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Bioprocess strategies for mass multiplication of and metabolite synthesis by plant growth promoting pseudomonads for agronomical applications

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ABSTRACT

The exponential substrate feeding (open-loop) and automated feedback substrate feeding (closed loop) strategies were developed to obtain high cell densities of fluorescent pseudomonad strains R62 and R81 and enhanced production of antifungal compound 2,4-diacetylphloroglucinol (DAPG) from glycerol as a sole carbon source. The exponential feeding strategy resulted in increased glycerol accumulation during the fed-batch cultivation when the predetermined specific growth rate (μ) was set at 0.10 or 0.20 h^{-1} ($<\mu_m = 0.29\ h^{-1}$). Automated feeding strategies using dissolved oxygen (DO) or pH as feedback signals resulted in minimal to zero accumulation of glycerol for both the strains. In case of DO-based feeding strategy, biomass productivity of 0.24 g/(L h) and 0.29 g/(L h) was obtained for R62 and R81, respectively. Using pH-based feeding strategy, biomass productivity could be increased to a maximum of 0.51 and 0.54 g/(L h), for the strains R62 and R81, respectively, whereas the DAPG concentration was enhanced to 298 mg/L for R62 and 342 mg/L for R81 strains. These yields of DAPG are thus far the highest reported from GRAS organisms.

1. Introduction

Fluorescent pseudomonads are a group of plant growth promoting rhizobacteria (PGPR) which rigorously colonize roots and provide beneficial effects to plant development [1,2]. It has been well established that bioinoculant preparations using the culture broth of such pseudomonads offer excellent combination of disease control and plant growth promotion [1,2]. Most of the studies have been focused on development of such bioinoculant formulations and enumeration of growth characteristics when applied to plants in both controlled conditions and in the fields. Pseudomonads have been grown to high cell density using exponential, DO- and/or pH-based feeding strategies [3–10]. A pH-based feeding was coupled with fill-and-draw methodology to achieve stable repeated fed-batch technique for enhancing rhamnolipid production in *Pseudomonas aeruginosa* S2 strain [10]. Sun et al. [7] observed that pH- and DO-based feeding strategies were found to be superior over traditional exponential feeding during fed-batch cultivation of *Pseudomonas putida* KT2440 for achieving high cell densities. Suzuki et al. [9] made dual use of pH signal where feeding was done based on pH controller's output of upper limit of pH set point while pH control via base addition was done using lower limit of the pH set point. Feed pump control by this strategy may add feed pulses of variable magnitude into the bioreactor. In all these cases fed-batch cultivation based on DO- and/or pH-signals has been exploited to achieve high cell, protein or metabolite productivities. However, the studies pertaining to mass cultivation of pseudomonads for agronomical purposes are almost absent. The current study addresses this issue by developing bioprocess strategies required for mass multiplication of two such pseudomonad strains, and a strategy for the addition of constant magnitude feed pulse.

2,4-Diacetylphloroglucinol (DAPG) is a widely studied antibiotic produced by fluorescent pseudomonads in the suppression of diseases of the plants [2,11–13]. We have recently shown that the bioinoculant pseudomonads' formulations which contained DAPG improved the shelf life of such formulations [14]. It was observed that the presence of 10 mg DAPG per gram of formulation (which also contained 54.6 μg siderophore per gram formulation) suppressed the contamination proliferation when such formulations were under shelf storage at 28 °C [14]. This would mean that the culture broth should contain at least 50 mg DAPG/L when 20 mL of the broth is used for preparing 100 g bioinoculant formulation. The

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native *Pseudomonas fluorescens* usually produces less than 20 mg DAPG/L when grown on nutritional liquid medium [15]. In order to achieve this minimum value of 50 mg DAPG/L in the culture broth along with high cell density, fed-batch cultivations were realized. Although the broth can be concentrated to achieve the desired levels of DAPG, optimization of the process for enhanced DAPG production would significantly reduce the intermediate concentration steps. Moreover, the broth obtained from fed-batch cultivation can be diluted to achieve the desired levels of cell and DAPG concentrations so as to increase the throughput of the process.

Fed-batch cultivation process is considered as efficient method to achieve high cell density, which is often necessary for high yield and productivity of a desired product [3,16]. Fed-batch cultivation of pseudomonads has been widely studied and reported for non-agronomical applications [4–7]. As the objective in the present study is different from those studies, different feeding strategies have been enumerated in the process of achieving the desired goal. Initial fed-batch studies were done using a simple open-loop strategy such as exponential feeding of nutrients using predetermined rate and later the shortcomings were addressed using automated closed loop strategies. In the closed loop strategies, DO- and pH-based feeding schemes were used separately as feedback parameters.

2. Materials and methods

2.1. Bacterial strains

Fluorescent pseudomonad strains R62 and R81 used in the present investigation were obtained from Dr. A K Sharma of Department of Biological Sciences, GB Pant University of Agriculture and Technology (GBPUAT), Pantnagar, India. These strains belong to PGPR group and could be potentially used in bioinoculant formulations due to their plant growth promoting characteristics [17,18]. The strains were isolated from the rhizosphere of wheat (variety UP 2338) from Budaun District, Uttar Pradesh, India. The bacterial cultures were maintained as 50% glycerol stocks at–20 °C in King's B medium [19]. All chemicals (extra pure grade) used in this study were obtained from Merck (Mumbai, India).

2.2. Culture medium and pre-culture preparation

The fluorescent pseudomonads R62 and R81 were cultivated in GA-optimized Schlegel's medium [20]. The trace elements solution was prepared according to Aragno et al. [21]. Pre-cultures were grown overnight from −20 °C glycerol stocks on GA-optimized medium in baffle-less 500 mL Erlenmeyer flasks with 100 mL working volume. The cultivation for preculture was carried out for 30 h at 28 °C in an orbital shaker (Scigenics Biotech, India) at 240 rev/min.

2.3. Bioreactor operations

A 5 L bioreactor (Minifors AG, Switzerland), equipped with inbuilt controller for pH, DO and temperature was interfaced with a personal computer for data acquisition, and control of feed pump interactively based on acquired pH or DO signals using an in-house developed software. The base pump output of the built-in controller was directly used for pH control at the lower limit of set point, while output of upper limit of set point was not used for both batch and fed-batch cultivations. Batch cultivations were carried out at 2 L working volume, and fed-batch culture operations were implemented at the end of batch phase based on different feeding strategies at 28 °C. GA-optimized medium [20] was used as a reference medium in batch cultures for further modifications to enhance DAPG production. In batch phase of fed-batch cultivations, a modified GA-optimized medium (Table 1) developed in present work was used. The feed medium composition was varied according to feeding strategy (Table 1). In the fed-batch cultures, the transient phase between batch and fed-batch phases was carried out by manual addition of a few feed pulses. Thereafter, the feeding was switched over to automated mode based on the feeding strategies implemented. The set point for dissolved oxygen was 30% of the saturation value. It was controlled around the set point by using a double cascade control in which the stirrer speed was first increased from 550 rpm to a maximum of 700 rpm, followed by a gas mix-mode where the air (0.1 vvm constant) was enriched with variable pulses of pure oxygen automatically. Antifoam-A (Sigma–Aldrich) was added manually on the onset of foam formation. The measurement and control of the pH, temperature, dissolved oxygen, and agitation speed was performed by an in-built digital measuring and control system of the bioreactor. Internal PID control with set point values was used for DO control with respect to agitation and/or aeration rates. The pH was controlled at the set point of 7.0 ± 0.05 by automatic addition of 2 N KOH/2 N H_3PO_4 for DO-based fed-batch cultures. For pH-based fed-batch cultures, the pH was controlled at the set point of 6.9 ± 0.05; 4 N ammonia water was used instead of 2 N KOH.

Masterflex model 7518-02 (Cole-Parmer Instrument Company, USA) was used as the feed pump. The remote mode of the pump was turned "ON" during the fed-batch mode of cultivation. The pump operated between 2 and 10 V and received the signal from the PCI card 1716L (Advantech, Taiwan), which was fitted in the PCI slot of the personal computer (HP Compaq dx2000MT). Masterflex 06402-16 norprene tubing (Saint-Gobain, USA) was used for feed discharge. The pump was calibrated using the feed composition, and a 7-point standard plot between the feed-rate and the output voltage was established. Due to constraints in the existing in-built software, customized control software was developed using LabVIEW® 9 platform (National Instruments, Austin, TX, USA). The connection between the bioreactor and the personal computer was established through a RS-232 serial-port. The detailed information on data acquisition and control protocols is beyond the scope of this communication. During cultivation the samples were taken at frequent intervals to measure cell density, residual glycerol and DAPG.

2.3.1. Feeding strategies during fed-batch cultivation

2.3.1.1. Exponential feeding. During constant specific growth rate fed-batch cultivation, the exponential feeding was done according to the following formula [16]:

$$F(t) = \frac{((\mu/Y_G) + m_s)x_i V_i e^{\mu t}}{S_o} \quad (1)$$

where $F(t)$ is the feeding rate (L/h) at any time (t), μ is the specific growth rate (h^{-1}), t is the elapsed time (h) after the feeding started, S_0 is the substrate (glycerol) concentration in feed (g/L), x_i is the biomass concentration at the start of feeding

Table 1
The media composition fed-batch cultivation of pseudomonads R62 and R81.

Component	Composition of modified GA-optimized medium for batch cultivation[a]	Feed composition for fed-batch cultivation	
		Exponential and DO-based	pH-based
Glycerol (g/L)	15.00	800	800
Na_2HPO_4 (g/L)	4.56	0	0
KH_2PO_4 (g/L)	2.82	0	0
NH_4Cl (g/L)	1.75[b] 2.36[c]	74.23	[d]
KCl (g/L)	0.50	0	0
$MgSO_4 \cdot 7H_2O$ (g/L)	0.91	48.52	48.52
$ZnSO_4 \cdot 7H_2O$ (g/L)	0.058	0	0
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (g/L)	0.124	0	0
Ammonium ferric citrate (AFC)	800 μg/L	25.6 mg/L	25.6 mg/L
Trace elements solution (mL/L)	0.47	25	25

[a] Tryptophan in GA-optimized medium was excluded in the modified GA-optimized medium and zinc sulphate and ammonium molybdate were enhanced to the levels indicated here.
[b] The value represents the concentration of NH_4Cl taken in batch medium for exponential and DO-based fed-batch cultivation.
[c] The value represents the concentration of NH_4Cl taken in batch medium for pH-based fed-batch cultivation.
[d] 4 N ammonia solution was used as a nitrogen source as well as to maintain pH in pH-based fed-batch cultivation.