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Development of Detection Methods for Cellulolytic Activity of *Auricularia auricula-judae*

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To obtain basic information on the detection of cellulolytic activity in *Auricularia auricula-judae*, the influences of dye reagent, pH, and temperature were assessed. Chromogenic dye (congo red, phenol red, remazol brilliant blue, and trypan blue) was individually incorporated into a medium containing either carboxymethyl-cellulose, Avicel, or D-cellobiose as a polysaccharide carbon substrate. The other assessments utilized pHs ranging from 4.5 to 8.0 and temperatures from 15~35°C. Overall, when *A. auricula-judae* species were transferred onto media contained Congo red and adjusted pH 7.0 and then incubated at 25°C for 5 days, the clear zone indicative of cellulolytic activity was more pronounced.

KEYWORDS : *Auricularia auricula-judae*, Cellulolytic enzyme, Chromogenic media, Congo red

Auricularia auricula-judae, which is also known as wood ear, free ear, black ear mushroom, and free jelly fish, is an edible mushroom that is cultivated mainly in China, Taiwan, Thailand, and Indonesia. The mushroom is reddish brown, gelatinous, usually wrinkled or veined surface and without distinctive smell [1, 2].

A. auricula-judae is consumed medicinally as it demonstrates significant antitumor, cardiovascular, and hypercholesterolemic effects [3-5]. It grows singly or usually in clusters on coniferous and deciduous wood, and so is classified as a wood-decaying fungus [6, 7]. *A. auricula-judae* may also be useful industrially, given the prolific and high-quality production of cellulase and carboxymethyl cellulase by *Pleurotus sajor-caju* and *Pholiota nameko*, respectively [8-10].

Since *A. auricula-judae* is a wood-decaying fungus, we hypothesized that it may produce cellulolytic enzymes capable of degrading the components of wood cell including cellulose (cellulase), hemicelluloses (hemicellulase), and lignin (ligninase). To address this hypothesis, it was necessary to screen for optimal media conditions for the detection of cellulolytic enzymes. For this aim, we selected a method based on a dye coupled to a polysaccharide. When the dye or dye-polymer complex is hydrolyzed, pale or colorless haloes are produced [11, 12]. Cellulose was presently used as the carbon substrate as it is typically used for the detection of fungi that secrete cellulolytic enzymes. *Trichoderma* and *Saccharomyces* were used as a positive and negative control for cellulolytic enzyme production, respectively.

A total of three *A. auricula-judae* species and control cultures were obtained from the Korean Agricultural Culture Collection (KACC, Suwon, Korea) and the authors' laboratory, respectively. All the cultures were prepared at 25°C for 5~7 days on potato dextrose agar (Difco, Detroit, MI, USA). The basic medium was comprised of 0.1% yeast nitrogen base (Difco) as a nitrogen source and 1.5% agar powder. In addition, one of three polysaccharides, carboxymethyl (CM)-cellulose (Sigma-Aldrich, St. Louis, MO, USA), Avicel (Fluka, Buches, Switzerland), and D-cellobiose (Sigma-Aldrich), was also added to the medium as the carbon substrate. After incubation, each plate was positioned on a white light box the diameter of the pale or colorless haloes (hereafter referred to as the clear zone) around each fungal colony was measured using the aided eye; the halo results from the reaction between the enzymes secreted by fungi and the dye or dye-polysaccharide complex.

To select the best dye to detect cellulolytic activity, 0.5% of a chromogenic dye (congo red, phenol red, remazol brilliant blue, or trypan blue; Sigma-Aldrich) was added the uninoculated medium. Cultures were transferred to each chromogenic medium and incubated at 25°C for 5 days. To evaluate the effect of pH, the chromogenic medium judged to be superior was prepared at pH of 4.0~8.0 and, following inoculation, incubation was carried out at 25°C for 5 days. Lastly, the aforementioned media were incubated at temperatures of 15~35°C. Each assay was repeated three times.

The results of cellulolytic activity test of the three *A.*

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Table 1. Comparison of chromogenic reaction by *Auricularia auricula-judae* on media containing different dyes

Species	Congo red			Phenol red			Remazol brilliant blue			Trypan blue		
	CM	Avi	Cel	CM	Avi	Cel	CM	Avi	Cel	CM	Avi	Cel
A. auricula-judae GBAA-01	+	+	+	+	+	+	+	+	+	–	–	+
A. auricula-judae ASI 6009	+	+	+	+	+	+	–	–	+	–	–	–
A. auricula-judae ASI 6021	+	+	+	+	+	+	–	–	–	–	–	–
Trichoderma (positive control)	+	+	+	–	–	–	–	–	–	–	–	–
Saccharomyces (negative control)	+	+	+	–	–	–	–	–	–	–	–	–

+, clear zone detection; –, no clear zone detection.
CMC, carboxymethyl cellulose; Avi, Avicel; Cel, D-cellobiose.

auricula-judae species are given in Table 1. The clear zone of *Trichoderma* and *Saccharomyces*, used as the positive and negative controls, respectively, only appeared in the congo red-containing medium; the reaction exhibited by *Trichoderma* was particularly pronounced. In contrast, all three *A. auricula-judae* species consistently formed clear zones on congo red- and phenol red-containing media, with clear zones appearing only occasionally on remazol brilliant blue- and trypan blue-containing media. Fig. 1 shows the clear zone formed due to β-glucosidase activities of *Trichoderma* and *A. auricula-judae* GBAA-01. The clear zone produced in the presence of the D-cellobiose carbon source was superior to those evident using the other polysaccharide carbon sources. The clear zone was also more clearly formed in the congo red-containing medium than the other media consistent with the use of the dye in the detection of extracellular enzymes in various fungi [13, 14].

Congo red changes color from blue to red at pH 3.0~5.2. As the growing fungi degrade cellulose to organic acids, the medium pH declines and a series of reactions lead to a medium change in color from red to light purple or light gray. Presently, congo red was used as the chromogenic dye in media that varied in pH from 4.5~8.0. The different pH influenced the color of the medium and the appearance of the clear zone (Fig. 2). Especially, pH 4.5 and 5.0 media were very dark in color. The clear zones evident at pH 4.5, 5.0, and 6.0 appeared to have an uncertain boundary line, and the zone at pH 8.0 was smaller than the one detected at pH 7.0. Subsequent experiments were conducted at pH 7.0. Regarding the carbon substrates, recognition of clear zones in the CM-cellulose-containing medium was hindered by the presence of black spots. Among the five tested temperatures, well-formed clear zones were evident at 25°C and 30°C, with the lower temperature producing a superior appearing zone

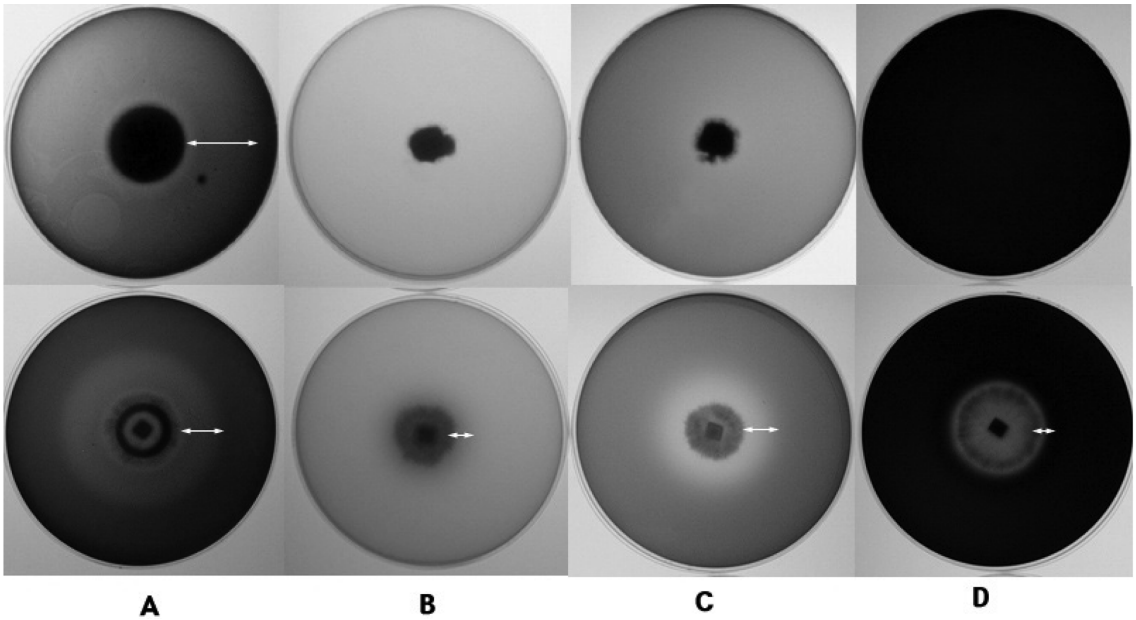

Fig. 1. Examples of clear zones formed in media containing D-cellobiose and dye (A, congo red; B, phenol red; C, remazol brilliant blue; D, trypan blue). Top row, after incubation of *Trichoderma* used as positive control. Bottom row, after incubation of *Auricularia auricula-judae* GBAA-01. Arrows indicate clear zones.

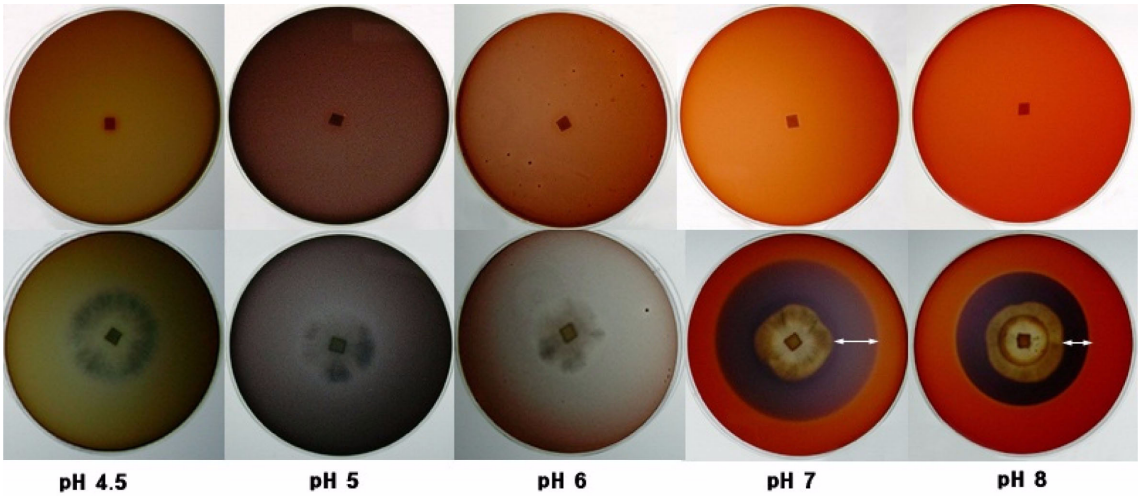

Fig. 2. Examples of clear zones formed in pHs ranging from 4.5 to 8.0. Media contain D-cellobiose and congo red as carbon substrate and chromogenic dye, respectively. Top row, before incubation. Bottom row, after incubation of *Auricularia auricula-judae* GBAA-01. Arrows indicate clear zones.

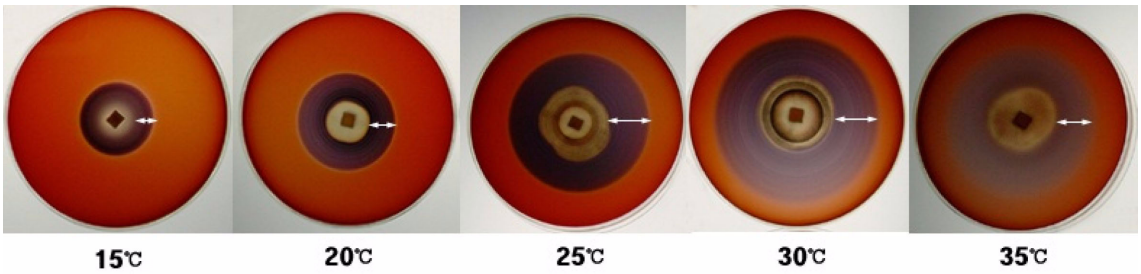

Fig. 3. Result of chromogenic reaction of *Auricularia auricula-judae* GBAA-01 on the congo red containing, pH 7.0 medium incubated at various temperatures. D-cellobiose was used as the carbon substrate. Arrows indicate clear zones.

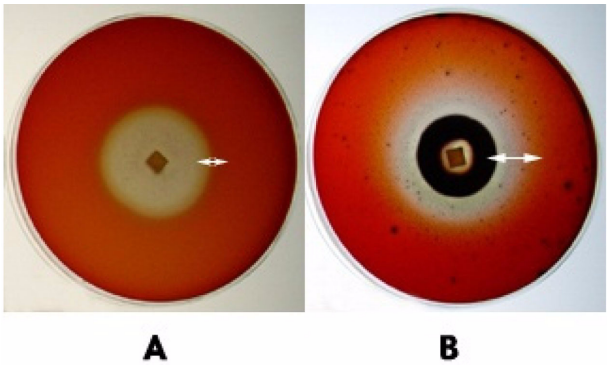

Fig. 4. Clear zone formed on congo red-containing, pH 7.0 medium by chromogenic reaction of *Auricularia auricula-judae* GBAA-01. Carbon substrates used were Avicel (A), and carboxymethyl-cellulose (B). Arrows indicate clear zones.

than at 30°C (Fig. 3). The shape of the clear zones was also different depending on the added carbon substrate (Fig. 4).

The present results reveal that detection of cellulolytic enzyme activity in *A. auricula-judae* species is best accomplished in a congo red-containing, pH 7.0 medium incubated at 25°C for 5 days. Although further studies on different large-scale assessments are needed, the present results should aid those who are studying extracellular enzyme activity in mushrooms.

Acknowledgements

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