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Combination of Colony Formation and Congo Red Reaction for Detecting Intra-and Extra-Cellular Cellulolytic Activities

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세포내외 섬유소 분해능 검출을 위한 Colony 형성과 Congo Red반응의 병용

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A selective medium which allows growth of only cellulolytic bacteria was developed. The medium composed of 0.5% carboxymethylcellulose (CMC), 0.005% yeast extract, minerals and agar. Colony formation on this medium indicates overall activities of cellulose utilization. A subsequent test with Congo Red dye could distinguish extracellular cellulolysis from intracellular type.

Hydrolysis of cellulose by bacteria has been demonstrated by growing cells on nutrient agar having carboxymethylcellulose (CMC); subsequent flooding of the medium with Congo Red solution resulted in the formation of visible halos around the colonies[3, 7, 10, 13] Congo Red binds tightly to high molecular -glucans but not to oligosaccharides containing less than five contiguous -linked D-glucopyranosyl units[3, 13]. After the dye binded with the polysaccharides the agar surface is washed with NaCl solution to remove unbinded dye moecules. If the polysaccharide is hydrolyzed to oligosaccharides by the cellulase excreted from bacterial cells discolored zones (halos) will appear around the colonies where no polysaccharide molecules are remained to hold the dye molecules. The Congo Red dye test is, therefore, applicable for the detection of only extracellular type cellulolytic activity.

In the present study we have developed a selective medium which allows growth of, cellulolytic bacteria of both extra- and intra-cellular types exclusively. Subsequent application of Congo Red dye test could distinguish the two types of cellulolytic activities from each other.

Materials and Methods

Bacterial strains and culture media. Five species of *Bacillus; B. subtilis, B. cereus, B. megaterium, B. amyloliquefaciens* HI, and *B. stearothermophilus* were used. As *B. subtilis,* three strains such as RM125 (originated from T. Uozumi.[11]), BD170 (originated from D. Dubnau,[4]) and SBS202 (originated from S.D. Ehrlich,[5]) were used. *Escherichia coli* HB101 (originated from H. W. Boyer,[2]) and *Cellulomonas* sp. CS1-1 were also used. All strains were from the KAIST culture collections.

The bacteria were grown routinely in L broth (10 g Difco Bacto tryptone, 5 g Difco yeast extract, 5 g NaCl per liter adjusted to pH 7.2) or LB agar solidified by the addition of Difco

Table 1. Composition of three known media developed for studies of *Cellulomonas*, *Bacillus* and *Escherichia* strains.

Ingredients	Basal media* originally developed for		
	Cellulomonas[6, 9, 12]	*Bacillus*[8]	*Escherichia*[1]
$NaNO_3$	0.1		
$(NH_4)_2So_4$		0.2	
NH_4Cl			0.1
K_2HPO_4	0.1	1.4	
KH_2PO_4		0.6	0.3
Na_2HPO_4			0.7
NaCl			0.05
KCl	0.05		
$CaCl_2$			0.001
$MgSO_4 \cdot 7H_2O$	0.05	0.02	0.012
Na-citrate$\cdot 2H_2O$		0.1	
Yeast extract	0.05		
Glucose	0.1	0.5	0.4
Agar	1.1	1.5	1.5

* Adjusted pH to 7.0 for all media.
Basal medium for *Cellulomonas*, *Bacillus* and *Escherichia* is called as cellulomonas basal medium, Spizizen minimal medium and M 9 medium, respectively.

agar (15 g/l). Spizizen minimal medium[8], M9 medium[1], and Cellulomonas basal medium[6, 9, 12] were also used for the development of a new selective medium for cellulolytic bacteria.

Detection of cellulolytic activity. Washed cells of bacteria were inoculated on the newly developed selective medium by streaking or spreading. The plates were incubated at 30°C except for *E. coli* which was incubated at 37°C. The appearance of colonies on this selective medium indicates overall utilization of cellulose by the organism. The surfaces of the plates were then flooded with 0.1% aqueous solution of Congo Red for 30 min. Reflooding with 1 M NaCl for 15 min was followed to remove unbinded dye molecules. The visible halos formed around the colonies indicate production of extracellular type cellulase.

Results

Evaluation of existing media. Three known media which have originally been developed for studies of *Cellulomonas* (Cellulomonas basal medium,[6, 9, 12]), *Bacillus* (Spizizen minimal medium,[8]), and *Escherichia* (M9 medium,[1]), having compositions shown in Table 1 were tried to use as basal media for detecting cellulolytic activity. The glucose contents of these media were replaced by 0.1% of CMC before tested. As test organisms *B. subtilis* RM125, *Cellulomonas* sp. CS1-1, and *E. coli* HB101 were used.

It was found that when the glucose was replaced by CMC in M9 medium, none of the three test strains could grow. By fortifying with 0.005% of yeast extract the strains of RM125 and CS1-1 could grow remaining HB101 not grown. In the CMC-replaced spizizen medium all of the three test organisms including non-cellulolytic *E. coli* could grow. The growths of three organisms, however, disappeared when citrate was eliminated from the CMC-replaced medium. Apparently the growth of non-cellulolytic organism on the CMC-replaced Spizizen minimal medium was due to the presence of citrate as energy source. When the citrate content was replaced by 0.005% of yeast extract only cellulolytic strains, CS1-1 and RM125, could grow. All of the three test organisms also grew on the Cellulomonas basal medium in which 0.1% of glucose was replaced by 0.1% of CMC. The growth of *E. coli* was ceased when the yeast extract content of 0.05% was reduced to 0.005%.

When Congo Red dye was applied on the surface of the plate formation of halo around the colonies could be observed as shown in Figure 1. The halos were formed, however, around the colonies of only *B. subtilis* which is known to be

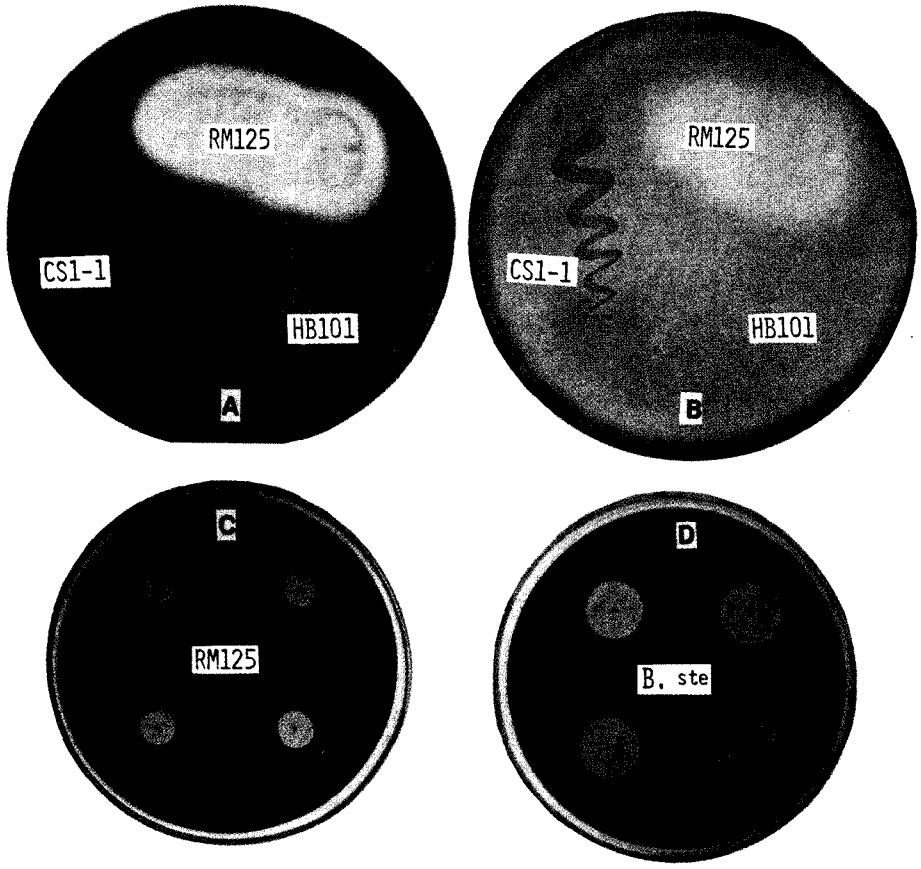

Fig. 1. Colony- and halo- formation of *B. subtilis* RM 125, *Cellulomonas* sp. CS1- 1, *E. coil* HB101, and *B. stearothermophilus* on Cellulomonas basal medium (A), Spizizen minimal medium (B), and CMC minimal medium newly developed in this study (C, D; Table 3). For C and D cells were inoculated simultaneously by toothpicking and incubated for 24 hours at 30℃. *B. stearothermophilus* (B. ste) shows larger halos indicating higher cellulolytic activity than RM 125,

extracellular cellulolytic organism. The halos were more clear on Cellulomonas basal medium than on Spizizen minimal medium or M9 medium (picture not shown). The clearness of halos was further increased by increasing the 0.1% CMC content to 0.5% in the Cellulomonas basal medium.

Formulation of a selective medium. A minimal amount of yeast extract in Cellulomonas basal medium which helps utilization of CMC but does not support the growth of organisms without carbon sources was tried to find. As shown in Table 2 when the yeast extract in the Cellulomonas basal medium was limite α to 0.005% non of the three test organisms could grow without carbon sources added. By adding 0.5% of CMC, cellobiose, or cellulose under the same yeast extract content both *Cellulomonas* and *Bacillus* could grow with exception of *E. coli* which is known to be non-cellulolytic. When CMC was replaced by starch only the *Bacillus* test organism could grow indicating that the other two test organisms are lack of starch hydrolytic activity.

Based upon the data appeared in Table 2 a new medium on which only cellulolytic organisms can grow was formulated and shown in Table 3.

Relation between growth and halo formation. On the newly developed selective medium nine strains of bacteria were tried to grow and Congo Red dye tests were followed. As shown in Table 4 the results of Congo Red dye tests were not always corelate with those of colony formation. All of the three *Bacillus subtilis* strains tested which are known to be extracellular cellulolytic organisms showed both growth and halo formation. However, *B. cereus* and the *Cellulomonas* strain showed colony formation but not halo formation. The

Table 2. Effect of yeast extract and carbon sources added to the mineral components of the Cellulomonas basal medium * on the growth of *Cellulomonas* sp., *B. subtilis* RM 125, and *E. coli* HB101.

Additives (%)						Growth		
Yeast extract	Glucose	Cellobiose	CMC	Cellulose	Starch	*Cellulomonas* sp.	*B. subtilis*	*E. coli*
0.5						++	++	++
0.05						+	+	+
0.01						±	+	±
0.005						−	−	−
	0.5					++	++	++
		0.5				+	+	−
			0.5			−	−	−
				0.5		−	−	−
					0.5	−	−	−
0.005	0.5					+++	+++	+++
0.005		0.5				++	++	−
0.005			0.5			++	++	−
0.005				0.5		+	+	−
0.005					0.5	−	++	−

* Each growth factor needed has been supplemented previously for the auxotrophic test organisms at final level of 20 μg/ml.

Table 3. Composition of a new medium* to be used for detecting cellulolytic activity of bacteria.

Ingredient	Concentration (%)
$NaNO_3$	0.1
K_2HPO_4	0.1
KCl	0.05
$MgSO_4 \cdot 7H_2O$	0.05
Yeast extract	0.005
CMC	0.5
Agar	1.1

* Adjusted pH to 7.0

Table 4. Tests of various bacteria for their cellulolytic activities of extra- or intra-cellular types.

Bacteria	Colony formation	Congo Red reaction
E. coli HB101	−	−
Cellulomonas CS1-1	+	−
B. subtilis RM125	+	+
B. subtilis BD170	+	+
B. subtilis SB202	+	+
B. cereus	+	−
B. megaterium	−	−
B. amyloliquefaciens Hl	+	+
B. stearothermophilus	+	+

two non-cellulolytic organisms, *E. coli* and *B. megaterium* showed neither colony nor halo formations.

Discussion

A precise detection of cellulolytic activity in microorganisms is essential for manipulation of cellulase genes as well as for biochemical or taxomonic studies. Congo Red dye which binds to polysaccharide molecules having more than five contiguous β-linked D-glucopyranosyl units has been developed[3, 7, 10, 13] and used widely. The use of the dye tests, however, has some limitations. Both *Cellulomonas* sp. CS1-1 and *B. cereus* showed negative response on the Congo Red dye tests indicating non-cellulolytic activities (Table 4). However, these organisms could grow on our selective medium having CMC as sole carbon source as appeared in the same table. Apparently the two organisms should be listed as cellulolytic organisms notwith standing the negative results of the Congo Red dye

tests. Another limitation related to this dye test is the fact that a positive result from the test does not always mean the organism tested can grow on cellulose as a sole source of carbon. It may need further ability to use oligosaccharides delivered from the cellulose.

It may be concluded, therefore, that initial tests for overall cellulolytic activities in an organim should be relied on the observation of colony formation on an selective medium such as the one we have developed in this study. For further tests to distinguish the types of cellulolysis, intra- or extracellular, the Congo Red dye tests should be followed.

요 약

섬유소 분해능이 있는 bacteria를 분리 동정하거나 유전자 재조합에 의한 형질 전환주중에서 이 분해능을 갖는 재조합주를 신속하고도 확실하게 분리해내는 일은, 섬유소 분해능을 가진 유전자를 복제 및 발현시키려는데 있어서 일차적인 조건이 된다. 한정된 양(0.005%)의 yeast ext를 함유한 기본배지에 0.5%의 CMC를 첨가 시킴으로써, 오직 섬유소 분해능이 있는 bacteria만을 분리할 수 있었고, 뒤이은 Congo red dye처리에 의해 세포벽 밖으로 분비 가능한 섬유소 분해효소를 생산하는 bacteria를 다시 구별해낼 수 있었다. 한편, 개발된 CMC 기본배지는 CMC대신 다른 탄소원 중합체를 대치시킴으로써, 다른 종류의 탄수화물 분해능을 갖는 bacteria도 쉽게 분리해 낼 수 있음을 알 수 있었다.

References

1. Anderson E. H.: Proc. Natl. Acad. Sci. USA, 32, 120 (1946)
2. Boyer H. W. and D. Roulland-Dussoix: J. Mol. Biol, 41, 459 (1969)
3. Cantwell B. A. and McConnell D. J.: Gene, 23, 211 (1983)
4. Dubnau D.: Proc. Natl. Acad. Sci. USA, 75, 1428 (1978)
5. Ehrlich S. D.: Proc. Natl. Acad. Sci. USA, 75, 1433 (1978)
6. Hitchner E. V. and Leatherwood J. M.: Appl. Environ. Microbiol., 39, 382 (1980)
7. Mahasneh A. M. and Stewart D. J.: J. Appl. Bacteriol., 48, 457 (1980)
8. Spizizen J.: Proc. Natl. Acad. Sci. USA, 44, 1072 (1958)
9. Stewart B. J. and Leatherwood J. M.: J. Bacteriol., 128, 609 (1976)
10. Teather R. M. and Wood P. J.: Appl. Environ. Microbiol., 43, 777 (1982)
11. Uozumi T.: Mol. Gen. Genet., 152, 65 (1977)
12. Whittle D. J., Kilburn D. G., Warren R. A. J. and R. C. Miller Jr.: Gene, 17, 139 (1982)
13. Wood P. J.: Carbohydr. Res., 85, 271 (1980)