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Optimization of Bench-Scale Production of Biosurfactant by *Bacillus licheniformis* R2

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Abstract

The fermentative production of biosurfactant by *Bacillus licheniformis* R2 strain in bench-scale bioreactor under different dissolved oxygen tensions was investigated. The statistically optimized minimal media was used for biosurfactant production, where glucose was used as a carbon source. The batch fermentations were carried out at different concentrations of DO - 30%, 50%, 70%, and initial 100% (with no further control thereafter) at 30°C and pH 6.5-7.2. In 30, 50 and 70% DO experiments, it was maintained during the course of the fermentation, using cascading mode with agitation. Adjusting the initial dissolved oxygen to 100% saturation, without any further DO control and collection of foam and recycle of biomass gave higher biosurfactant production. *B. licheniformis* R2 produced biosurfactant, thus reducing the surface tension and interfacial tension to 28mN/m and 0.5mN/m respectively in less than 10 hours. The results are indicative of the potential of the strain for the development of scaled-up biosurfactant production.

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Keywords: Bacillus licheniformis; bench-scale bioreactor; dissolved oxygen concentration; surface tension; interfacial tension

1. Introduction

Biosurfactants are biologically produced surfactants, which have several advantages as compared to chemically produced surfactants: lower toxicity, better activity under harsh conditions, biodegradability, and production using cheaper renewable substrates to list a few. Thus biosurfactants showed potential for various

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environmental applications such as bioremediation of heavy-metal contamination, oil-spills, in enhancing oil recovery (EOR) amongst many others [1, 2]. For these environmental applications biosurfactants are required in huge quantities, thus scale-up processes are required to be optimized. Depending upon the nature of the biosurfactant and the producing organisms, several patterns of biosurfactant production are possible, and are generally affected by nutrients and environmental and physical parameters. Supply of sufficient dissolved oxygen and mechanical agitation has been shown to improve lipopeptide biosurfactant production [3-5]. However, the vigorous agitation and aeration leads to severe foaming and causing unstable and inefficient fermentor operation as well as the requirement of antifoam addition [3, 4]. Therefore, the aeration and agitation strategies need to be optimized not only to meet the requirement of sufficient oxygen and mass transfer, but also to minimize the side effects of intensive foaming. Meanwhile, the bioreactor should also be tailored to cope with the foaming problems, avoiding massive addition of costly and probably cell growth-inhibiting antifoam agents [6]. We describe the modification of design of batch bioreactor used as a conventional fermentor by integrating it with a foam collector from its gaseous outlet, and also connecting it to a cell recycler. The biosurfactant fermentation was carried out under different combinations of aeration and agitations rates, to identify the optimal aeration conditions for biosurfactant production by *B. licheniformis* R2 and to develop feasible, cost effective, and commercially viable fermentation technology for biosurfactant production.

2. Experimental procedures

B. licheniformis R2 (NCBI GenBank accession no: DQ922950) was isolated from oil contaminated desert site. Statistically optimized minimal production media was used for biosurfactant production by *B. licheniformis* R2 [7]. Composition of media was (g/l): NH_4NO_3, 1.0; Glucose, 34.0; KH_2PO_4, 6.0; Na_2HPO_4, 2.7; $MgSO_4.7H_2O$, 0.1; $CaCl_2$, 1.2 X 10^{-3}; $FeSO_4.7H_2O$, 1.65 X 10^{-3}; $MnSO_4.4H_2O$, 1.5 X 10^{-3} and Na-EDTA, 2.2 X 10^{-3}.

For shake flask studies, 2% (v/v) seed culture (overnight grown culture in LB - OD_{660} - 0.8-0.9) was used as an inoculum into 50 ml of production medium in a 250 ml Erlenmeyer flask. The flasks were incubated on an incubator shaker (160 rpm) at 30 °C for 72 h, and intermittent samples were withdrawn to check growth as dry cell weight (DCW), surface tension (ST) and interfacial tension (IFT).

The bioreactor experiments were carried out in a New Brunswick Scientific BIOFLO 110 bench top fermentor (New Jersey, USA) with maximum working volume of 5 l, with an agitation rate of 300 rpm (constant in 100% DO experiment; otherwise as cascading mode with DO in the range of 150-650 rpm) and an aeration rate of 1.0 vvm. An overnight grown culture in LB (OD_{600} - 0.8-0.9) was added to the fermentor to a final concentration of 2% (v/v) [3]. The inoculum was pumped into the fermentor using peristaltic pump. Dissolved oxygen (DO) concentration was controlled by cascading with agitation in 30%, 50% and 70% DO experiments, whereas in 100% DO experiment initially medium was saturated with 100% DO and then it was not controlled throughout the batch. The experiments were conducted at 30°C, and pH was maintained at 6.5-7.2 by addition of 0.5 N NaOH/5% (v/v) o-phosphoric acid for automatic control. The foam generated in the vessel was introduced into the foam collector. The liquid culture accumulated on the bottom of the foam collector was completely recycled into the fermentor with a peristaltic pump, while the overflowed foams were collected aseptically in another vessel and no antifoam agent was added during the course of fermentation. Samples were analyzed for 72 h at different time intervals for cell growth (DCW), pH, temperature, dissolved oxygen, ST and Critical Micelle Dilutions (CMD) were monitored as a function of time.

Growth was monitored by measuring the biomass obtained by drying the pelleted cells overnight at 105 °C after centrifugation at 11,292 X g for 20 min. ST was determined with a Du-Nouy's tensiometer (Khushboo Sci. Co., Mumbai, India). IFT measurements against crude oil (API 25) were performed in a spinning drop

tensiometer (Model 510, Temco Int., USA). The IFT can be calculated from following equation, for drops with length greater than 4 times the width:

$$\gamma = 3.42694 \text{ X } 10^{-7} (\rho_h - \rho_d) \omega^2 D^3$$

Where, γ = IFT, mN/m; ρ_h = Density of heavy (Outer phase), g/ml; ρ_d = Density of lighter phase (Drop), g/ml; ω = Rotational velocity (RPM), rpm; **D** = Measured drop width (Diameter), mm.

3. Results and Discussion

B. licheniformis R2 biosurfactant, showed ST <30 mN/m in 24-36 h without any change till 72 h and reduced IFT to 0.53 and 0.63 (of 1:40 diluted broth) at shake flask level studies. ST value of the uninoculated media was 70-72 mN/m, and IFT value of crude oil against formation water was 12.5mN/m. The biosurfactant production was scaled up to 3 L in a fermentor and effects of different aeration conditions were optimized. The foam was found to be vigorously and continuously produced from the initial log phase of the growth (10-12 h) in 100% DO whereas, excessive foaming was observed at 26-27 h with 70% DO, at 34-36 h with 50% DO and at 40-43 h with 30% DO. Mechanical foam breaker was ineffective and it was impossible to use antifoaming agents to break the excess foam because it inhibited the biosurfactant production, and these agents showed surfactant activity. The foam that overflowed through the air-exhaust line was continuously collected and recycled to the fermentor (Fig 1). Figure 2 (A-D) illustrates the profiles of biosurfactant production (ST, CMD^{-1} and CMD^{-2}) and growth of *B. licheniformis* R2. Maximum growth (dry cell weight >5.0) and biosurfactant production in terms of ST (<30 mN/m) and CMD^{-2} was achieved in a shorter time with initial 100% DO saturation with no further DO control throughout the batch, followed by that with 70%, 50% and 30% DO. In all the experiments, reduction in ST observed was <30 mN/m and was maintained throughout the batch experiment. The biosurfactant production occurred mainly in the first 8-12 h as shown in figure 2 (D), the DO dropped to almost zero by 12 hours.

Fig. 1. Biosurfactant production by *B. licheniformis* R2, as batch fermentation in NBS BIOFLO 110 fermentor, showing excessive foam during the batch and collection and recycling of foam in the fermentor.

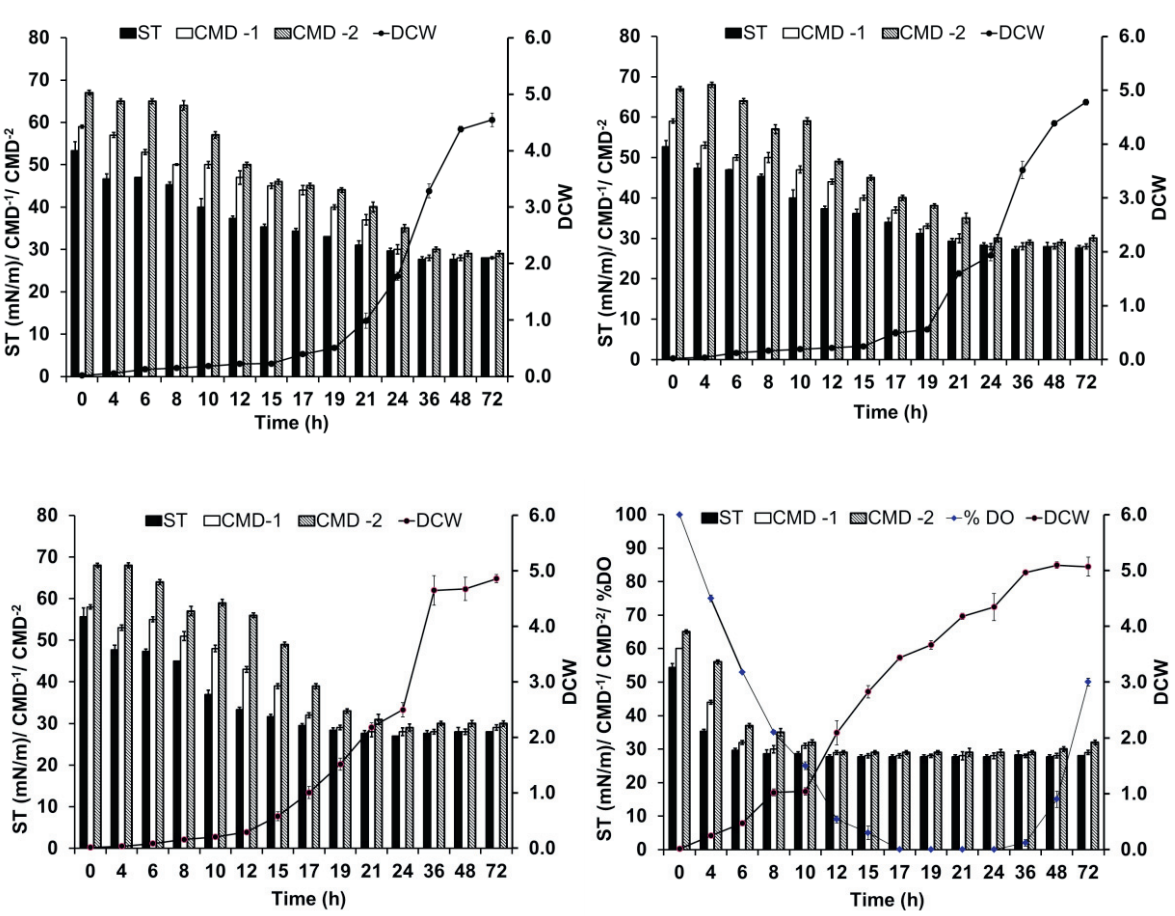

Fig. 2. Growth and surface activity (ST, CMD^{-1}, and CMD^{-2}) of *B. licheniformis* R2 under different % DO saturation in fermentor: **(A)** 30%; **(B)** 50% **(C)** 70% and **(D)** initial 100% DO saturation and no further DO control.

Studies were conducted to improve production efficiency and recovery bioprocesses in order to optimize yields. It is commonly observed that aerobic bacteria, like *B. subtilis,* need sufficient oxygen supply for growth and metabolite production. The high degree of foaming during fermentation suggested that the biosurfactant production may be affected by the extent of agitation and aeration. Both dissolved oxygen (DO) concentration in the medium and excessive oxygen supply has been reported to be unfavourable for growth and production of lipopeptide surfactants like lichenysin [8]. It was therefore required to maintain the DO in the medium to match the oxygen consumption rate of cells to avoid poor cell growth and hence inefficient biosurfactant production, because the high rate of aeration and agitation promoted cell growth and foam production in short time. Without addition of a large amount of antifoam agent, the rapid foam production resulted in overflow of the culture broth and a short fermentation time due to swift decrease in culture volume. This appeared to lead to a low level of biomass yield and biosurfactant production. The fermentor therefore was modified with foam collector and culture recycler. Hence, we have used a foam and cell recycler, wherein

the broth was recycled to the fermentor vessel under sterile conditions, during the course of fermentation. We observed that the best operating condition for biosurfactant production was by using initial DO saturation of the medium as 100% and no control thereafter (agitation rate = 300 rpm; aeration rate = 1.0 vvm; pH = 6.8-7.2 and temperature = 30 °C), yielded high biosurfactant concentration in less time, and no change in ST or CMD were observed up to 72 h. In this study, a bench-scale process in 5 L fermentor with 3 L working volume was optimized as using initial DO saturation of the medium as 100% and no control thereafter with maintaining agitation rate, yielded high biosurfactant concentration as 100 X CMD, in less time (10-12 h and no change in ST or CMD were observed up to 72 h), for biosurfactant production by *B. licheniformis* R2.

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