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FULL PAPER

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# Ribonuclease production of *Rhizopus oryzae* IFO 4697 under metal ion stress in liquid culture

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**Abstract** *Rhizopus oryzae* IFO 4697 was found to produce intracellular ribonuclease (RNase), and its growth and activity could be regulated under selected metal ion stress. The addition of $Fe^{2+}$, $Mg^{2+}$ and $Zn^{2+}$ to the SLSR medium was essential to growth and RNase production. $Ca^{2+}$ and $Mo^{6+}$ stimulated RNase production. It is concluded that the addition of 100 mg/ml $Ca^{2+}$, 5 mg/ml $Mo^{6+}$, 0.7 mg/ml $Zn^{2+}$, 2 mg/ml $Fe^{2+}$, and 49 mg/ml $Mg^{2+}$ to the SLSR medium was the best condition for producing RNase in high specific activity (3780 U/mg protein). This result indicates that a metal ion-regulated liquid medium is an efficient culture method for RNase production.

**Key words** Liquid culture · Metal ion · Mycelial growth · *Rhizopus* · Ribonuclease activity

## Introduction

RNases are important analytical enzymes that catalyze the cleavage of nucleotides in RNA. They play a major role in the determination of RNA structure and also have been used for the removal of RNA in single-cell protein preparations. RNases are produced by various microorganisms, usually cultured in a liquid state medium. For *Rhizopus* strains, most investigations of RNase had been conducted on the purification capabilities and properties of RNase from Gluczyme, which is a commercial amylase agent, and prepared by extracting the solid culture of the *Rhizopus* strain (Tomoyeda et al. 1969; Komiyama and Irie 1971; Woodroof and Glitz 1971; Sanda et al. 1979). Thompson and Eribo (1984) selected a solid medium for RNase production, because a solid culture of *Rhizopus* strain is superior to a liquid culture in terms of productivity.

In contrast, in previous papers (Fujio and Morita 1996; Morita and Fujio 1997, 1999; Morita et al. 1998, 1999), we used a so-called metal ion-regulated liquid medium and achieved a high level of glucoamylase and polygalacturonase production. We suggested that a metal ion-regulated liquid medium was superior to a wheat bran solid culture medium for glucoamylase and polygalacturonase production.

In this article, we have investigated the effects of various metal ions as supplementation on RNase production of the strain *Rhizopus oryzae* IFO 4697, and established a liquid culture system for RNase production using a metal ion-regulated liquid medium. We also compared RNase productivities of the present medium with a wheat bran solid medium (widely used in *Rhizopus* fermentation).

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## Materials and methods

### Microorganisms

A total of 46 *Rhizopus* strains (33 *Rhizopus* sp. and 13 *Rhizopus oryzae* strains) were used in this study (see Table 3). From the preliminary screening of the best producer of RNase on the potato dextrose agar slant incubated at 30°C for 5 days, *Rhizopus oryzae* IFO 4697 was selected (data not shown), and we used it in the following experiments.

### Cultivation

#### *Liquid culture*

A basal medium SLSR medium consisted of 2 g liquefied cassava starch, 0.4 g ammonium acetate, 0.1 g dipotassium

hydrogen phosphate, and 0.33 g citric acid dissolved in 100 ml deionized water. The pH was adjusted to 6.0 with sodium hydroxide. The medium was sterilized at 121°C for 20 min in a shaking flask. The *Rhizopus* strains were precultured on a slant of potato dextrose agar at 30°C for 7 days. Then, 2 ml of the spore suspension was inoculated into the shaking flask. The culture was incubated at 30°C for 2 days on a reciprocal shaker with agitation at 300 strokes per minute.

*Solid culture*

Solid cultivation was performed according to the method of Elegado and Fujio (1993). The solid medium consisted of 20 g wheat bran, 2 g cassava starch, and 20 ml tap water in a 500-ml Erlenmeyer flask stoppered with a cotton plug. After autoclaving the medium (121°C, 20 min), 2 ml of the spore suspension was inoculated into the medium, and cultivation was carried out at 30°C for 5 days, which was determined to be the best conditions for cultivation of the solid culture (data not shown).

Preparation of enzyme solution

*Liquid culture*

The fungal mycelia were filtrated through filter paper (no.7; Toyo Roshi, Tokyo, Japan). The cell mass from 100 ml culture was suspended with 20 ml 1.0% (w/v) sodium dodecyl sulfate (SDS). The enzyme was extracted by gentle stirring at 4°C for 24 h. After centrifugation, the supernatant was used as a crude enzyme solution.

*Solid culture*

One hundred milliliters 1.0% (w/v) SDS was added to the culture flask. The mixture was stirred for 12 h at 4°C and then centrifuged at 12 000 *g* for 20 min. The supernatant was used as a crude enzyme solution.

*Determination of the dry mycelium weight*

The dry mycelium weight (DMW) was determined after drying the mycelia in a 105°C oven for 24 h. DMW was defined as mycelial weight per 100 ml of culture medium.

*Protein assay*

Protein was measured using the method of Bradford (1976) with reagents from Bio-Rad (Bio-Rad protein assay; Bio-Rad, Hercules, CA, USA). Bovine serum albumin was used as a standard.

*Determination of RNase activity*

A modified Horitsu method (Horitsu et al. 1974) was used. The reaction mixture contained 0.5 ml 1% (w/v) yeast RNA, 1.0 ml 0.05 M acetate buffer (pH 4.5), and 0.5 ml diluted crude enzyme solution. After incubation for 15 min at 40°C, the reaction was stopped with 0.5 ml 0.75% (w/v) lanthanum nitrate and 25% (v/v) perchloric acid. After standing for 30 min in an ice bath, the precipitate was removed by centrifugation. Then, 0.1 ml of the supernatant solution was pipetted out, diluted to 5.0 ml with distilled water, and the absorbance was measured at 260 nm. One unit of enzyme activity (U) was defined as 1.00/15-min increase in absorbance under the conditions described above. RNase productivities in liquid and solid cultures cannot be directly compared based on the activity (U) per milliliter of enzyme solution, because in solid culture RNase must be extracted from the culture media using a liquid elution mixture. In the present investigation, the RNase productivity was expressed in terms of RNase activity per protein in the enzyme solution (specific activity, U/mg protein).

Table 1 shows the effects of $Fe^{2+}$, $Mg^{2+}$, and $Zn^{2+}$ supplementation on RNase activity and DMW of *Rhizopus oryzae* IFO 4697. *Rhizopus oryzae* IFO 4697 neither grew nor produced RNase in the SLSR medium, nor did the addition of two of these ions to the SLSR medium in combination stimulate RNase production. Addition of all three ions to the basal medium resulted in better RNase production than any combination of two of the three metal ions. The optimum concentration of $Fe^{2+}$, $Mg^{2+}$, and $Zn^{2+}$ for RNase production and mycelial growth was 2, 49, and 0.7 mg/ml, respectively (data not shown). These results are similar to those of the previous papers regarding glucoamylase and polygalacturonase production (Fujio and Morita 1996; Morita et al. 1999; Morita and Fujio 1999). Foster and Waksman (1939) also mentioned that $Zn^{2+}$ was essential for the growth of *Rhizopus nigricans*.

Table 2 shows the effects of other metal ion supplementation on the RNase production of *Rhizopus oryzae* IFO 4697 when SLSR medium supplemented with 2 mg/ml $Fe^{2+}$, 49 mg/ml $Mg^{2+}$, and 0.7 mg/ml $Zn^{2+}$ was used. Of 10 metal ions tested, $Mo^{6+}$ and $Ca^{2+}$ exerted remarkable effects on

**Table 1.** Effects of $Fe^{2+}$, $Mg^{2+}$, and $Zn^{2+}$ supplementation on RNase activity and dry mycelium weight (DMW) of *Rhizopus oryzae* IFO 4697

| Combination | RNase activity (U/ml) | DMW (g/100 ml) |
|---|---|---|
| SLSR basal medium | 0 | 0 |
| SLSR + Fe[a] | 0 | 0.06 |
| SLSR + Mg[b] | 0 | 0.08 |
| SLSR + Zn[c] | 2 | 0.11 |
| SLSR + Fe + Mg | 23 | 0.23 |
| SLSR + Fe + Zn | 11 | 0.17 |
| SLSR + Mg + Zn | 15 | 0.21 |
| SLSR + Zn + Mg + Fe | 92 | 0.46 |

[a] Fe, 2 mg/ml $Fe^{2+}$
[b] Mg, 49 mg/ml $Mg^{2+}$
[c] Zn, 0.7 mg/ml $Zn^{2+}$

**Table 2.** Effects of supplementation by various metal ions on RNase activity of *Rhizopus oryzae* IFO 4697

| Metal ion | Relative activity[a] (%) at: Concentration mg/ml | | |
|---|---|---|---|
| | 1 | 10 | 50 |
| $Ag^{+}$ | 100 | 36 | 0 |
| $Cu^{2+}$ | 97 | 94 | 91 |
| $Ni^{2+}$ | 98 | 100 | 87 |
| $Mn^{2+}$ | 101 | 106 | 0 |
| $Cd^{2+}$ | 36 | 8 | 0 |
| $Ca^{2+}$ | 113 | 114 | 117 |
| $Pb^{2+}$ | 103 | 106 | 101 |
| $Co^{2+}$ | 100 | 102 | 100 |
| $Al^{3+}$ | 94 | 90 | 91 |
| $Mo^{6+}$ | 123 | 127 | 81 |

[a] Relative activity was compared with a control medium consisting of SLSR medium supplemented with $Fe^{2+}$ (2 mg/ml), $Mg^{2+}$ (49 mg/ml), and $Zn^{2+}$ (0.7 mg/ml)

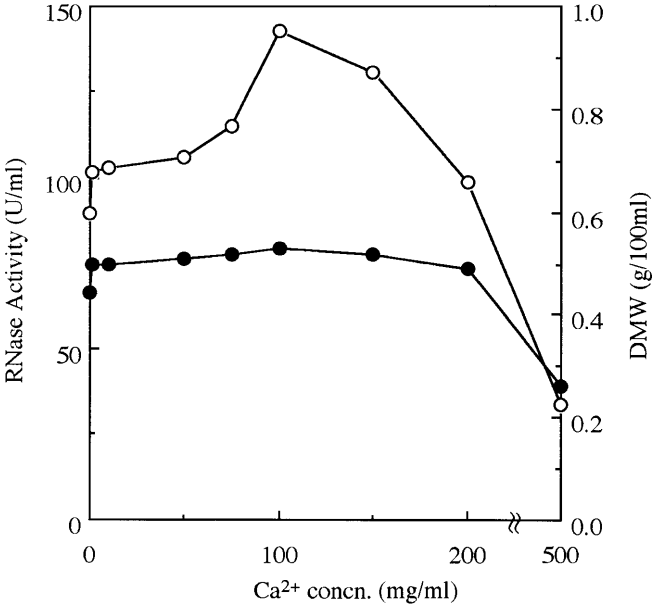


**Fig. 1.** RNase production and dry mycelium weight (*DMW*) of *Rhizopus oryzae* IFO 4697 under different concentration of calcium ions ($Ca^{2+}$). The basal medium was SLSR medium supplemented with 2 mg/ml $Fe^{2+}$, 49 mg/ml $Mg^{2+}$, and 0.7 mg/ml $Zn^{2+}$. Shaking cultivation was performed at 30°C for 2 days. *Open circles*, RNase activity; *filled circles*, DMW

RNase production. The addition of 10 mg/ml $Mo^{6+}$ increased relative activity to 127% compared with the control. The addition of 50 mg/ml $Ca^{2+}$ increased the relative activity to 117% compared with the control.

Figure 1 shows RNase activity under different concentrations of $Ca^{2+}$ in the SLSR medium supplemented with 2 mg/ml $Fe^{2+}$, 49 mg/ml $Mg^{2+}$, and 0.7 mg/ml $Zn^{2+}$. Supplementation by $Ca^{2+}$ stimulated RNase production of *Rhizopus oryzae* IFO 4697. $Ca^{2+}$ at 100 mg/ml was the optimal concentration to produce RNase; this resulted in RNase activity of 143 U/ml.

Figure 2 shows the effect of $Mo^{6+}$ for RNase production. The basal medium was the same as in Fig. 1. Supplementation with $Mo^{6+}$ also stimulated RNase production. $Mo^{6+}$ at 5 mg/ml was the optimal concentration to produce RNase; this resulted in RNase activity of 120 U/ml.

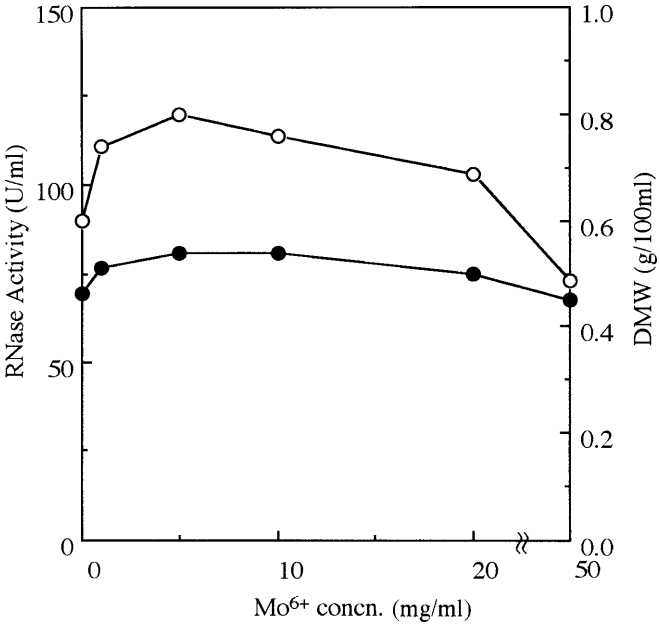


**Fig. 2.** RNase production and DMW of *Rhizopus oryzae* IFO 4697 under different concentration of molybdenum ions ($Mo^{6+}$). The basal medium was SLSR medium supplemented with 2 mg/ml $Fe^{2+}$, 49 mg/ml $Mg^{2+}$, and 0.7 mg/ml $Zn^{2+}$. Shaking cultivation was performed at 30°C for 2 days. *Open circles*, RNase activity; *filled circles*, DMW

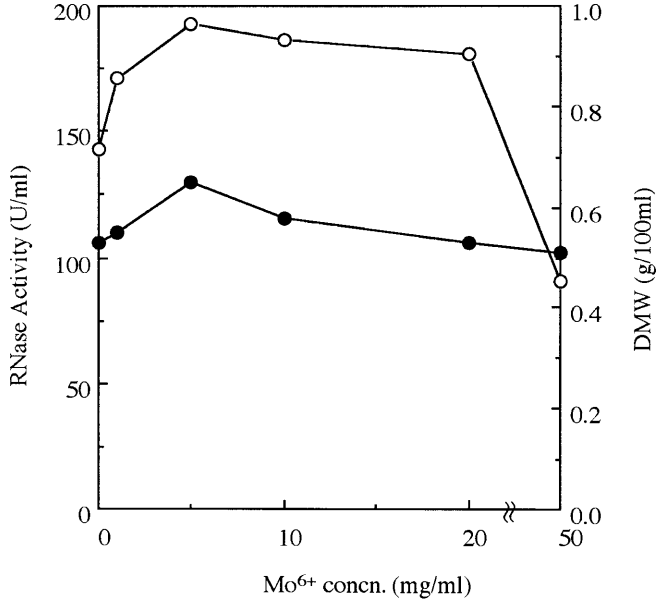


**Fig. 3.** Effects of molybdenum ions ($Mo^{6+}$) for RNase production and DMW of *Rhizopus oryzae* IFO 4697 when 100 mg/ml $Ca^{2+}$ was added to SLSR medium supplemented with 2 mg/ml $Fe^{2+}$, 49 mg/ml $Mg^{2+}$, and 0.7 mg/ml $Zn^{2+}$. Shaking cultivation was performed at 30°C for 2 days. *Open circles*, RNase activity; *filled circles*, DMW

Figure 3 shows RNase production under various concentrations of $Mo^{6+}$ added when the optimal $Ca^{2+}$ concentration (100 mg/ml) was added to the SLSR medium

**Table 3.** RNase production[a] of various *Rhizopus* strains

| Strain (origin) | RNase activity (U/ml) |
|---|---|
| *Rhizopus* sp. MKU4 (soil) | 128 |
| *Rhizopus* sp. MKU7 (soil) | 23 |
| *Rhizopus* sp. MKU8 (soil) | 78 |
| *Rhizopus* sp. MKU10 (soil) | 56 |
| *Rhizopus* sp. MKU11 (soil) | 48 |
| *Rhizopus* sp. MKU12 (soil) | 59 |
| *Rhizopus* sp. MKU17 (soil) | 52 |
| *Rhizopus* sp. MKU18 (soil) | 111 |
| *Rhizopus* sp. MKU21 (soil) | 27 |
| *Rhizopus* sp. MKU24 (soil) | 62 |
| *Rhizopus* sp. MKU32 (soil) | 27 |
| *Rhizopus* sp. MKU38 (soil) | 53 |
| *Rhizopus* sp. MKU40 (soil) | 62 |
| *Rhizopus* sp. MKU41 (ragi) | 57 |
| *Rhizopus* sp. F60 (ragi) | 48 |
| *Rhizopus* sp. F61 (ragi) | 106 |
| *Rhizopus* sp. F62 (ragi) | 89 |
| *Rhizopus* sp. F64 (ragi) | 26 |
| *Rhizopus* sp. F67 (ragi) | 73 |
| *Rhizopus* sp. F68 (ragi) | 52 |
| *Rhizopus* sp. F89 (ragi) | 21 |
| *Rhizopus* sp. F94 (ragi) | 10 |
| *Rhizopus* sp. F98 (ragi) | 42 |
| *Rhizopus* sp. ON (ragi) | 95 |
| *Rhizopus* sp. UM (ragi) | 25 |
| *Rhizopus* sp. WJ (ragi) | 20 |
| *Rhizopus* sp. Rh3 (ragi) | 39 |
| *Rhizopus* sp. LKN (tempeh) | 27 |
| *Rhizopus* sp. A-11 (ragi) | 102 |
| *Rhizopus* sp. G6 (ragi) | 24 |
| *Rhizopus* sp. G7 (ragi) | 36 |
| *Rhizopus* sp. G82 (ragi) | 34 |
| *Rhizopus* sp. UQM186F (soil) | 49 |
| *Rhizopus oryzae* TISTR3001 | 28 |
| *Rhizopus oryzae* TISTR3052 | 38 |
| *Rhizopus oryzae* TISTR3079 | 17 |
| *Rhizopus oryzae* TISTR3155 | 22 |
| *Rhizopus oryzae* TISTR3165 | 70 |
| *Rhizopus oryzae* TISTR3189 | 88 |
| *Rhizopus oryzae* TISTR3211 | 26 |
| *Rhizopus oryzae* TISTR3241 | 48 |
| *Rhizopus oryzae* TISTR3324 | 33 |
| *Rhizopus oryzae* TISTR3327 | 12 |
| *Rhizopus oryzae* IFO4697 | 193 |
| *Rhizopus oryzae* IFO5441 | 141 |
| *Rhizopus oryzae* IFO5442 | 108 |

*Rhizopus* sp. strains from ragi and tempeh were isolated by Elegado and Fujio (Elegado and Fujio 1993)
MKU, Department of Microbiology, Kasetsart University, Thailand; UQM, University of Queensland, Australia; TISTR, The Thailand Institute of Scientific Technological Research, Thailand; IFO, The Institute for Fermentation, Osaka, Japan
[a] The culture medium was SLSR medium supplemented with 2 mg/ml $Fe^{2+}$, 49 mg/ml $Mg^{2+}$, 0.7 mg/ml $Zn^{2+}$, and 100 mg/ml $Ca^{2+}$

supplemented with 2 mg/ml $Fe^{2+}$, 49 mg/ml $Mg^{2+}$, and 0.7 mg/ml $Zn^{2+}$. The addition of 5 mg/ml $Mo^{6+}$ to the basal medium was the optimum for RNase production, resulting in RNase activity and DMW of 193 U/ml and 0.65 g/100 ml, respectively.

Table 3 shows the RNase activity of various *Rhizopus* strains cultured in the metal ion-regulated liquid medium (SLSR medium supplemented with 2 mg/ml $Fe^{2+}$, 49 mg/ml $Mg^{2+}$, 0.7 mg/ml $Zn^{2+}$, 100 mg/ml $Ca^{2+}$, and 5 mg/ml $Mo^{6+}$). Among the strains tested, *Rhizopus* sp. MKU 4, MKU 18, F 61, A-11, IFO 4697, IFO 5441, and IFO 5442 showed high RNase activity, more than 100 U/mg protein. The highest producer of RNase was *Rhizopus oryzae* IFO 4697.

Our previous papers regarding glucoamylase and polygalacturonase production (Fujio and Morita 1996; Morita et al. 1999; Morita and Fujio 1999) also indicated that $Ca^{2+}$ stimulated enzyme production and that the optimal $Ca^{2+}$ concentration was 75 mg/ml and 250 mg/ml, respectively. $Mo^{6+}$ did not stimulate glucoamylase and polygalacturonase production. Many enzymes require cofactors to work; a cofactor being an organic molecule or a metal ion that is associated with the enzyme, often forming part of the catalytic mechanism. Neither $Fe^{2+}$, $Mg^{2+}$, $Zn^{2+}$, $Ca^{2+}$, or $Mo^{6+}$ acted as a cofactor of RNase (data not shown). The increase in RNase activity is not always directly proportional to the increase in mycelial growth. However, RNase production may increase along with mycelial growth.

The RNase productivity of *Rhizopus oryzae* IFO 4697 in the metal ion-regulated liquid medium (the SLSR medium supplemented with 2 mg/ml $Fe^{2+}$, 49 mg/ml $Mg^{2+}$, 0.7 mg/ml $Zn^{2+}$, 100 mg/ml $Ca^{2+}$, and 5 mg/ml $Mo^{6+}$) was 3780 U/g protein. The value on the wheat bran solid medium was 180 U/g protein.

Horitsu et al. (1974) reported on RNase production of *Aspergillus niger* in liquid culture. The strain produced 130 U/mg protein. Our culture medium consists of only artificial composition, except for cassava starch. The medium achieved a high specific RNase activity. It is possible that RNase from *Rhizopus oryzae* IFO 4697 in the liquid culture is easily purified and valuable as an industrially useful enzyme. Thompson and Eribo (1984) reported that a solid culture of *Rhizopus* strain is superior to a liquid culture in terms of RNase production. However, we established a liquid culture system for RNase production with much higher specific activity by controlling some specified metal ion concentrations in a liquid medium.

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