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Optimal Media Conditions for the Detection of Extracellular Cellulase Activity in *Ganoderma neo-japonicum*

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To determine the optimal media conditions for the detection of the extracellular cellulase activity in *Ganoderma neo-japonicum,* we varied three media conditions: dye reagent, pH, and temperature. 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 tested media ranging from 4.5 to 8.0. 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 neo-japonicum* transfer onto media containing Congo red with a pH of 7.0, followed by incubation at 25°C for 5 days. Our results will be useful to researchers who study extracellular enzyme activity in *Ganoderma neo-japonicum.*

KEYWORDS : Cellulase, Chromogenic media, Congo red, *Ganoderma neo-japonicum*

Many studies about fungi in Korea have been done 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, cellolytic, 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 [1]. Hong *et al*. [2] have isolated a cellulase from *Pleurotus sajor-caju*, and Hashimoto [3] have extracted carboxy methylcellulase from *Pholiota nameko,* an edible mushroom. *Ganoderma neo-japonicum* is found throughout Japan and so is called Japanese fungi. This fungus is famous as a traditional and folk medicine and is used to cure various diseases and cancers alone or in combination with other herbal medicines [4]. *G. neo-japonicum* is a high-class, wood-decaying fungus. Once *G. lucidum* mycelia enter woody tissue, the fungus grows continuously using components extracted the cells of the wood. Wood-decaying fungi can degrade cellulose, hemicellulose, and lignin, the main components of the wood cell wall, by secreting enzymes such as cellulase, hemicellulase, and ligninase [5, 6]. In this regard, we expected that *G. neo-japonicum* has cellulolytic activity and tested it.

A plate assay is a frequently used as a 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 colourless haloes. This method is cost-efficient, simple, and convenient [7, 8].

For this assay, we wanted to determine the optimal media conditions for the detection of extracellular enzyme activity of *G. neo-japonicum*. We chose cellulose as the carbon substrate as it is the most plentiful substrate on the planet and has been commonly used for the screening of cellulase producing fungi. The aims of this study were: 1) to determinate the best dye to detect the extracellular cellulase activity of *Ganoderma neo-japonicum*, and 2) to screen for and select the most suitable pH and temperature.

Two *Ganoderma* genera 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, Franklin Lakes, NJ, USA) at 25°C for 5 days. To correctly identify extracellular cellulase activity, *Trichoderma* was used as a positive control and *Saccharomy-*

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Table 1. Comparison of clear zone formation by *Ganoderma neo-japonicum* 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
Ganoderma neo-japonicum	+	+	+	+	+	+	+	+	+	-	-	-
Ganoderma lucidum (GBGL-01)	+	+	+	+	+	+	+	+	+	-	-	-
Trichoderma (positive control)	+	+	+	-	-	-	-	-	-	-	-	-
Saccharomyces (negative control)	+	+	+	-	-	-	-	-	-	-	-	-

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

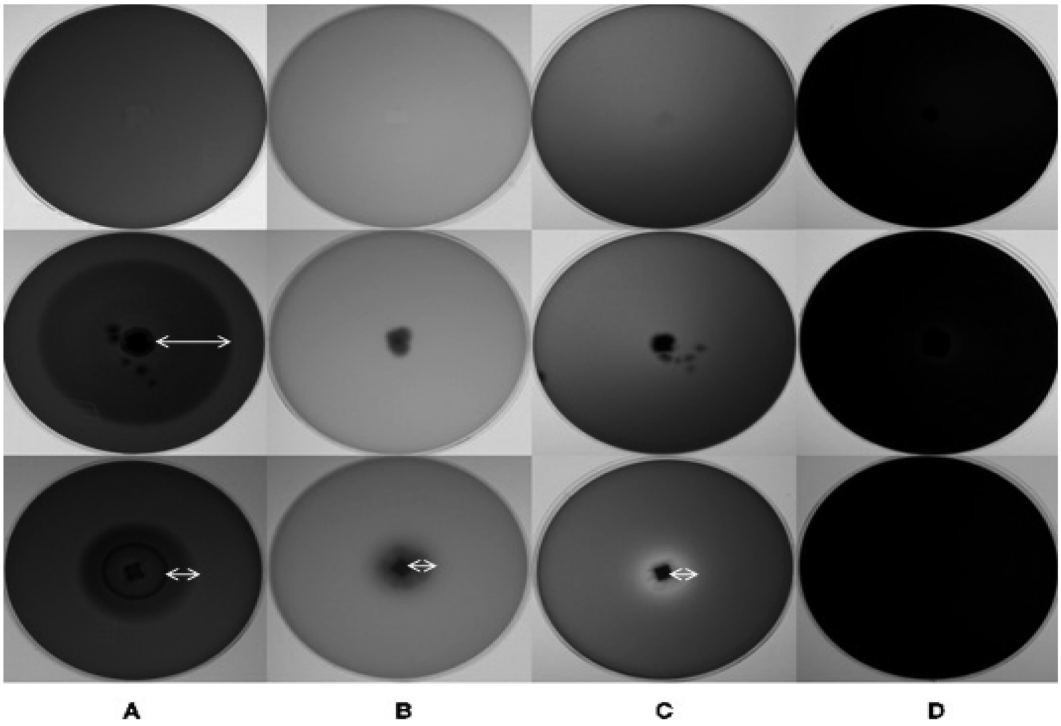

Fig. 1. Examples of clear zones detected in chromogenic media containing D-cellobiose and different dyes (A, Congo red; B, phenol red; C, remazol brilliant blue; D, trypan blue). Top row: before incubation. Middle row: after incubation of *Trichoderma*. Bottom row: after incubation of *Ganoderma neo-japonicum.*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

ces was used as a negative control. To detect extracellular cellulase activity, the precultures were transferred onto chromogenic media. The chromogenic media consisted of 0.1% yeast nitrogen base (Difco) as a nitrogen source and 1.5% agar powder. In addition, the media contained 0.5% Congo red, phenol red, remazol brilliant blue, or trypan blue (Sigma, St. Louis, MO, USA) as the chromogenic dye linked to one of three polysaccharides: CM-cellulose (Sigma) to measure CM-cellulase activity, Avicel (Fluka, Ireland) for avicelase, and D-cellobiose (Sigma) 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 on a white light box. To evaluate the effect of pH on the chromogenic reaction, the cultures were transferred to Congo red-containing media of 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 transferred to Congo red media maintained at pH 7.0, and incubated for 5 days at different temperatures ranging from 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 *Saccaromyces* (the positive and negative controls, respectively), clear zones were only detected in the Congo red media. Media containing any of the three carbon substrates, CM-cellu-

lose, avicel, or D-cellobiose, showed clear zones. The clear zone of *Trichoderma* was the largest, but no significant clear zone was detected for *Saccharomyces*. *G. neo-japonicum*, on the other hand, formed a clear zone on both Congo red-containing media and remazol brilliant blue-containing media. *G. neo-japonicum* and *G. lucidum* (GBGL-01) formed clear zones in media containing CM-cellulose, avicel, or D-cellobiose. 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 obvious in the Congo red-containing media than in the other three dye-containing media. For this reason, we used Congo red for subsequent analyses.

Congo red is a pH indicator that changes from blue to red at pH 3.0~5.2. Fungi first degrade cellulose into cellobiose, and 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, resulting in the Congo red media changing color from red-orange to light gray, with light purple or light blue. Therefore, we made media with pHs ranging from 4.5 to 8.0. The pH difference affected the color of the media and the formation of the clear zone (Fig. 2). Clear zones were not

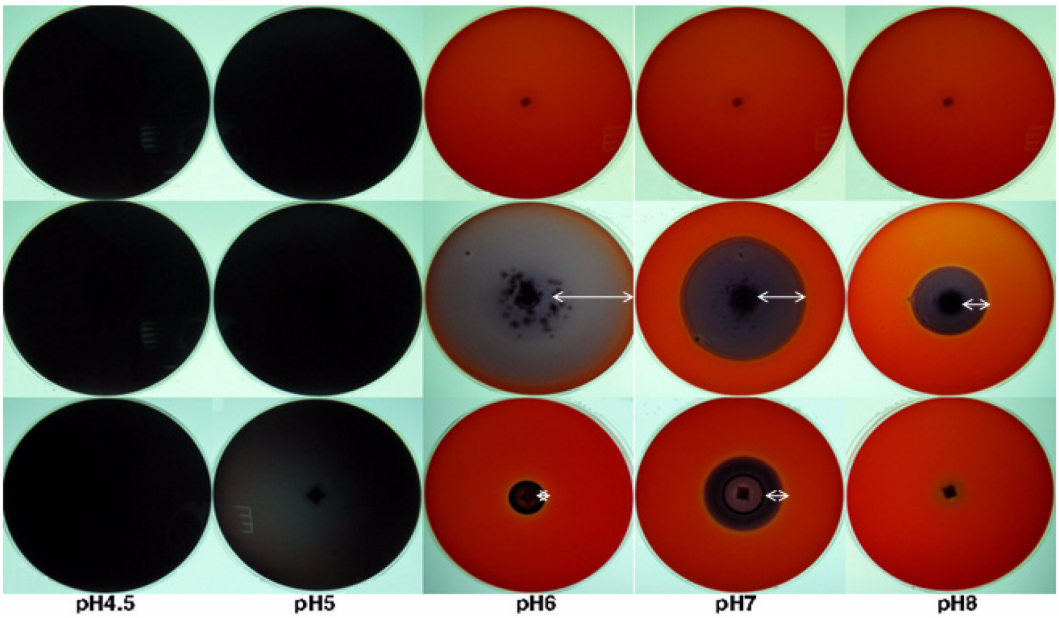

Fig. 2. Examples of chromogenic reactions on media containing Congo red and D-cellobiose over a range of pH. Top row: before incubation. Middle row: after incubation of *Trichoderma*. Bottom row: after incubation of *Ganoderma neo-japonicum*. Arrows indicate clear zones.

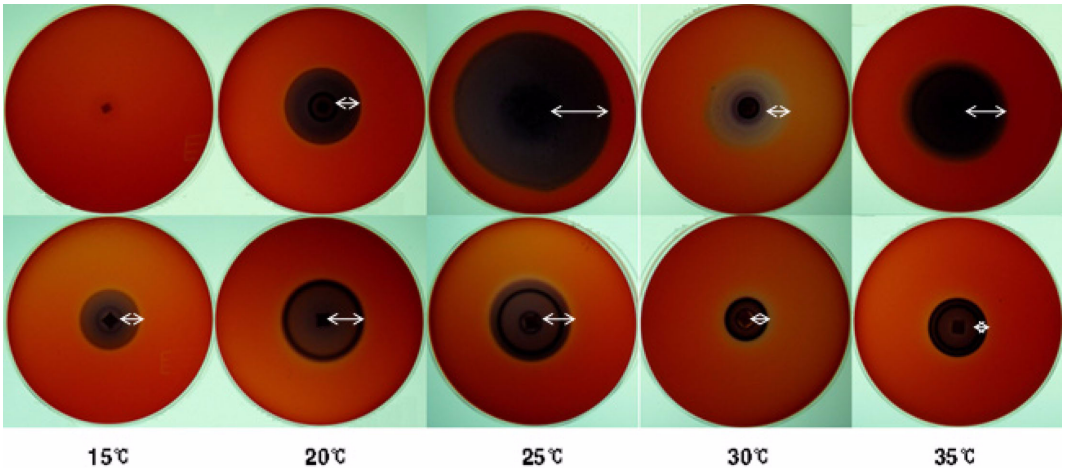

Fig. 3. Examples of chromogenic reactions on media containing Congo red at pH 7.0 over a range of temperatures. D-cellobiose was used as the carbon substrate. Top row: after incubation of *Trichoderma*. Bottom row: after incubation of *Ganoderma neo-japonicum*. Arrows indicate clear zones.

clearly observed in media of pH 4.5 or 5.0 because of the dark color of the media. The clear zone at pH 6.0 had an uncertain boundary line, and the clear zone at pH 8.0 was smaller than the one observed at pH 7.0. As a result, we selected pH 7.0 for the detection of the extracellular cellulase activity in *G. neo-japonicum*. For the carbon substrates, media containing avicel and D-cellobiose showed clear zones with a distinct color change and boundary line. Media with CM-cellulose showed a clear zone with an uncertain boundary line but a clear change of color. In addition, we tested in different temperatures ranging from 15 to 35°C, to determine how 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 media conditions, dye reagent, pH, and temperature for assays detecting the extracellular cellulase activity of *G. neo-japonicum*. Congo red has been used successfully in the detection of cellulolytic enzymes in *Ophiostoma* and *Leptographium* species [9]. Yoon *et al*. [10] also showed that Congo red can be used in the detection of extracellular cellulase in various fungi. We confirmed that Congo red was the most suitable dye for the detection of the extracellular cellulase activity in *G. neo-japonicum*. The clear zone at pH 7.0 was much more clear than at other pHs and 25°C was the best temperature. Therefore, the best method calls for *G. neo-japonicum* transfer onto media containing Congo red at pH 7.0, followed by a 5 day incubation at 25°C. We expect that our results will be useful to researchers who study extracellular enzyme activity in *G. neo-japonicum.*

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