmol/l, $MgSO_4$ 0.1 mmol/l, $CuSO_4$ 12.8 μmol/l, $FeSO_4$ 1 μmol/l and $CaCl_2$ 0.5 μmol/l (pH 7.0).

Flask and bioreactor culture conditions

In the primary inoculum, a loop of colonies from a fresh potato dextrose agar-slant was transferred into 50 ml BPY medium in shaken flasks (250 ml) and cultured at 37 °C and 180 rpm for 12 h. For the secondary inoculation, 10 ml of the primary culture was inoculated into 200 ml BPY medium in shaken flasks[11], and cultured in the same conditions as those in the primary inoculum.

Bioreactor cultivation was performed in a 19 l bioreactor containing 12 l of medium (L1523, Bioengineering AG, Switzerland); the temperature was controlled at 32 °C and the pH was maintained at 7.0 with the automatic addition of 4.0 mol/l NaOH. Three control strategies were adopted in batch cultivation to investigate the effects of agitation and aeration rates on f_{out} and surfactin enrichment in foam from overflowing cultures. The agitation and aeration rates were controlled as shown in Table 1. In control 1, the agitation and aeration rates were controlled at 200 rpm and at 0.66 vvm respectively. In control 2, the agitation and aeration rates were controlled at a relatively high level; the agitation rate was 300 rpm and the aeration was increased from 1.2 to 2.66 vvm as the foam overflowed and the medium volume in the bioreactor was reduced.

Analytical methods

The colony forming units (CFU) were calculated using plate colony-counting methods. Dry cell weight (DCW) was obtained by collecting the fermentation broth with different

Table 1 Control strategy of agitation and aeration rates

Control	Agitation rate (rpm)	Aeration (vvm)
1	200	0.66
2	300	1.2–2.66
3	150–250	1.8

incubation times in the IBM medium. After centrifugation at 1000 × *g* for 15 min, the collected pellets were dried for 8 h at 90 °C and the dry cells weighed[8]. Then, the dry cell weight (DCW) was determined using a pre-determined standard curve relating the number of colony forming units (CFU) to the dry weight: DCW = 2.43 × log CFU/ml − 18.63, with an R^2 value of 0.92. Results were represented as CFU per milliliter.

Culture samples were precipitated and then extracted with methanol as described by Cooper *et al.*[3] in the extraction of surfactin. Surfactin concentration in crude samples was determined by reverse phase HPLC (U-3000, Dionex, United States) equipped with an Agilent C18 column (4.5 mm × 250 mm, Agilent, United States) and a UV detector. About 20 μl of the surfactin sample was injected into the column and then eluted with acetonitrile with 0.1 % TFA at a flow rate of 0.84 ml/min. Eluent absorbance was monitored at 210 nm[17]. Quantization was performed based on a standard curve using a standard sample of surfactin (Sigma–Aldrich Co., United Sates). The standard curve equation is: $y = 17.771x - 38.742$ ($R^2 = 0.999$). The equation for the calculation of the specific growth rate (μ/h) was: $\mu = dX/(X \cdot dt)$; the equation for the calculation of the specific growth rate ($qp/g/(g^*h)$) was: $qp = dP/(X \cdot dt)$, *X*: cell yield (g/l); *P*: product yield (U/ml); *t*: fermentation time (h).

Results and discussion

Effect of the control strategy on cell specific growth rate

The effect of agitation and aeration rates on cell growth in the three control strategies adopted in this study in batch culture is shown in Figure 1. In controls 1 and 2, *B. amyloliquefaciens* fmb50 in the bioreactor reached up to 10^9 CFU/ml after 15 ± 0.2 h culture, and then the cell growth went into stationary phase (Fig. 1A). However, in control 3, cell growth declined from 12 h of cultivation. That may be due to the agitation rate changing between 150 and 250 rpm; normal cell growth seemed to be inhibited and the biomass decreased due to the simultaneous increase of the overflow. *B. amyloliquefaciens* fmb50 reached its maximum specific cell growth rate at 6 h of cultivation in control 1, which was 4 h earlier than in control 2 (Fig. 1B). In control 3, where cell growth was disturbed, a lower specific cell growth rate was observed. The maximum specific cell growth rate was lowered nearly by half with respect to controls 1 and 2. Although the increase in the agitation rate promoted quadratic cell growth, this one was not delayed.

Surfactin production is essentially associated with cell growth[7,16], although high biomass does not necessarily mean high surfactin production. It seems that a lower specific growth rate is more conducive to the production of biosurfactants such as mycosubtilin[4]. The effect of agitation and aeration rates on cell growth and cell specific growth rate was studied in this paper and the best control strategy was chosen to enhance surfactin production.

Effect of the control strategy on f_{out} and the concentration and production of enriched surfactin in foam

Foam began to overflow outside the bioreactor after 6 h of cultivation (Fig. 2A). The maximum f_{out} in control 1 was 0.3 l/h while in control 2 it was 1.3 l/h, which indicated that the foam overflow was significantly affected by the agitation and aeration rates used in different strategies. The surfactin concentration in the overflow foam also changed with time. As shown in Figure 2B, different trends were observed in the three control strategies. In control 3, surfactin concentration increased linearly and showed the highest specific production rate, reaching a maximum of 0.426 l/h (Fig. 2C).

The linear increase of surfactin concentration and the relatively high specific production rate in control 3 (Fig. 2B and C) indicated that high surfactin enrichment in foam

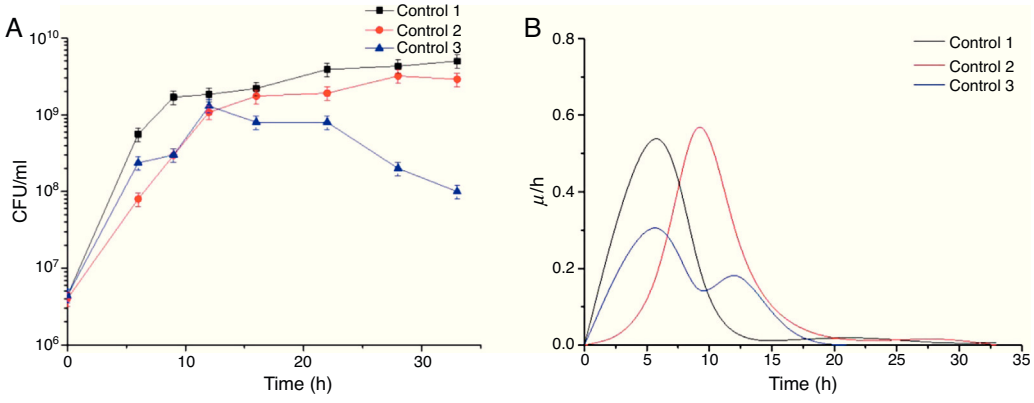

Figure 1 Time courses of CFU (A) and cell specific growth rate (B) by *Bacillus amyloliquefaciens* fmb50 in batch culture with three control strategies. In control 1, the agitation rate was 200 rpm, and the aeration rate was 0.66 vvm; in control 2, the agitation rate was 300 rpm, and the aeration rate increased from 1.2 to 2.66 vvm; in control 3, the agitation rate changed between 150 and 250 rpm to keep the overflowing flow rate at a relatively moderate level, and the aeration rate was kept at 1.8 vvm.

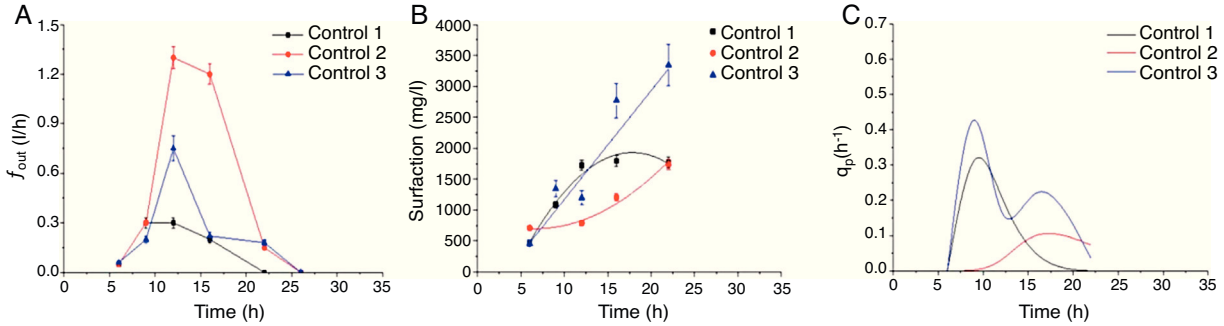

Figure 2 Time courses of foam overflowing flow rate (A), surfactin concentration in the foam (B) and specific production rate (C) in batch culture of *Bacillus amyloliquefaciens* fmb50 with three control strategies. In control 1, the agitation rate was 200 rpm, and the aeration rate was 0.66 vvm; in control 2, the agitation rate was 300 rpm, and the aeration rate increased from 1.2 to 2.66 vvm; in control 3, the agitation rate was changed between 150 and 250 rpm to keep f_{out} at a relatively moderate level, and the aeration rate was kept at 1.8 vvm.

may be achieved by a combined control of the agitation and aeration rates at a reasonable level. As shown in Figure 2B, the maximum surfactin concentration in foam reached 3.342 g/ml and the maximum surfactin production in foam reached 2.525 g (approximately overflowing 7.6 l foam) in control 3. However, maximum surfactin concentration and maximum surfactin production were 1.8 g/ml and 8.46 g (approximately overflowing 4.7 l of foam) in control 1, respectively. These results in control 3, which show the linear increase of surfactin concentration and the relatively high specific production rate, are similar to those in the study by Davis *et al.*[6], the enrichment of surfactin in the foam was enhanced to 0.44 g/l with the time going by supplementing culture broth in the stationary phase[6]. Foam stopped overflowing at 22 h in control 1 and at 26 h in controls 2 and 3. This was partly due to the medium level in the bioreactor dropping as overflowing foam: however; the surfactin in the foam was thus separated from the broth almost completely[6]. Apparently, continuous surfactin production in the bioreactor ensured an increasing trend of surfactin concentration in the foam. With the agitation and aeration rate controlled under different strategies, the surfactin production presented a different kinetic trend. Control 3, under which f_{out} was below 0.7 l/h, indicated that surfactin enrichment may be improved through stir speed and aeration control.

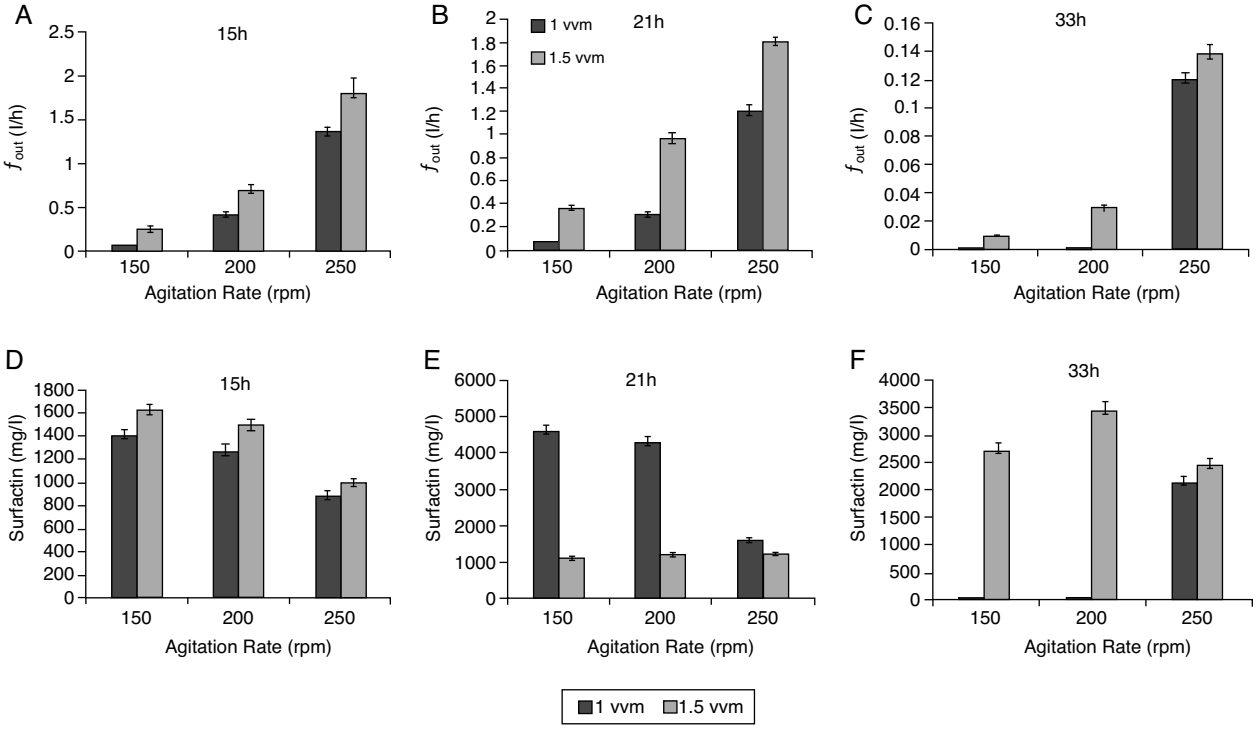

Figure 3 Effect of agitation and aeration rate on foam overflowing flow rate (up) and surfactin concentration in the overflowing foam (down) at 15 h (A and D), 21 h (B and E), and 33 h (C and F) by *Bacillus amyloliquefaciens* fmb50 in batch culture.

Influence of agitation and aeration rates on f_{out} and concentration of enriched surfactin

The influence of agitation and aeration rates on f_{out} and surfactin enrichment in the overflowing foam were investigated in batch culture experiments conducted using a series of combinations of aeration and agitation rates. Foam overflow began after 6 h of cultivation (Fig. 2A). As indicated in Fig. 3(A–C), f_{out} tended to increase with an increased aeration rate from 1 to 1.5 vvm and agitation rate from 150 to 250 rpm. At 33 h, f_{out} was extremely low, only a maximum 0.14 l per hour (Fig. 3C); however, raising the aeration rate to 250 rpm, the f_{out} could increase to a maximum 1.76 per hour.

Agitation and aeration rates significantly affected the concentration of surfactin in the foam. In the early stage of fermentation (i.e. at 15 h; Fig. 3A), an increase in agitation rate increased f_{out}; however, the surfactin concentration was at a relatively low level and decreased from 1.6 g/l to 1 g/l with the agitation increase. At 21 h, the maximum surfactin concentration of 4.7 g/l occurred at 150 rpm agitation and 1 vvm aeration. With the increase of agitation to 250 rpm, the surfactin concentration was reduced to 1.5 g/l. Interestingly, the change in the agitation rate had no effect on the surfactin concentration at 1.5 vvm aeration (Fig. 3E). At 33 h, the surfactin concentration in the foam was maintained at >2.5 g/l with an aeration rate of 1.5 vvm and it did not drop with an increasing agitation rate. However, under the condition of aeration rate at 1 vvm, the surfactin concentration in the foam was 2.5 g/l only with the agitation rate of 250 rpm. Apparently, the agitation and aeration rates significantly affected the concentration of surfactin enriched in the foam.

The agitation and aeration rates in the aerobic fermentation greatly affected the foam formation rate and the surfactin concentration in the foam. Similar to our case, a previous study observed that a higher stirring speed or aeration rate led to a higher f_{out}[6]. However, surfactin enrichment in this study presented a different kinetic relationship with f_{out} compared to that in the basic study by Davis *et al.*[6], in which the foam continuously overflowed under a constant agitation rate. In the late stages of batch FOFC, such as at 33 h (Fig. 3F), the surfactin concentration in the foam was lower than at an earlier stage (21 h), because > 50% of the culture broth in the bioreactor was gone. Thus, the broth level in the bioreactor was too low to maintain the overflowing foam and further foam formation was also limited because almost all the surfactin had already been drained away in the foam. Both the surfactin concentration in the foam and f_{out} were determined by the surfactin produced in the broth. Furthermore, the f_{out} level affected the surfactin enrichment, and at the same time, f_{out} was influenced by the agitation and aeration rates.

Fed-batch culture with controlled agitation and aeration rates

A fed-batch culture process was also adopted in this study. Figure 4 shows that foam began to overflow after 6 h of cultivation. Afterwards, f_{out} gradually dropped from 0.6 l/h to 0.2 l/h by real-time control of the agitation and aeration rates and was maintained at this rate. From 15 h, the IBM medium was fed into the bioreactor at a 0.2 l/h feed rate (matching f_{out}). In this culture, the biomass was kept above 10^9 CFU/ml. Foam continued to overflow until the cultivation terminated and surfactin was enriched in the foam with a concentration >4 g/l; the maximum concentration reached 4.7 g/l. At the end of the fermentation, the surfactin concentration in the culture broth in the bioreactor was very low (data not shown), which means that almost all the surfactin was transferred into the foam.

In other studies of foam overflowing cultures, although the product was enriched and separated in foam, the foam overflowed spontaneously, and thus the surfactin concentration in it fluctuated[6,10,16]. For this reason, surfactin in the foam was not enriched to a very high level; in previous studies, in which surfactin was produced by potato process effluent, it was 1.67 g/l and 0.9 g/l[6,10]. Thus, controlled foam overflow in this work achieved almost 3 and 4 times the surfactin concentration compared with the previous studies, respectively[6,10]. Furthermore, the medium feeding in our

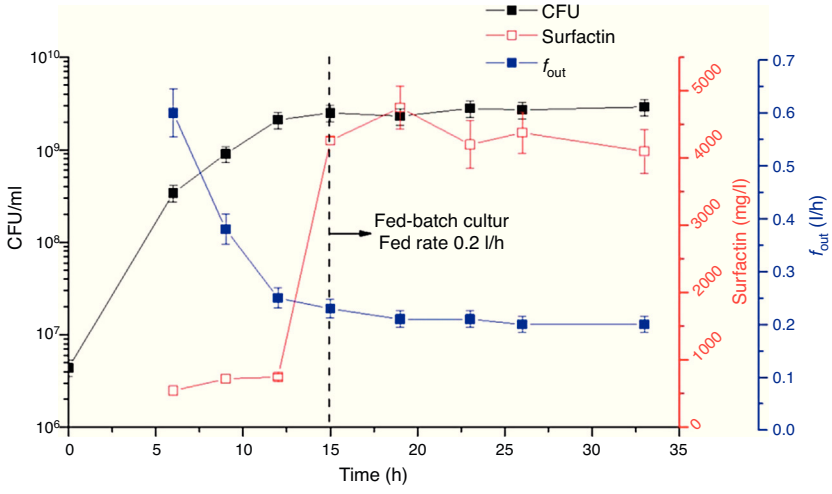

Figure 4 Time course profiles of CFU, surfactin concentration in foam, and foam overflowing flow rate (f_{out}) during fed-batch fermentation of *Bacillus amyloliquefaciens* fmb50 under controlled agitation and aeration rate to keep f_{out} constant.

study overcame disruption of cell growth, which ensured that surfactin was continuously produced. Combined with control of f_{out} at a low level, the surfactin concentration in the foam was maintained above 4 g/l, which will significantly reduce production costs and improve industrial production capacity.

Conclusions

Our study focused on the control of agitation and aeration rates in foam overflowing fermentation to improve surfactin enrichment and continuous high-level production. In batch mode, the agitation and aeration rates were found to have a close relationship with cell growth and surfactin production. In further research, our study revealed that f_{out} does not always negatively impact on the surfactin concentration in the foam. The broth level in the bioreactor and the surfactin residue in the broth also affect both f_{out} and the surfactin enrichment of the foam.

Through feeding the medium into the bioreactor, a fed-batch fermentation process was successfully established in which foam overflowed at a controlled flow rate of 0.2 l/h, and surfactin in the enriched foam was kept at a level above 4 g/l.

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Conflict of interest

The authors declare that they have no conflicts of interest.

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