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Comparison of Dyes for Easy Detection of Extracellular Cellulases in Fungi

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To evaluate which dye is effective in a plate assay for detecting extracellular cellulase activity produced by fungi, four chromogenic dyes including remazol brilliant blue, phenol red, congo red, and tryphan blue, were compared using chromagenic media. For the comparison, 19 fungal species belonging to three phyla, ascomycota, basidiomycota, and zygomycota were inoculated onto yeast nitrogen-based media containing different carbon substrates such as cellulose (carboxylmethyl and avicel types) and cellobiose labeled with each of the four dyes. Overall, the formation of clear zone on agar media resulting from the degradation of the substrates by the enzymes secreted from the test fungi was most apparent with media containing congo red. The detection frequency of cellulase activity was also most high on congo red-supplemented media. The results of this study showed that congo red is better dye than other three dyes in a plate assay for fungal enzyme detection.

KEYWORDS: Cellulase, Chromogenic media, Congo red, β-Glucosidase

Fungi are very good sources of producing diverse enzymes that can degrade natural polymeric compounds such as cellulose, pectin and starch. Since these polysaccharides are widely used in various industries such as food and dairy, pulp and paper, textile, animal feed, pharmaceutical, detergent, cosmetic, and chemical-synthesis processes, development of enzyme systems that break down the polysaccharides has been looked for. For this purpose, fungi have been collected and screened for their ability of producing extracellular enzymes.

For the screening the method should not be expensive, tedious, and inconvenient. Especially, when we have to screen large numbers of sampled fungi, efficient screening methods are a prerequisite (Leonid *et al.*, 2004). Plating assay is one of the screening methods that have been frequently used. A number of plate-screening methods for the detection of polysaccharide-degrading microorganisms have been described in the literature. These methods are usually based on the complex formation between polysaccharides and dyes (Teather and Wood, 1982) and the use of soluble and insoluble dye-labeled polysaccharides to generate visible clear halo zone around the colony-derived enzyme (Castro *et al.*, 1995). The plate screening methods with chromogenic substrates provide an array of relatively straightforward and simply applicable tools for specific detection of polysaccharide degrading fungi. Reactive dyes such as congo red, phenol red, remazol brilliant blue and tryphan blue have been known for making chromogenic media. However, no comparative data has been available that show which dye is better in the use of chromogenic media for the evaluation of the ability of secreting extracellular enzymes in diverse groups of fungi.

Therefore, in this study we compared the four commonly used chromogenic dyes to determine which dye is favorable for the detection of extracellular enzyme activities in diverse fungal species. For the comparison, we chose cellulose as substrate that is the most abundantly produced biopolymer on the earth. Here we report sound data that shows congo red is recommended chormogenic dye in a plate assay for the selection of cellulose-degrading fungi.

Materials and Methods

Fungal cultures. A total of 19 fungal species belonging to three phyla, ascomycota, basidiomycota, and zygomycota, were tested for cellulase activity in chromagenic media. Some fungal cultures were obtained from Korean Agricultural Culture Collection (KACC) and American Type Culture Collection (ATCC), others were field isolates from the authors' laboratory (Table 1). All the cultures were maintained on potato dextrose agar (Difco, USA).

Cellulolytic activity test. The fungal isolates were precultured on 2% potato dextrose agar or 2% malt extract agar (Difco, USA) at 25°C for 5 days. For the observation of fungal extracellulase activity, the precultures were transferred onto the media containing each of 0.5% CM-cellulose (Sigma, USA), D-cellobiose (Sigma, USA), Avicel (Fluka, Ireland) as enzymatic carbon source, 0.1% yeast nitrogen base (Difco, USA) as its fundamental nitrogen

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Table 1. Comparison of chromogenic reaction by fungal extracellular cellulases on medium containing different dyes

Phylum	Species	Congo red			Phenol red			Remazol brilliant blue			Tryphan blue		
		CMC	Avi	Cel	CMC	Avi	Cel	CMC	Avi	Cel	CMC	Avi	Cel
Ascomycota	*Ampelomyces quercinus*	+	−	+	−	−	−	−	−	+	−	−	−
	Aspergillus lucknowensis	+	+	+	−	−	+	−	−	−	−	−	−
	Botryotinia fuckeliana	−	−	+	−	−	−	−	−	+	−	−	+
	Botrytis elliptica	+	−	+	+	+	+	+	−	+	−	−	−
	cladosporium cladosporioides	+	−	+	−	−	−	+	−	+	−	−	+
	Epicoccum nigrum	−	+	+	−	−	−	−	+	+	−	−	−
	Magnaporthe grisea	+	+	+	+	+	+	−	+	−	−	−	−
	Fusarium solani	+	+	+	−	+	+	−	+	+	−	−	−
	Penicillium aurantiogriseum KACC 41338	−	−	+	−	−	+	−	−	−	−	−	−
	P. daleae KACC 41627	+	+	+	+	+	+	−	−	−	−	−	−
	P. soppii	−	−	+	−	−	−	−	−	−	−	−	−
	Trichoderma atroviride KACC 40774	−	−	+	−	−	+	−	−	−	−	−	−
	T. harzianum KACC 40787	−	−	+	−	+	+	−	−	−	−	−	−
	T. reesei KACC 40745	−	−	+	−	+	+	−	−	−	−	−	−
	T. reesei ATCC 56765 (positive control)	+	−	+	−	−	+	−	−	−	−	−	−
	Saccharomyces cerevisiae (negative control)	−	−	-	−	−	−	−	−	−	−	−	−
Basidiomycota	*Coprinus radians*	−	−	+	−	−	−	−	−	−	−	−	−
	Lentinus edodes	+	+	+	−	−	+	−	−	+	−	−	+
Zygomycota	*Mortierella* sp.	+	+	+	−	+	+	−	−	−	−	−	−
	Mucor mucedo	−	−	+	−	−	−	−	+	−	−	−	−

CMC, Carboxymethyl-cellulose; Avi, Avicel; Cel, Cellobiose. +, clear zone detection; −, no clear zone detection.
KACC: Korean Agricultural Culture Collection. ATCC: American Type Culture Collection.

source, 0.5% dyes (Congo Red, Phenol Red, Remazol Brilliant Blue and Tryphan Blue, Sigma, USA) for chromogenic reaction, and 1.5% agar powder. After 5~7 days of culturing at 25°C, evaluation of enzyme activity was performed by observing clear zone (plaque) formed around the fungal colony by reaction between the enzymes secreted by the fungi and chromogenic substrates. Clear zone was observed by naked eyes or documented by taking a photo. Photos were taken after mounting the plate onto a light box. Moreover, to estimate the effect of pH on chromogenic reaction, cultures were inoculated on congo red-contained media having different pHs, and incubated at 25°C for 5 days. To make certain the presence of enzyme activity, *Trichoderma reesei* (ATCC56765), a known cellulolytic fungal isolate, was used as a positive control. *Saccharomyces cerevisiae* was used as a negative control for no enzyme activity detection.

Results and Discussion

It is widely accepted that cellulases secreted by fungi consist of three major components: endoglucanases, cellobiohydrolases and β-glucosidases. These enzymes are known to be involved in cellulose and cellobiose degradation. Therefore we used these carbon substrates to assess which chromogenic dye is better for a plate assay of detecting fungal extracellular cellulases.

All the detection results of cellulolytic activity based on the formation of clear zone were given at Table 1. Among the four dyes used, positive detection of clear zone was most highly observed in the medium contained congo red. In case of D-cellobiose substrate, clear zone that showing the presence of β-glucosidase activity could be detected from all the media contained the 4 dyes tested. The representative example of clear zone detection was shown in Fig. 1. Among the four positive reactions, clear zone was more clearly observed by naked eyes from the media containing congo red than from the media containing other three dyes. Based this observation, we selected congo red for further analysis of cellulases. As we could see Fig. 1A, the color of congo red contained media was changed from red-orange to light gray with light purple or light blue depending on fungal species. We questioned what could effect on color change. Congo red is known that can be used a pH indicator due to a color change from blue at pH 3.0 to red at pH 5.2 and vise versa, red to blue. Fungi can degrade cellobiose into glucose and routinely metabolize glucose to organics acids that are acidic compounds. We suspected the change of medium color could result from the lowering of medium pH by the secreted organic acids by the fungi. Thus, we made media with acidic and alkaline pHs and inoculated them with *Trichoderma reesei* (ATCC56765), a known β-glucosidase producing culture. The pH variation had effect on the change of medium color (Fig. 2). In the media with pH 4.5, 5.0, and 6.0 the color was light blue, while in the

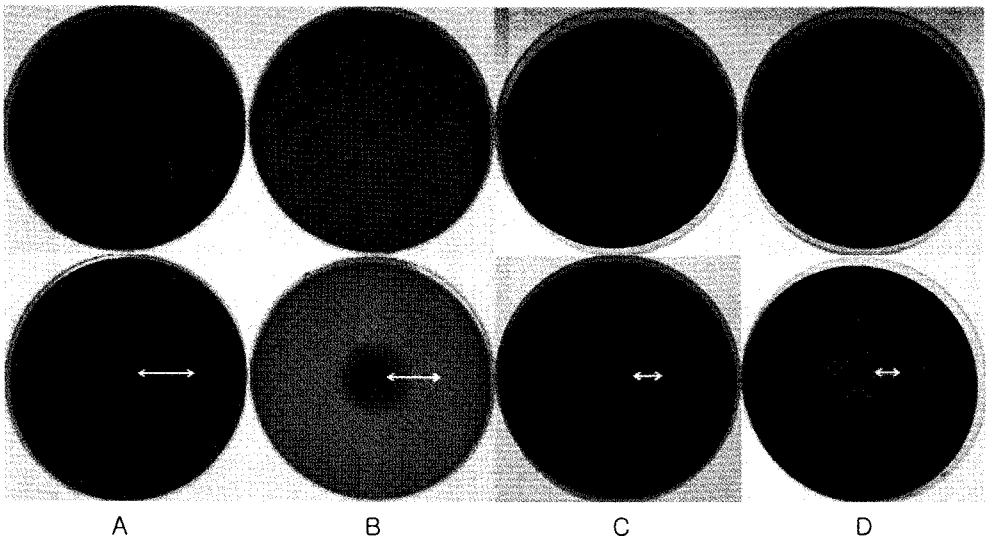

Fig. 1. Examples of positive detection of fungal β-glucosidase activity by chromogenic reaction in media containing D-cellobiose and different dyes (A, congo red; B, phenol red; C, remazol brilliant blue; D, tryphane blue). Top row: before incubation. Bottom row: after incubation. Arrows indicate clear zone formed.

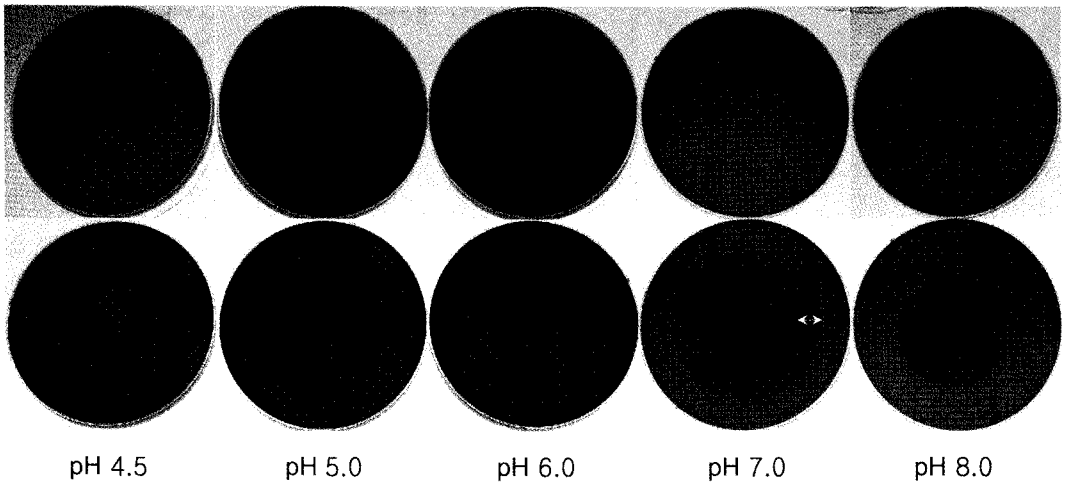

Fig. 2. Observation of chromogenic reaction on congo red media in different pH range. Top row: before fungal inoculation. Bottom row: after fungal growth. *Trichoderma reesei* ATCC 56765 was used as the test fungus.

media with pH 7.0 and 8.0 the color was orange. The fungus inoculated could grow in all the tested media with different pH. Clear zone detection was apparent at pH 7.0 with good color contrast among substrate zone (orange colar), clear zone (light blue), and fungus growing zone. Thus, pH 7.0 was used for the preparation of chromagenic media with cellulose substrate.

For the plate assay with cellulose substrate, we used two different celluloses, CM-cellulose and avicel. Because these cellulose are commercially available, and have been routinely used as growth substrate for the screening of cellulase-producing fungi. Regarding CM-cellulose substrate, 10 fungal species belonging to Ascomycota, Basidiomycota, and Zygomycota produced clear zone in congo red-contained media, while 2~3 fungal species belonging to Ascomycota produced clear zone in phenol red or remazol brilliant blue contained media. All the names of fungal species that showed enzyme activity in both the media contained phenol red and remazol brilliant blue were found in the names of the 10 fungal species that showed activity in congo red-contained media No clear zone was produced on tryphan blue-contained media. These results indicate that congo red is most efficient dye for the detection of CM-cellulose degrading enzyme in the fungi tested.

When it comes to avicel substrate, 7 species produced clear zone in the media contained congo red and phenol red, respectively. Among these 7 species, only 4 species showed enzyme activity in both the two dye-added media. The remained 3 species favored either congo red or phe-

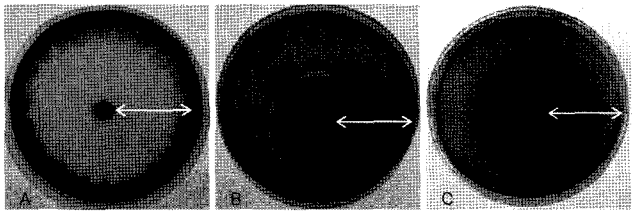

Fig. 3. Clear zone formed in chromogenic media containing congo red by enzyme activity of *Lentinus edodes*. Carbon substrates used are CM-cellulose (A), avicel (B), and D-cellobiose (C). Arrows indicate clear zone formed.

nol red contained medium. No clear zone was observed in tryphan blue contained media. Thus, to detect fungal enzyme activity that degrades avicel type of cellulose, both congo red and phenol red dyes could be used.

Overall, our results with diverse fungal species and three different cellulase substrates show that the clarity and detection rate of clear zone (enzyme activity) is apparent with the medium contained congo red. The successfully use of congo red in the detection of fungal cellulolytic activity was reported in *Aspergillus* species by Onsori *et al.* (2004) and *Ophiostoma* and *Leptographium* species by Hyun *et al.* (2006). These reports also support our work. We expect the use of chromagenic method including con-red could be extended to the detection of fungal extracellular enzymes that degrade other polymeric substrates such as pectin and starch.

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