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SHORT COMMUNICATION

Manipulation of culture conditions for extensive extracellular catalase production by *Exiguobacterium oxidotolerans* T-2-2^T

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Abstract A hydrogen peroxide (H_2O_2)-resistant bacterium, *Exiguobacterium oxidotolerans* T-2-2^T, secretes extracellular catalase. A low shaking frequency (60 rpm) was employed following the observation that a higher amount of extracellular catalase was produced under this condition compared with the previous shaking frequency (120 rpm). The cell concentration reached approximately 4.5×10^6 cells mL^{-1}, at which the initiation of extracellular catalase production was triggered, while the production of the extracellular catalase was largely dependent on that of the intracellular catalase. The best initial aeration condition for extracellular catalase production was 7.5 mg O L^{-1}, which is 95 % of that of the air-saturated state measured under the low shaking frequency. In addition, although the total productivity of catalase was unchanged, introduction of aminolevulinic acid and Tween 60 promoted extracellular production. The extracellular and intracellular catalase productivities were 16,000 and 6,000 U mL^{-1} of spent culture medium, respectively.

Keywords *Exiguobacterium oxidotolerans* · Catalase · Extracellular production · Aeration · Low rotation rate

Catalase is reported to play a role in decomposing the H_2O_2 produced as a byproduct of oxygen metabolism. In addition, catalase is important for the elimination of H_2O_2 existing in extracellular environments. For example, only pathogenic species belonging to the genus *Mycobacterium* produce extracellular catalase (Raynaud et al. 1998). On the other hand, a symbiotic bacterium in squid, *Vibrio fisheri*, eliminates H_2O_2 produced by the host squid using catalase to create a niche in the light organ of the host (Visick and Ruby 1998). Catalase from *Bacillus subtilis* (KatA) can be induced by H_2O_2 as a cellular response to oxidative stress (Naclerio et al. 1995). In *B. subtilis*, simultaneous production of extracellular catalase with induction of catalase by H_2O_2 stimulation was observed. However, because the signal peptide gene sequence does not exist upstream of *katA* (Naclerio et al. 1995), the secretion system may be different from general secretion systems (Zhou and Zhang 2004). A small amount of extracellular catalase is produced by *B. subtilis* even under ordinary growth conditions. This may be due to the fact that catalase production is limited in the absence of H_2O_2 stimulation. To enhance the production of extracellular catalase in *B. subtilis*, a recombinant catalase expression system has been developed (Shi et al. 2008). In addition to the example of *B. subtilis*, extracellular catalase secretion has been reported in several bacteria (Raynaud et al. 1998; Bendtsen et al. 2005; Dumas et al. 2008). However, studies on the enhancement of extracellular catalase production by the manipulation of culture conditions have not yet been carried out.

It can be speculated that H_2O_2-tolerant microorganisms surviving in H_2O_2-containing wastewater might be good candidates for the production of catalase. We have isolated H_2O_2-tolerant microorganisms from the wastewater of a fish processing plant that uses H_2O_2 as the bleaching agent for fish eggs (Yumoto et al. 1998, 1999, 2004, 2010; Kimoto et al. 2012) and have characterized the catalases from these isolates (Yumoto et al. 2000; Hara et al. 2007; Kimoto et al. 2012). The high catalase activity detected in the supernatant of the spent culture medium will facilitate purification of the catalase by eliminating the need for a the cell disruption step to obtain intracellular catalase and a large amount of intracellular background proteins. In previous studies, we found that

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Exiguobacterium oxidotolerans T-2-2^{T} can secrete intracellular catalase (EktA) in the medium (extracellular EktA) (Takebe et al. 2007; Hanaoka et al. 2013). In this study, we estimated the time course of the production of intracellular and extracellular catalases and found a relationship between initiation of extracellular catalase production and viable cell count. Furthermore, extracellular catalase productivity was increased by manipulating the growth conditions.

Exiguobacterium oxidotolerans T-2-2^{T} was cultivated aerobically at 27 °C in PYS-3 broth (pH 7.5) containing (L^{-1} of distilled water) 8.0 g polypeptone (Nihon Pharmaceuticals, Tokyo, Japan), 3.0 g yeast extract (Kyokuto), 5.0 g sodium succinate. Although we used shake culture at 120 rpm for the cultivation of *E. oxidotolerans* T-2-2^{T} (Takebe et al. 2007), we discovered that a low shaking speed of 60 rpm increased the extracellular catalase productivity in our preliminary experiment. To determine the effect of aeration on the production of extracellular catalase, the cells were cultured in 100–700 mL PYS-3 broth in a 2-L nonbaffled Erlenmeyer flask at 60 rpm by rotary shaking. The cell suspension adjusted to OD_{650}=0.5 was inoculated as one part per thousand to the medium volume. The inoculated medium was incubated for 20 h and 48 h for purification of intracellular and extracellular catalases, respectively. Aminolevulinic acid (ALA), which is a natural amino acid, can be a preliminary precursor for porphyrin biosynthesis during heme biosynthesis. A 1 M ALA water solution was sterilized by passing through a sterilized 0.22-μm pore size filter and added to the culture medium at a final concentration of 0.1–2.0 mM when a seed of the culture was inoculated. The accumulation of catalase in the inner circumference (S-layer) in the mid-stationary growth phase of strain T-2-2^{T} was reported. In addition, it has been demonstrated that the accumulated catalase can be dissociated by treatment with Tween 60 ($C_{18}E_{20}$) (Hanaoka et al. 2013). To facilitate extracellular catalase production by dissociation of catalase accumulating in the S-layer into the extracellular space, a nonionic detergent, Tween 60, was added to the culture medium. To prevent hydrolysis of Tween 60 during cultivation of strain T-2-2^{T}, the detergent (0.1 %) was added after 48 h of cultivation. A 20 % (w/v) Tween 60 aqueous solution was sterilized by passing through a sterilized 0.22-μm pore size filter and added to the culture medium to a final concentration of 0.1 % at 5 min prior to the end of the cultivation. Catalase activity was measured spectrophotometrically using a Hitachi U-3210 spectrophotometer by monitoring the initial decrease in absorbance at 240 nm caused by the disappearance of H_2O_2 per min, at 25 °C. The H_2O_2 concentration was determined on the basis of the extinction coefficient of 43.6 M^{-1} cm^{-1} (Hildebrant and Roots 1975). The standard reaction mixture for the assay contained 50 mM potassium phosphate buffer (pH 7.0), 30 mM H_2O_2 and 10 mL catalase solution in a total volume of 1.0 mL. The amount of enzyme activity that decomposed 1 mol of H_2O_2 per min was defined as 1 U. The enzyme activities are expressed as the mean of at least four independent measurements. To examine the intracellular catalase activity, the cell suspension was applied to a multibead shocker (Yasui-Kikai,

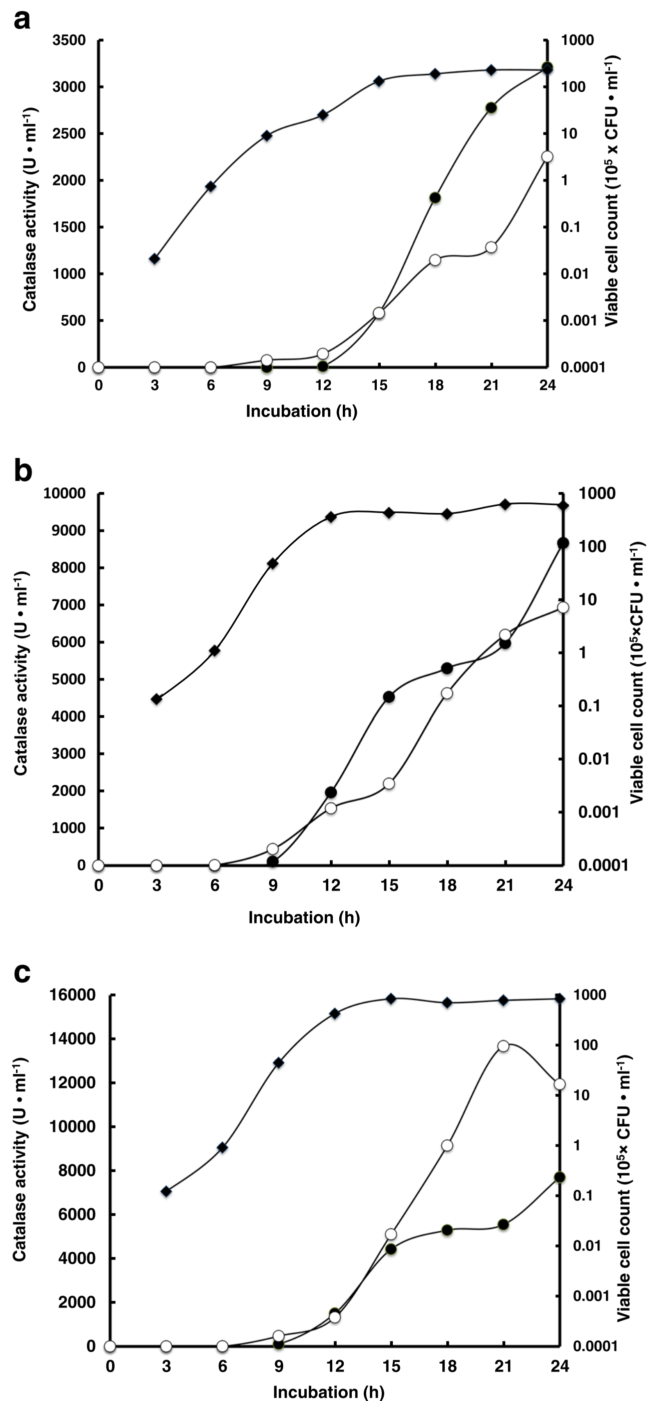

Fig. 1a–c Changes in extracellular and intracellular catalases of *Exiguobacterium oxidotolerans* T-2-2^{T} during cultivation under different aeration conditions. **a** 500 mL broth, **b** 300 mL broth, **c** 200 mL broth in a 2-L nonbaffled Erlenmeyer flask. Extracellular (*filled circles*) and intracellular (*open circles*) catalase activities were estimated using culture supernatant and cell extract, respectively. Cell growth was expressed as colony forming units (CFUs; *filled diamonds*)

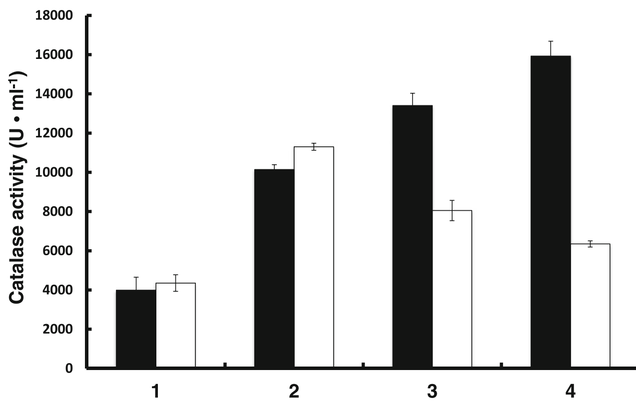

Fig. 2 Effects of aeration, addition of aminolevulinic acid (ALA; 2.0 mM) and Tween 60 (0.1 %) on the production of extracellular and intracellular catalases. Extracellular (*black bars*) and intracellular (*white bars*) catalase activities were measured using the culture supernatant and cell extract, respectively, in a 48 h culture of *E. oxidotolerans* T-2-2^T in a 2-L nonbaffled Erlenmeyer flask. Conditions: *1* Medium volume 500 mL; *2* medium volume 200 mL; *3* medium volume 200 mL and with ALA added to the medium; *4* as condition *3* but with addition of Tween 60 after 43 h of cultivation (5 h before the end of cultivation). Values are mean±standard deviation (*bars*) of results

Osaka, Japan) at 2,500 rpm for 6 min to disrupt the cells. Bovine serum albumin (BSA) dissolved in an equal volume of a mixture of milli-Q water and acetonitrile was used as the standard. Protein content was determined using a BCA protein assay reagent kit (Pierce, Rockford, IL) with BSA as the standard.

To estimate the initiation period of extracellular catalase production, the time course of extracellular catalase production was estimated under different aeration conditions depending on the volume of the culture medium. When the medium volumes were 200 and 300 mL, extracellular catalase production began after 9 h of culture, corresponding to the late logarithmic or early stationary phase (Fig. 1). The extracellular catalase activity was higher in 200 mL medium (7,700 U mL^{-1}) than in 300 mL (6,900 U mL^{-1}) and 500 mL (3,200 U mL^{-1}) medium. Similarly, the intracellular catalase activity was much higher in 200 mL medium (14,300 U mL^{-1}) than in 300 mL (9,100 U mL^{-1}) and 500 mL (2,400 U mL^{-1}) medium. Thus, the productivity of the extracellular catalase depended largely on the productivity of the intracellular catalase. On the other hand, the initiation of extracellular catalase production was triggered by the cell concentration reaching approximately 4.5×10^6 cells mL^{-1}. Therefore, it can be speculated that the extracellular catalase production was triggered in a cell-density-dependent manner by an autoinducer (Biswa and Doble 2013). The extracellular catalase activity was maintained after 24 h to at least up to 72 h (data not shown), indicating that the culture period does not need to be exact for the harvest of extracellular catalase from spent medium. High intracellular catalase production was observed under low aeration conditions using a nonbaffled Erlenmeyer flask compared with that under high aeration conditions in *E. oxidotolerans* T-2-2^T using a baffled Erlenmeyer flask (Takebe et al. 2007); 500 mL medium in a nonbaffled Erlenmeyer flask with 120 rpm of rotary shaking was employed for the production of extracellular catalase (Takebe et al. 2007). Extracellular catalase productivity was 1,500 U mL^{-1} spent culture medium under these latter conditions. Extracellular catalase production increased proportionally according to the decrease in the medium volume from 600 mL (1,500 U mL^{-1} spent culture medium) to 200 mL (the best condition; 11,000 U mL^{-1} spent culture medium) with shaking at 60 rpm. Under the best conditions, the initial broth aeration was 7.5 mg OL^{-1}, which is 95 % of that of the air-saturated state. The aeration for 100 mL medium in a 2-L volume was considered to be too high (7.8 mg O L^{-1}) for the effective production of catalase. The activity at this medium volume increased 2.5 times compared with that in 500 mL medium (6.8 mg O L^{-1}), which is regarded as the standard condition (4,000 U mL^{-1} of spent culture medium; Fig. 2, column 2). It can be concluded that both low shaking frequency (60 rpm) and appropriate aeration

Table 1 Comparison of productivities of catalase from different sources

Source	Gram positive or negative	Location[a]	Catalase activity (U)[b]	Reference
Exiguobacterium oxidotolerans T-2-2^T	Positive	Extracellular	16,000	This study
		Extracellular + intracellular	22,000	This study
Micrococcus luteus	Positive	Intracellular	6,900	Nakayama et al. 2008
Rhizobium radiobacter 2-1	Negative	Intracellular	16,000	Nakayama et al. 2008
Recombinant *Bacillus subtilis*	Positive	Extracellular	3,500	Shi et al. 2008
Vibrio rumoiensis S-1^T	Negative	Intracellular	1,200	Yumoto et al. 2000
Psychrobacter piscatorii T-3	Negative	Intracellular	11,000	Kimoto et al. 2008
Escherichia coli	Negative	Intracellular	15	Nakayama et al. 2008

[a] Extracellular enzyme recovered from supernatant of spent culture medium. Intracellular enzyme recovered from disrupted cells harvested from spent culture medium

[b] Catalase activity mL^{-1} of spent culture medium

(7.5 mg O L^{-1}) are necessary for obtaining the highest extracellular catalase productivity, because aeration alone cannot account for the high productivity.

It has been reported that ALA can aid the growth of plants by facilitating the biosynthesis of chlorophyll from porphyrin (Nadler and Granick 1970). Therefore, it is expected that ALA acts as a precursor in heme biosynthesis for catalase production. When 2.0 mM ALA was added to the culture medium for strain T-2-2^T, extracellular catalase production became 1.3 times higher than in the absence of ALA after 48 h of cultivation. However, there was no pronounced difference in the production of total catalase (extracellular plus intracellular) between medium without ALA (control) and with 2.0 mM ALA (Fig. 2, column 3). The extracellular catalase production increased 1.2 times in comparison with that of the control culture (no Tween 60 addition), whereas intracellular catalase production decreased. This result demonstrates that Tween 60 is useful for facilitating the removal of catalase found on the cell surface of strain T-2-2^T. On the basis of the above results, strain T-2-2^T was cultivated in 200 mL PYS-3 broth containing 2.0 M ALA (initially) and 0.1 % Tween 60 (added prior to the end of the cultivation) in a 2-L flask. Under these conditions, catalase productivity increased approximately four times in comparison with levels observed under standard conditions (Fig. 2, column 4).

The production of extracellular EktA was enhanced by manipulating the rotary shaking frequency, the aeration conditions, and by adding a heme precursor and a nonionic detergent. Increased extracellular catalase production through low-frequency rotary shaking may be due to using only minimal shaking to disperse propagated cells in the culture fluid. Strong shaking may accelerate the diffusion of extracellular enzymes, preventing the higher production of extracellular enzymes. On the other hand, an optimal dissolved oxygen concentration results in a good proliferation rate for the production of the extracellular enzyme. In addition, although the total productivity of catalase was unchanged, the addition of additives promoted the extracellular production of catalase.

From the results, 16,000 U catalase mL^{-1} of medium can be obtained from the culture supernatant. This corresponds to an activity of 4,400 U mg^{-1} protein. Considering that activities of commercially available catalases of *Aspergillus niger* and bovine liver are 2,000–4,000 and 5,000–40,000 U mg^{-1}, respectively, the activity of our culture supernatant is extremely high. The productivities of catalases per milliliter of spent culture medium from the following sources were compared: *Micrococcus luteus* (Nakayama et al. 2008), which produces a commercially available product (intracellular catalase from cell extract); *Rhizobium radiobacter* strain 2-1 (Nakayama et al. 2008), which was discovered as a high-catalase-producing bacterium (intracellular catalase from cell extract); and recombinant extracellular-catalase-producing *B. sublitis* (Shi et al. 2008). The productivity of extracellular EktA was much higher than those of *M. luteus* (2.3 times higher) and the recombinant *B. subtilis* (4.6 times higher) and was equivalent to that of intracellular *R. radiobacter* (Table 1). Furthermore, the extracellular *plus* intracellular catalase activity in the manipulated culture condition is 22,000 U mL^{-1} of spent culture medium. Other hydrogen-peroxide-tolerant bacteria that exhibit high catalase activity could be candidates for low-cost catalase production. Indeed, we have isolated other hydrogen-peroxide-tolerant bacteria, namely, *Vibrio rumoiensis* S-1^T (Yumoto et al. 1998, 1999, 2000) and *Psychrobacter piscatorii* T-3 (Kimoto et al. 2008, 2012). Although their intracellular-catalase-producing abilities are very high, the extracellular catalase productivity of *E. oxidotolerans* T-2-2^T is higher than that of intracellular catalase (Table 1). *V. rumoiensis* S-1^T and *P. piscatorii* T-3 do not produce as much extracellular catalase as the intracellular enzyme. This is probably due to the complexity of the cell surface structure of Gram-negative bacteria. It is considered that, for the attainment of an extremely high productivity of extracellular catalase, Gram-positive hydrogen-peroxide-tolerant bacteria, which produce extraordinarily high catalase activity, should be used.

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