JOURNAL OF BIOSCIENCE AND BIOENGINEERING
Vol. 105, No. 6, 683–686. 2008
DOI: 10.1263/jbb.105.683

Stable Repeated-Batch Production of Recombinant Dye-Decolorizing Peroxidase (rDyP) from *Aspergillus oryzae*

Mozaffar Shakeri,[1] Yasushi Sugano,[1] and Makoto Shoda[1]*

Chemical Resources Laboratory, Tokyo Institute of Technology, R1-29, 4259 Nagatsuta, Midori-ku, Yokohama, 226-8503, Japan[1]

Received 10 October 2007/Accepted 7 March 2008

Recombinant *Aspergillus oryzae* expressing a dye-decolorizing peroxidase gene (*dyp*) was cultivated for repeated-batch production of recombinant dye-decolorizing peroxidase (rDyP) using maltose as a carbon source. High-level rDyP activity in limitation of carbon and nitrogen sources was maintained stably for 26 cycles of repeated 1-d batches of *A. oryzae* pellets without any additional pH control. Cultures maintained at 4°C for 20 d resumed rDyP production following a single day of incubation. One liter filtrated crude rDyP containing 4600 U rDyP decolorized 5.07 g RBBR at the apparent decolorization rate of 17.7 mg l^{-1} min^{-1}.

[**Key words:** dye-decolorizing peroxidase, recombinant *Aspergillus oryzae*, repeated-batch, Remazol Brilliant Blue R]

The colored effluent from textile and dyestuff manufacturing is generally visible xenobiotic pollutants. It is estimated that 10% (w/w) of the total dyestuff utilized worldwide (1), or about 7×10^7 t (2), is released annually into the environment. Conventional activated sludge systems do not effectively decolorize dye-containing effluents (3, 4), making this an increasingly important environmental issue.

A newly isolated fungus, *Thanatephorus cucumeris* Dec1 (formerly called *Geotricum candidum* Dec1), produces DyP, a new type of peroxidase, that can decolorize a wide variety of synthetic dyes (5). The *dyp* gene encoding DyP expressed in *Aspergillus oryzae* (6) and the productivity of rDyP by *A. oryzae* in solid culture is 3000-fold higher than that by the original Dec 1 (7). DyP is the only fungal peroxidase which has been transformed successfully to the host microorganism, *A. oryzae* and the direct use of crude rDyP to decolorization is of primary concern.

Among the available bioreactor strategies, repeated-batch operation reduces nonproductive down-times such as the lag phase, time for reactor clean up, and bioreactor sterilization which are associated with batch operation (8). Moreover, repeated-batch operation minimizes the accumulation of detrimental products such as proteases.

We reported previously repeated-batch production of rDyP by *A. oryzae* using complex media containing wheat brans (9). However, only 7 cycles of repeated-batch culture were possible mainly because composition differences in each lot of wheat brans such as phosphate and the excessive amount of proteins in the materials caused the fluctuation of productivity of the enzyme. In addition to, compared to complex media, defined media support a more reproducible cultivation performance, permits process control and monitoring, is less sensitive to sterilization condition and thus minimizing scaling up problem (10). However, defined media is more expensive than complex media. Therefore, to increase the number of cycles and avoid the drawback from unstable rDyP activity in complex substrate, we examined rDyP production in repeated-batch culture of *A. oryzae* pellets using defined medium containing maltose as carbon source. Maltose induces Taka-amylase A and *A. oryzae* was transformed with the gene *dyp* from Dec1 using the promoter of *amyB* (11). We then examined the decolorization of an anthraquinone dye, Remazol Brilliant Blue R (RBBR; Nippon Kayaku, Tokyo) by a crude enzyme solution obtained from the repeated-batch culture.

Recombinant *A. oryzae* (7) was maintained at ambient temperature on a Czapek-Dox slant. The cells were transferred from the slant to a maltose-agar plate containing 2 g $NaNO_3$, 1 g K_2HPO_4, 0.5 g $MgSO_4\cdot7H_2O$, 0.5 g KCl, 30 g maltose, 10 g Polypepton (Nippon Seiyaku, Tokyo) and 15 g agar in one liter of distilled water.

The media shown in Table 1 were used for preculture, batch, and repeated-batch cultures after sterilization for 20 min at 121°C. All media were adjusted to pH 5.5–6.2, prior to use.

From a 7-d old *A. oryzae* culture grown on maltose-agar plates, a plug (0.5×0.5 cm) was cut using a platinum loop, and transferred to 50 ml of sterile distilled water. The mixture was vortexed for 5 min, and 1 ml of the prepared spore suspension was transferred to 150 ml preculture medium in a 500 ml flask and incubated at 30°C at 120 strokes per min (spm) for 30 h. This preculture (3 ml) was added to 150 ml of batch culture medium and incubated at 30°C at 120 spm. When the rDyP activity reached more than 2 U ml^{-1}, the daily repeated-batch culture was initiated. On each day, the cells were statically sedimented so that all the pellet cells remained at the bottom of the flasks. Then, 100 ml of culture supernatant was removed and replaced with the same amount of fresh repeated-batch culture medium. The first

* Corresponding author. e-mail: mshoda@res.titech.ac.jp
phone: +81-(0)45-924-5274 fax: +81-(0)45-924-5976

TABLE 1. Composition of preculture, batch culture, and repeated-batch culture media (%) (w/v)

Cultures	$NaNO_3$	K_2HPO_4	$MgSO_4 \cdot 7H_2O$	KCl	$FeSO_4 \cdot 7H_2O$	Maltose	Yeast extract	$CaCO_3$
Preculture	0.2	0.1	0.05	0.05	0.002	3	1	–
Batch culture	0.2	0.1	0.05	0.05	0.002	3	–	0.1
Repeated-batch culture	0.05–0.1	0.1	0.05	0.05	0.002	1	–	0.1

six cycles of repeated-batch culture were performed using medium containing 0.1% (w/v) $NaNO_3$, and subsequent cycles were performed using medium containing 0.05% (w/v) $NaNO_3$ because previous studies have shown that nitrogen limitation improves the production of dye-decolorizing enzymes (5, 12). Preliminary experiments showed that 1% (w/v) maltose led to high production of rDyP in the range of 0.5% to 3% (w/v) (data not shown). Therefore, we used 1% (w/v) maltose as a carbon source in repeated-batch culture. The average productivity of rDyP (U ml^{-1} d^{-1}) was defined as the sum of the increase in rDyP activity in each batch (U ml^{-1})/incubation time (d). The incubation time in repeated-batch culture includes the time of initial batch culture.

For decolorization test of the anthraquinone dye, RBBR by crude rDyP, a 52 d-repeated-batch culture supernatant containing 4.6 U ml^{-1} rDyP activity (Fig. 1) was harvested by filtration through gauze. This crude rDyP was adjusted to pH 5, which is optimal for rDyP stability (13). Then 50 ml of crude rDyP solution was mixed with RBBR in a 150-ml Erlenmeyer flask and incubated at 30°C at 100 spm. Dye decolorization was initiated by addition H_2O_2. After each 0.24 mM (150 mg/*l*) dye addition at 2–10 min time intervals, 0.4–0.64 mM H_2O_2 was added at 2–8 min time intervals for total addition of 8.6 mM of RBBR and 20 mM of H_2O_2. The RBBR decolorization ratio (%) was calculated as $\{(C_i - C_f)/C_i\} \times 100$ and the apparent decolorization rate was calculated by $(C_i - C_f)/t$, where C_i and C_f are the initial and final concentrations of the dye, respectively, and t is the time.

For rDyP activity assay, samples (3 ml) were centrifuged for 5 min at 15000×*g*, and the supernatant was assayed for rDyP activity at 30°C in a 3-ml cuvette containing 15 µl of 40 mM H_2O_2, 2945 µl of 25 mM citrate buffer (pH 3.2), 25 µl of the culture supernatant and 15 µl of 25 mM anthraquinone dye, reactive blue 5 dye (RB5). Decolorization was monitored spectrophotometrically at 600 nm (maximum absorbance for RB5). One unit of enzyme activity was defined as the amount of enzyme required to decolorize 1 µmol of RB5 in the reaction mixture in 1 min.

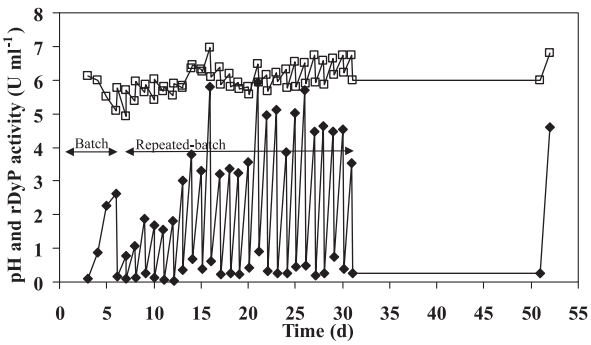

FIG. 1. Changes in rDyP activity (closed diamonds) and culture pH (open squares) during batch and repeated-batch cultures of *A. oryzae*.

The rDyP activity and pH variation in the initial batch and consecutive repeated-batch cultures are shown in Fig. 1. The experiments were conducted in duplicate, and the results are the average of duplicate samples. Formation of *A. oryzae* pellets and expression of rDyP activity were observed in the initial batch starting on the third day of cultivation. When the rDyP activity reached 2.6 U ml^{-1} by day 6, daily repeated-batch cultures were initiated with fresh medium containing 0.1% (w/v) $NaNO_3$ and 1% (w/v) maltose. A previous study showed that rDyP is rapidly inactivated at pH values above 7 (7). During repeated-batch culture, the pH was maintained lower than 7. Then, we used 0.05% (w/v) $NaNO_3$ after the first six cycles of repeated batch to reduce nitrogen source, whereupon the activity increased, resulting in more than 3 U ml^{-1} of rDyP activity between days 11 and 31. The repeated-batch is expected to continue further, but the following experiment was conducted.

To investigate the stability of *A. oryzae* pellets, on the 31st day of cultivation, we inoculated *A. oryzae* pellets separated from the culture supernatant to fresh repeated-batch culture medium, and maintained the culture at 4°C for 20 d. Thereafter, the sample was returned to the above-mentioned active culture conditions and rDyP production and its activity were measured. The results revealed that rDyP production was resumed within a day, with an activity of approximately 4.6 U ml^{-1} (Fig. 1). The average productivity of rDyP in batch culture was 0.43 U ml^{-1} d^{-1}, but it was increased to 2.82 U ml^{-1} d^{-1} in repeated-batch culture.

Only two-third of culture supernatant volume was exchanged with fresh medium. However, the residual rDyP activity just after medium replacement decreased more than two-third of initial rDyP activity. This inconsistency between theoretical and experimental data is mainly because of inhibitory effect of $FeSO_4 \cdot 7H_2O$ and $NaNO_3$ on rDyP activity of replaced medium (data not shown). Disappearing of inhibitory effect of mentioned compounds on rDyP activity proceeded by their consumption in each culture.

Formation of stable *A. oryzae* pellets and quick cell sedimentation for cell reuse in the repeated-batch culture appeared to be associated with active and high rDyP production. Several other methods have been proposed to separate and reuse cells, including a cell retention bioreactor (8), immobilization of cells (14), cross-flow microfilteration (15), and the use of ultrasonic filtration (16). However, the sedimentation of fungal cell pellets in this study is simple, making it optimal for future industrial applications.

The pH values (the initial value and range) affect pellet formation (17, 18). In this study, the pH of the culture was maintained in range of 5 to 7 by daily repeated-batch culture without any additional pH control. $CaCO_3$ in the media prevented sudden pH decrease as a consequence of organic acid production from living cells metabolism. Thus, maintaining