Optimal Medium Conditions for the Detection of Cellulolytic Activity in *Ganoderma lucidum*

Woo-Sik Jo^{1*}, Soon-Hwa Bae¹, Doo-Hyun Cho¹, So-Deuk Park¹, Young-Bok Yoo² and Seung-Chun Park³

¹Department of Agricultural Environment, Gyeongbuk Agricultural Technology Administration, Daegu 702-320, Korea

²Mushroom Research Division Department of Herbal Crop Research National Inst. of Horticultural & Herbal Science, RDA, Suwon 404-707, Korea

³College of Veterinary Medicine, Kyeongbuk National University, Daegu 702-701, Korea

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To determine the optimal medium conditions for the detection of the cellulolytic activity in *Ganoderma lucidum*, we varied three media conditions: dye reagent, pH, and temperature. First, we evaluated the use of four dyes, Congo Red, Phenol Red, Remazol Brilliant Blue, and Trypan Blue. To observe the effect of pH on the chromogenic reaction, we also made and tested various media spanning acidic and alkaline pHs, ranging from 4.5 to 8.0. Furthermore, in order to research the effect of temperature on the clear zone and the fungus growing zone, we tested temperatures ranging from 15 to 35°C. On the whole, the best protocol called for *Ganoderma lucidum* transfer onto media containing Congo red with pH adjusted to 7.0, followed by incubation at 25°C for 5 days. Our results will be useful to researchers who aim to study extracellular enzyme activity in *Ganoderma lucidum*.

KEYWORDS : Cellulolytic enzyme, Chromogenic media, Congo red, *Ganoderma lucidum*

Studies about fungi in Korea have been reported extensively with regard to their classification, nutritional components and effective components. In particular, due to the rapid development of fungal enzymology during the past three decades, amylolytic, cellulolytic, proteolytic, and pectolytic enzymes have been adopted for use in many fields, including industry, medicine, pharmacy, and agriculture. As a result, development of methods to purify high-quality enzymes from fungi is a rapidly progressing area of study (Park *et al.*, 1986). Hong *et al.* have isolated a cellulase from *Pleurotus sajor-caju*, and Hashimoto has extracted carboxymethyl cellulase from *Pholiota nameko*, an edible mushroom (Hashimoto, 1972; Hong *et al.*, 1984).

Ganoderma lucidum is distributed in Asia and is called Young Zhi, Ling Zhi, and Mannentake (or Reishi) in Korea, China, and Japan, respectively. In these countries, this fungus has been famous as a traditional and folk medicine used for the cure of various diseases and cancers solely or in combination with other herbal medicines (Kim and Kim, 1999). Also, *Ganoderma lucidum* is a high-class, wood-decaying fungus. Once wood-decaying fungi mycelia enter woody tissue, the fungi grow continuously using components extracted the cells of the wood. The wood-decaying fungi can degrade cellulose, hemicellulose, and lignin, the main substrates of the wood cell wall, by secreting enzymes such as cellulase, hemicellu-

lase, and ligninase (Abraham *et al.*, 1998; Shin *et al.*, 1991).

A plate assay is a frequently used screening method for detection of microorganisms that secrete extracellular enzymes. From a number of possible plate assays for polysaccharide activity, we selected a method based on a dye coupled to a polysaccharide. When the dye-polymer complex is hydrolysed, the dye or polysaccharide-dye molecules diffuse from the colony zone, producing pale or colorless haloes. This method is cost-efficient, simple, and convenient (Castro *et al.*, 1993; Hejgaard and Gibbons, 1979).

For this assay, we wanted to determine the optimal medium conditions for the detection of cellulolytic activity in *Ganoderma lucidum*. We chose cellulose as the carbon substrate as it is the most plentifully presented substrate on the planet and has been commonly used for the screening of cellulase producing fungi. The aims of this study were: first, to determinate the best dye to detect the cellulolytic activity of *Ganoderma lucidum* and second, to screen and select the most suitable pH and temperature values.

A total of 3 *Ganoderma lucidum* species were obtained from the Korean Agricultural Culture Collection (KACC, Suwon, Korea), and others were prepared by the authors' laboratory. Preculturing of all the cultures was done on potato dextrose agar (Difco, USA) at 25°C for 5 days. To correctly identify cellulolytic activity, *Trichoderma* was used as a positive control and *Saccharomyces* as a nega-

*Corresponding author <E-mail : jowoosik@yahoo.co.kr>

tive control. To detect cellulolytic activity, the precultures were transferred onto chromogenic media. The basis of the chromogenic media consisted of 0.1% yeast nitrogen base (Difco, USA) as a nitrogen source and 1.5% agar powder. Besides this, the media also contained 0.5% Congo Red, Phenol Red, Remazol Brilliant Blue, or Trypan Blue (Sigma, USA) as the chromogenic dye linked to one of three polysaccharides: CM-cellulose (Sigma, USA) to measure CM-cellulase activity, Avicel (Fluka, Ireland) for avicelase, and D-cellobiose (Sigma, USA) for β -glucosidase. After incubation for 5 days at 25°C, estimation

of cellulase activity was conducted by observing the clear zone formed around each fungal colony, which results from the reaction between the enzymes secreted by the colony and the chromogenic substrates. The clear zone of each sample was observed with the naked eye and photographed while placed on a white light box. To evaluate the effect of pH on the chromogenic reaction, the cultures were transferred to Congo red-containing media in different pHs ranging from 4.5 to 8.0, and incubated at 25°C for 5 days. To evaluate the effect of temperature, the cultures were also transferred to Congo red media con-

Table 1. Comparison of clear zone formation by *Ganoderma lucidum* on media with different dyes

Species	Congo red			Phenol red			Remazol brilliant blue			Trypan blue		
	CM	Avi	Cel	CM	Avi	Cel	CM	Avi	Cel	CM	Avi	Cel
<i>Ganoderma lucidum</i> ASI 7092	+	+	+	–	–	–	–	+	+	–	–	–
<i>Ganoderma lucidum</i> ASI 7125	+	+	+	–	–	+	+	+	+	–	–	–
<i>Ganoderma lucidum</i> ASI 7127	+	+	+	–	+	–	+	+	+	–	–	–
<i>Trichoderma</i> . (positive control)	+	+	+	–	–	–	–	–	–	–	–	–
<i>Saccharomyces</i> . (negative control)	+	+	+	–	–	–	–	–	–	–	–	–

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

Fig. 1. Examples of clear zones detected in chromogenic media containing D-cellobiose and different dyes (A, Congo red; B, phenol red; C, remazol brilliant blue; D, trypan blue). Top row: before incubation. Middle row: after incubation of *Trichoderma*. Bottom row: after incubation of *Ganoderma lucidum* ASI 7125. Arrows indicate clear zones.

trolled at pH 7.0, and incubated for 5 days at different temperatures ranging between 15 to 35°C. These assays were each repeated three times.

The results of the chromogenic reaction for the four different dyes are given in Table 1. The clear zone appeared in the chromogenic media for Congo red, phenol red, and remazol brilliant blue, but no clear zone appeared in the media with trypan blue. Fig. 1 shows representative examples of the clear zones observed due to β -glucosidase activity. In the cases of *Trichoderma* and *Saccharomyces*, used as the positive and negative controls, respectively, the clear zone was only detected in the Congo red media. Media containing any of the three carbon substrates, CM-cellulose, avicel, and D-cellobiose, showed the clear zone. The clear zone of *Trichoderma* was the largest, but no significant clear zone was detected for *Saccharomyces*. *Ganoderma lucidum*, on the other hand, formed a clear zone not only on Congo red-containing media but also remazol brilliant blue-containing media. *Ganoderma lucidum* ASI 7125 and 7127 formed clear zones in media containing CM-cellulose, avicel, or D-cellobiose. In contrast, no clear zone was observed for *Ganoderma lucidum* ASI 7092 in media containing CM-cellulose. Also, in some cases, a clear zone was observed on the phenol red-containing media, but no clear zone was observed on trypan blue-containing media. Among the four dyes, the clear zone was much more clearly dis-

played in the Congo red-containing media than the other three dye-containing media. For this reason, we used Congo red for further analysis.

Congo red is known to be usable as a pH indicator, due to a color change from blue to red at pH 3.0–5.2. The fungi can first degrade cellulose into cellobiose, then break down cellobiose to form glucose, and finally metabolize glucose to organic acids. The organic acids secreted by the fungi lower the media pH and result in the Congo red media changing color from red-orange to light gray, with light purple or light blue. Therefore we made various media with pH ranging from 4.5 to 8.0, including both acidic and alkaline pHs. The pH difference affected the color of the media and the formation of the clear zone (Fig. 2). All in all, clear zones were not clearly observed in media with pH 4.5 or 5.0 because of the media's dark color. The clear zone at pH 6.0 appeared to have an uncertain boundary line, and the clear zone at pH 8.0 appeared to be smaller than the one observed at pH 7.0. As a result, we selected pH 7.0 for the detection of the cellulolytic activity in *Ganoderma lucidum*. For the carbon substrates media containing avicel or D-cellobiose clearly showed clear zones with a distinct color change and boundary line. However, media with CM-cellulose showed a clear zone with an uncertain boundary line but a clear change of color. In addition, we tested in temperatures ranging from 15 to 35°C, in order to research how

Fig. 2. Examples of chromogenic reactions on media containing Congo red and D-cellobiose over a range of pH values. Top row: before incubation. Middle row: after incubation of *Trichoderma*. Bottom row: after incubation of *Ganoderma lucidum* ASI 7127. Arrows indicate clear zones.

Fig. 3. Examples of chromogenic reactions on the Congo red containing, pH 7.0 media over a range of temperatures. D-cellobiose was used as the carbon substrate. Top row: after incubation of *Trichoderma*. Bottom row: after incubation of *Ganoderma lucidum* ASI 7125. Arrows indicate clear zones.

temperature affects the clear and fungus growing zones. Among the 5 tested temperatures, 25°C was the most suitable temperature for all three carbon substrates (Fig. 3).

Our work identifies the optimal medium conditions, dye reagent, pH, and temperature, for an assay detecting the cellulolytic activity of *Ganoderma lucidum*. Regarding the dye reagent for screen extracellular enzyme-producing fungi, Hyun *et al.* (2006) reported that Congo red was used successfully in the detection of cellulolytic activity in *Ophiostoma* and *Leptographium* species. Also, Yoon *et al.* (2007) proved that Congo red can be used in the detection of extracellular cellulase in various fungi. By the same token, we confirmed that the Congo red was the most suitable dye for the detection of the cellulolytic activity in *Ganoderma lucidum*. Also, clear zone at pH 7.0 was much more clearly than the other pH ranges media and 25°C was the most suitable temperature among 5 ranges of temperature. In general terms, the best method calls for *Ganoderma lucidum* transfer onto media containing Congo red at pH 7.0, followed by 5 day incubation at 25°C. We expect that our results will be useful to researchers who aim to study extracellular enzyme activity in *Ganoderma lucidum*.

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