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# $DCO_2$ on-line measurement used in rapamycin fed-batch fermentation process

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**Abstract**

A Streptomyces fed-batch fermentation was studied with on-line measurement of dissolved carbon dioxide ($DCO_2$). A selected cell strain R060107 of *Streptomyces hygroscopicus* was used in the production of rapamycin. pH was controlled only in the production phase at the low limit of 5.1 after its value dropped from an initial 6.5 to 5.1. $DCO_2$ was used to control the feed in the fed-batch fermentation of *S. hygroscopicus* R060107. The experiment investigated the relationship between rapamycin production and the evolution of $DCO_2$. A high limit of $DCO_2$ ($hDCO_2$) was used to control medium feed. In order to meet the needs for the dynamic growth and metabolism of cells in the process, the $hDCO_2$ was defined as a dynamic parameter, and was therefore calculated constantly in the process. A fermentation condition of limited nutrient supply was realized. It was observed that rapamycin was secreted when the glycerol concentration was well controlled in the fermentation condition. Experimental results showed that 500 mg/l of rapamycin was produced in 120 h of fermentation.


*Keywords:* $DCO_2$; Streptomyces; Antibiotic; Rapamycin; Fed-batch; Fermentation

## 1. Introduction

Rapamycin is produced from *Streptomyces hygroscopicus*. It was first reported as an antifungal agent in 1975 by Vezina et al. [1]. Although rapamycin was originally isolated as an antifungal agent, it was marketed as an immune suppressing drug to prevent organ rejection in transplant surgery. Researchers have found that rapamycin plays the role of a molecular marriage broker, linking two proteins that normally ignore each other into a complex called a heterodimer [2]. Scientists have utilized this property to develop new medicines. Rapamycin, also known as sirolimus, is a component of commercially available drug-eluting stents implanted into coronary arteries [3]. More recently it has been suggested that rapamycin may even assist in the treatment of heart attack [4]. *S. hygroscopicus* fermentation is currently used for the commercial production of rapamycin as a secondary metabolite of the cells.

Many macrolide antibiotics are produced by strains of Streptomycetes [5]. Those products are synthesized as secondary metabolites. Antibiotics are usually associated with the mycelium culture and isolation is carried out by extraction with organic solvents [5–7]. The switch from primary to secondary metabolism not only constitutes a complex biochemical differentiation, but also coincides with a process of morphological differentiation [7]. Formation of secondary metabolites is usually favored by suboptimal growth conditions. It is often suppressed by inorganic phosphate and by carbon and nitrogen sources. In the conventional batch process, this production phase is quite short. To maintain high synthesis activity for nutrient-limited microorganisms, the fed-batch mode has been introduced in an increasing number of those fermentation processes [8]. The major purpose of fed-batch operation is to control the nutrient concentration of the culture medium. To deal with the challenge of process optimization, researchers have focused on different control strategies. Several parameters have been used as an indirect feedback of process control, including dissolved oxygen (DO), respiratory quotient (RQ), pH, metabolite concentration, $CO_2$ in exhaust off-gas and

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in dissolved condition ($DCO_2$), carbon source concentration, and turbidity measurements [9–12]. Fermentation of Streptomyces challenges existing bioreactor design and process control due to its shear-sensitive filamentous morphology and, as in most antibiotic production, its high DO demand. Optimization of process control in a commercial bioreactor is always an interesting research project. McIntyre and McNeil reviewed gross effects of $CO_2$ on product and biomass concentrations in submerged fermentation processes especially in filamentous cultures [10]. Studies showed that dissolved carbon dioxide ($DCO_2$) could influence the morphology of filamentous microorganisms, and directly impacted product synthesis. Tulin et al. and other researches reported that different models including a pH-stat model were effectively used in the fed-batch process for the production of an enzyme product and rapamycin [11,12]. Fed-batch processes with indirect or direct feedback control utilize an observable parameter that is closely connected with the features of the microbial reaction.

On-line monitoring is important not only for developing control strategies but also for providing a greater insight into the dynamics of the bio-reactions that regulate production. The effects of carbon dioxide as well as oxygen on cellular metabolism are very important in aerobic fermentation. Carbon dioxide is produced in great quantities during the course of aerobic fermentations. Previous research has shown that dissolved carbon dioxide can both stimulate and inhibit the growth of microorganisms [11,13,14]. Low levels of $DCO_2$ (below 5%) are reported to stimulate cell growth, and its high levels have inhibitory effects. Shang et al. investigated the effect of $CO_2$ on the production of polyhydroxybutyric acid in the fed-batch process [15]. Shen et al. previously reported on the effects of $CO_2$ on the formation of flavor volatiles in a fermentation of immobilized brewer's yeast [16]. They observed that elevated $DCO_2$ concentrations could decrease the cell growth during the active fermentation stage of the yeast. Research showed that high $CO_2$ could inhibit cell growth, nutrient utilization, and product formation, in some cases, it altered protein glycosylation [10,17]. In submerged antibiotic fermentations, it could cause significant morphological and biochemical changes, such as lower growth rates, and lower antibiotic productivity. In those processes, the productivity was improved when the carbon dioxide was controlled.

Fermentation of filamentous microorganisms usually produces thick or viscous culture broth. In high-thickness or viscosity fermentation broth, $DCO_2$ concentration was observed reaching as high as 190% of the concentration level calculated by assuming equilibrium between the air coming out of the fermentor and the fermentation broth. For low-viscosity broth, the highest $DCO_2$ concentration was found to be about 133% of the equilibrium value [10,13]. Therefore, the concentration of $DCO_2$ is of particular interest in the case of mycelial fermentations of filamentous bacteria and fungi, affecting both the morphology and productivity. It is necessary to study and eventually to control $DCO_2$ on-line for the improvement of a fermentation process.

In the present study, a BioFlo 310 benchtop fermentor connected to a $DCO_2$ monitoring system was used in the rapamycin fermentation of a selected Streptomyces strain R060107. The experiment investigated the relationship between rapamycin production and the evolution of $DCO_2$. A high limit of $DCO_2$ ($hDCO_2$) was used to control medium feed. Since the cell concentration increased in the process due to growth, the $hDCO_2$ was defined as a dynamic parameter, which was calculated constantly in the process. Its value increased from 3.0% at the beginning to 5.3% at the end of the fed-batch run. Limited carbon source supply restricted growth and respiration, and affected cell metabolism. An optimized fermentation condition, with designed DO and pH, and less viscosity was maintained in the experiment. High fermentation productivity of 500 mg/l of rapamycin in 120 h of fermentation was obtained.

## 2. Materials and methods

*S. hygroscopicus* R060107 was used in the experiment. The strain was selected and mutated from ATCC 29253 cells. There were several techniques used in the cell selection and mutation, which included chemical, biological and physical methods. ATCC 11651 *Candida albicans* was used to select cell strains since rapamycin is very active against all strains of *C. albicans* as described in the previous publication [12]. Fermentation biomass was extracted with different solvents as described previously [1,12]. The production and purity of rapamycin were confirmed by HPLC (Millipore), using Waters Nova-pak C18 column. Glycerol concentration levels were determined using Sigma reagents (G-7793 and F-6428). A benchtop BioFlo 310 fermentor (New Brunswick Scientific Co., Inc., "NBS") of 5 l working volume was used for the fermentation (Fig. 1). An InPro 5000 $CO_2$ sensor and a Mettler Toledo 5100e transmitter were used to measure the $DCO_2$ level. Its 4–20 mA output was directly connected to the BioFlo 310 controller. A certified 5% $CO_2$ gas cylinder (Airgas Co.) was used to calibrate the $DCO_2$ monitor.

A seed culture was prepared in a 1-l Erlenmeyer flask containing 200 ml of growth medium. 1.0 ml of spore suspension inoculated the shaker flask. The composition of the shaker medium was same as used in the previous publication [12]. The culture was incubated at 28 °C for 40 h on a rotary shaker (NBS model Innova 4430) at 240 rpm. The entire inoculum was transferred to the BioFlo 310 vessel containing 4 l of fermentation medium. The compositions of the fermentation medium and its trace metal solution followed the previous publication [12]. The feed medium consisted of 50% glycerol mixed with trace metal solution (30 ml/l). Fermentation temperature was 28 °C, DO 30%, aeration rate 2 l/m (0.5 vvm), and agitation speed 200–1000 rpm. pH was not controlled until it dropped to 5.1. 29% $NH_4OH$ solution was used as base solution to control pH.

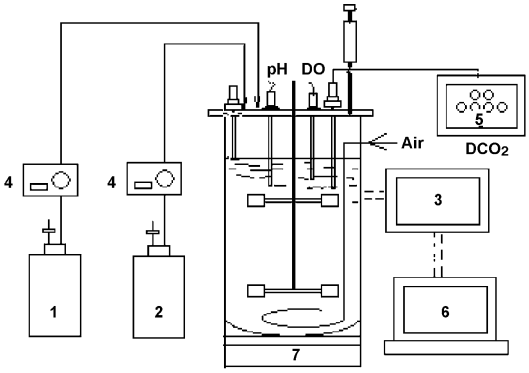


Fig. 1. BioFlo310 set-up with external instrument of $DCO_2$ measurement. (1) Base solution, (2) feed medium, (3) BioFlo310 controller, (4) pumps, (5) MT5100 $DCO_2$ measuring system, (6) PC with BioCommand OPC and (7) BioFlo310 vessel.