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Upscaling of RS87 endospore production process - from flask to semi-industrial scale cultivation

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ABSTRACT

Utilization of PGPR (plant growth promoting rhizobacteria) has been documented to enhance crop yield and reduce the usage of agrochemicals. *Bacillus cereus* strain RS87 has been proven for its PGPR properties as well as an ability for 50% chemical fertilizer substitution in several crops (i.e. rice, pepper, cucumber and tomato). Nonetheless, the conventional process for RS87 endospore production using 0.5xTSB (tryptic soy broth) in a flask-scale cultivation was not a cost-effectiveness and resulted in a low spore yield ($2x10^7$ spores/mL after 4 days). In order to make the process compatible for a large-scale production, an effective low-cost cultivation medium is inevitable. Beside, the cultivation conditions in a bioreactor-scale must be established. An optimized cultivation medium previously developed for RS87 by Jenjitwanich et al. (2018) or omCCY was proven to yield good growth and endospore production at a flask-scale. In this study, omCCY was employed in an upscaling cultivation using a 5 L-bioreactor. Effects of %dissolved oxygen (%DO) and mode of cultivation (batch *vs* fed-batch) on growth and endospore production of RS87 were investigated. The 24 h-batch cultivation with low %DO (10-20%) yielded a comparable maximum spore count ($3.4x10^8$ spores/mL after 24 h) to that of flask-scale cultivation. Maintaining of %DO at a moderate level (40-50%) allowed us to obtain the maximum spore count of $2.5x10^8$ spores/mL within only 12 h. Finally, the fed-batch cultivation with pulse additions of nutrients (glycerol + yeast extract) and divalent ions further improved the endospore yield to $5.2x10^8$ spores/mL (at 12 h).

Keywords: Upscaling cultivation, PGPR, *B. cereus* RS87, endospore

INTRODUCTION

According to the statistical data provided by the National Statistical Office of Thailand (NSO), 114.6 million rai (35.7% of Thailand's total area) was accounted as an agricultural area (NSO, 2013). Obviously, agriculture is one of the most important sector in Thailand.Various types of agrochemicals, including fertilizer, herbicide and pesticide, are heavily applied with an aim to improve crop yield. Unfortunately, excessive use of agrochemicals leads to an accumulation of these products in soil and water. The chemical residues raise the concern regarding their impact on human health and environment. Many biological approaches, therefore, are introduced as an alternative strategy to improve crop yield. Among them, utilization of plant growth promoting rhizobacteria (PGPR) was widely accepted as an environmental friendly and cost effective alternative. PGPR is a group of soil bacteria that live and interact with plant root either from an outside (extracellular PGPR, ePGPR) or an inside (intracellular PGPR, iPGPR) of the root. Both types of PGPR can enhance plant growth through several mechanisms including plant hormone production, nitrogen fixation, phosphate solubilization, siderophore production, ACC deaminase and an induction of plant defense mechanisms against plant pathogen.

Bacillus cereus strain RS87 was reported as a promising ePGPR possessing every property of PGPR previously mentioned. RS87 significantly enhanced growth of rice, cucumber, pepper and tomato (Jetiyanon and Plianbangchang, 2012; Jetiyanon et al., 2010) The *in vivo* safety assessment of RS87 via a gavage test with adult Wistar rat reported an LD_{50} of higher than $9x10^8$ cfu/kg (Lohitnavy, Jetiyanon, Plianbangchang & Wittaya - areekul, 2014). Therefore, RS87 was considered nontoxic when given orally in standard animal testing.

The conventional process for RS87 endospore production using 0.5xTSB in a flask-scale cultivation was not cost-effective and resulted in a low spore yield ($2x10^7$ spores/mL after 4 days). In order to make the process compatible for a large-scale production, an effective low-cost cultivation medium is greatly essential. Previously, in our medium optimization study using a response surface methodology (RSM), omCCY was developed based upon the formula of casein-casein-yeast (CCY) medium reported by Stewart (1981). omCCY supported good growth and endospore production of RS87

($5x10^8$ spores/mL within 24 h) and costed only 7.85 THB/L (comparing to 65.86 THB/L of 0.5xTSB originally used for RS87 cultivation).

In order to establish a process suitable for a large-scale production of RS87 endospore, this study aimed to optimize the cultivation conditions at a bioreactor-scale. Effects of %DO and mode of cultivation (batch *vs* fed-batch) on growth and endospore production of RS87 were investigated in detail.

MATERIAL AND METHODS

Materials

The chemicals and medium components were purchased from Sigma-Aldrich (USA), Merck (Germany), Asia Pacific Specialty Chemical (Australia), LOBA chemie (India), LabM (UK) and Lieber (Germany). *B. cereus* strain RS87 was previously isolated by Assoc. Prof. Kanchalee Jetiyanon from Faculty of Agriculture, Natural Resources, and Environment, Naresuan University (Jetiyanon, 2002). The strain was maintained as a working culture on tryptic soy agar (TSA). TSB with 20% glycerol was used for a long-term storage at -80°C.

Inoculum preparation and cultivation condition

The inoculum was prepared by transferring a single colony of RS87 into TSB and incubated overnight at 37°C, 150 rpm. Cells were harvested, washed with sterile normal saline and used as an inoculum. The flask-scale cultivation was performed using the omCCY (containing 2 g/L glycerol, 4 g/L yeast extract (YE), 1.768 g/L KH_2PO_4, 4.525 g/L K_2HPO_4, 0.1 g/L $MgCl_2 \cdot 6H_2O$, 2 mg/L $MnCl_2 \cdot 4H_2O$, 29 mg/L $CaCl_2 \cdot 2H_2O$, 7 mg/L $ZnCl_2$, and 13 mg/L $FeCl_3 \cdot 6H_2O$) in a 250 mL-flask at a working volume of 50 mL. An initial optical density at 600 nm (OD_{600}) was adjusted to 0.1 (corresponding to $7 x10^5$ spores/mL).

An upscaling cultivation was performed in a 5 L-bench-top bioreactor (Minifors, Infors, Switzerland) at a working volume of 1.5-2.5 L. Endospores suspended in sterile water was used as an inoculum (10^6 spores/mL). Samples were taken at a 6 h- or 12 h-interval and analyzed for total count (cfu/mL), spore count (spore/mL) and %sporulation. Briefly, the total count was determined using a serial dilution with spread plate technique. For spore count, the sample was heated at 80°C for 10 min in order to eliminate all vegetative cells before an analysis.

Bioreactor cultivation Run No.1: batch cultivation with low %DO

The batch cultivation was performed at a working volume of 1.5 L. In order to mimic a low aeration condition in a flask-scale cultivation, air was supplied at a rate of 1 vvm while an agitation speed was under a cascade control between 300–600 rpm in order to maintain %DO at 10-20%. The temperature was controlled at 37°C. Although the pH was not controlled, the buffer capacity of omCCY kept the pH in a neutral range (6.8 – 7.3) throughout an entire cultivation. Antifoam (Anti-foam 204, Sigma) was used to control excessive foaming.

Bioreactor cultivation Run No.2: batch cultivation with moderate %DO

The batch cultivation was performed in a similar manner to that of the previous experiment (Run No.1) except that pure oxygen gas was periodically supplied to maintain the %DO between 40% – 60% throughout the cultivation. Also, the working volume was increased from 1.5 L to 2 L and the cultivation period was extended from 24 h to 48 h. During the first 12 h, samples were taken at a 6 h-interval and analyzed for total count (cfu/mL), spore count (spore/mL) and %sporulation. After that, the sample was taken at a 12 h-interval until 48 h of cultivation.

Bioreactor cultivation Run No.3; fed-batch cultivation with moderate %DO

Fed – batch cultivation was performed with pulse feeding strategy. Concentrated mixtures (10x) of yeast extract, glycerol and $MgCl_2$ were fed into the bioreactor at 6, 12 and 20 h. %DO was maintained at a moderate level (40 - 60%) with an aeration at 1 -1.5 vvm, agitation speed of 300 -600 rpm and supplementation of pure oxygen gas. Samples were taken and analyzed as described in Run No. 2.

RESULTS

Previously, CCY was selected as the most suitable basal medium for growth and endospore formation of RS87 (Jenjitwanich et al., 2018). It was further modified mainly via supplementation of appropriate carbon and nitrogen sources. RSM experiment was performed in order to optimize the concentrations of key nutrients (glycerol and YE). Optimized medium (referred as omCCY) contained 2 g/L glycerol, 4 g/L YE, 1.768 g/L KH_2PO_4, 4.525 g/L K_2HPO_4, 0.1 g/L $MgCl_2 \cdot 6H_2O$, 2 mg/L $MnCl_2 \cdot 4H_2O$, 29 mg/L $CaCl_2 \cdot 2H_2O$, 7 mg/L $ZnCl_2$, and 13 mg/L $FeCl_3 \cdot 6H_2O$. omCCY supported

good growth and endospore production of RS87 ($5x10^8$ spores/mL within 24 h) and costed only 12% of 0.5xTSB originally used for RS87 endospore production. In this study, omCCY was employed in an upscaling cultivation and the cultivation parameters (mainly %DO and mode of cultivation) were investigated.

Bioreactor cultivation Run No.1; batch cultivation with low %DO

In order to mimic the flask-scale cultivation which aeration was considerably low, %DO was maintained at a low level. %DO drastically decreased to 10-20% within the first 1.5 h of cultivation and then gradually increased after 16 h (Figure 1), possibly as a consequence of the sporulation process. The maximum total count and spore count obtained at 24 h were $5x10^8$ cfu/mL and $3.4x10^8$ spores/mL (67% sporulation), respectively.

Bioreactor cultivation Run No.2; batch cultivation with moderate %DO

Maintaining of %DO at a moderate level (40-50%) seemed to enhance growth and endospore formation of RS87. Comparing to the low %DO ($5x10^8$ cfu/mL at 24 h), higher maximum total count ($1.2x10^9$ cfu/mL at 24 h) was observed (Figure 2). Surprisingly, while growth rate was greatly improved, the maximum spore count obtained ($2.5x10^8$ spores/mL at 12 h) was only comparable to that of low %DO. Therefore, a fed-batch cultivation with moderate %DO was employed in the next run.

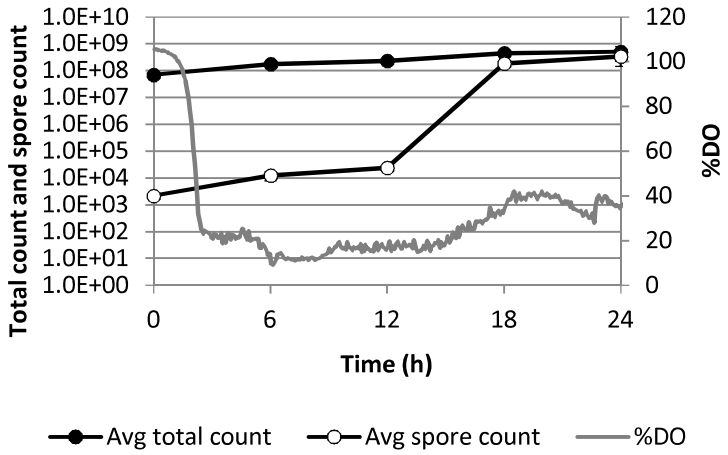

Figure 1. Total count (filled circle) and spore count (empty circle) from batch cultivation with low %DO (10-20%)

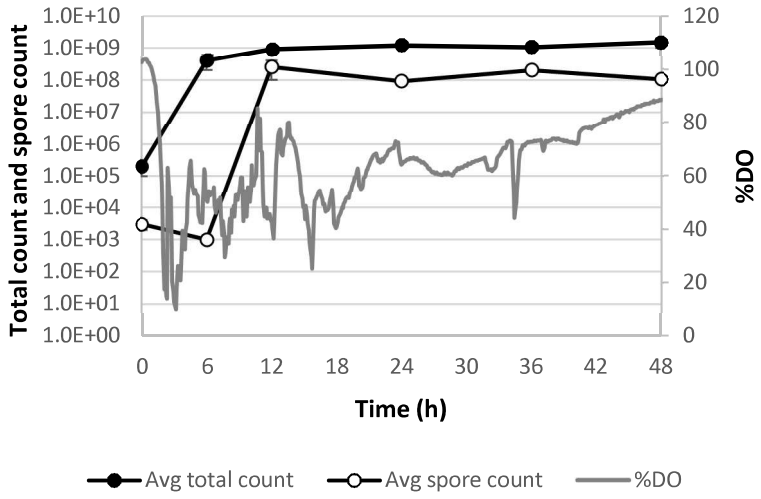

Figure 2. Total count (filled circle) and spore count (empty circle) from batch cultivation with moderate %DO (40-50%)

Bioreactor cultivation Run No. 3; fed-batch cultivation with moderate %DO

Fed-batch cultivation was performed with pulse feedings of glycerol, YE and $MgCl_2$ at 6, 12 and 20 h of cultivation. The cultivation parameters were maintained similar to Run No. 2. Despite a comparable maximum total count obtained ($1.4x10^9$ cfu/mL after 24 h), maximum spore count obtained was significantly improved ($5.2x10^8$ spores/mL after 12 h) comparing to the batch cultivation.

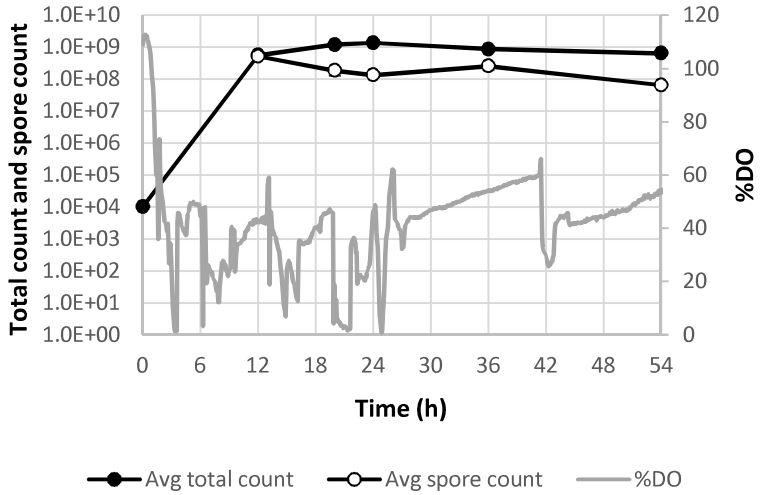

Figure 3. Total count (filled circle) and spore count (empty circle) from fed-batch cultivation with moderate %DO (40-50%)

DISCUSSIONS

The positive effect of moderate %DO on growth of RS87 agreed with several literatures, for example, an observation by Avignone – Rossa et al. (1992) that highest growth, spore production and toxin production of *Bacillus thuringiensis* (closely related to *B. cereus*) were obtained from a cultivation condition with high oxygen transfer rate (OTR). While DO at 26% was reported as an optimal growth and endospore formation (1.3×10^9 spores/mL after 16 h) of *Bacillus thuringiensis* var *israelensis* by Kraemer – Schafhalter and Moser, (1996). Fabrizio et al. (2011) reported that 50% DO resulted in the highest spore yield (3.8×10^9 spores/mL) and faster growth of *Bacillus thuringiensis* var. *israelensis*. Different responses towards oxygen level were frequently observed even between the subspecies or variant as *Bacillus* species was widely recognized for its high variation and diversity. A good example was the fact that moderate oxygen level (40–70%) enhanced sporulation of *B. thuringiensis* var. *kurstaki* (Ghribi et al., 2007) but not of *B. thuringiensis* var. *israelensis* (Sarafzadeh and Narvaro, 2006), in which the highest spore yield was obtained in the absence of oxygen during a two-step cultivation (20→0 %DO). In conclusion, the response of *Bacillus* species to oxygen level was likely to be strain specific and therefore must be optimized specifically for each strain.

Fed-batch cultivation was described by Minihane and Brown (1986) as the strategy to improve microbial growth by avoiding crab-tree effect, substrate inhibition, and catabolite repression. Fed-batch cultivation becomes an ideal method for obtaining high cell density. The fed-batch cultivation was applied for endospore production of *Bacillus thuringiensis* (Kang, Lee and Chang, 1992) as the technique allowed the sporulation to occur after high cell density was obtained. Furthermore, an intermittent fed-batch cultivation resulted in 3 times higher spore yield ($1.25x10^{10}$ spores/mL) than a continuous fed-batch cultivation. Another study on *B. thuringiensis* var. *kurstaki* by Vu, Tyagi, Valéro, and Surampalli (2009) revealed that an intermittent fed-batch cultivation resulted in higher spore yield than batch cultivation. Interestingly, the highest spore yield was observed when 2 intermittent feedings were applied and asporogeneous (Spo^-) variant was detected when 3 intermittent feedings were used. In this study, approximately 2-fold increase in the maximum spore count was obtained when intermittent feeding was applied. The presence of Spo^- mutant was, however, not detected in this study.

CONCLUSIONS

In this study, the optimization of cultivation condition at a bioreactor-scale was performed with the omCCY medium previously developed by Jenjitwanich et al. (2018). The upscale study (from flask-scale cultivation to 5 L-bioreactor) revealed the positive effect of a moderate %DO (40-50%) on growth of RS87. With a moderate %DO, higher maximum biomass could be obtained in shorter time. Intermittent fed-batch cultivation resulted in a significant improve in maximum spore count obtained. In summary, an intermittent fed-batch cultivation with medium %DO (40-60%) was recommended for a bioreactor-scale spore production of RS87.

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