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Characterization of cellulase production by carbon sources in two *Bacillus* species

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The induction of cellulase production in two *Bacillus* spp. was studied by means of measuring cellulase activities under the condition of different carbon sources. The results indicate that cellulase could not be induced by cellulose material as a sole carbon source. Instead, they could be induced by monosaccharide or disaccharide with reducing group. Moreover, the expression of cellulase components was synergistic. When cell wall/envelope enzyme and endoenzyme from two *Bacillus* spp. acted on these inducers, analysis of reaction products by high performance liquid chromatography (HPLC) revealed that cell wall/envelope enzyme and endoenzyme from two *Bacillus* spp. were inactive on these inducers. It also indicated that these inducers entered cells directly and served function of induction.

Key words: *Bacillus*, cellulase, induction, carbon source.

INTRODUCTION

Cellulase is a multi-enzyme complex, regulated and controlled by complicated metabolic process. Low yield and specific activity are major factors in preventing application of cellulase. Therefore, clarifying the induction mechanism of cellulase production and its regulating and controlling principles will be meaningful not only in theory, but also in providing clues and methods to enhance the cellulase yield as well as to design screen model which can select high yield strain. In the past, the studies of inducible and regulating mechanism mainly focused on some microbial strains such as *Trichoderma* sp. The synthesis of cellulase can be induced by many oligomeric and dimeric sugars (Hrmova et al., 1991; Magnelli and Forchiassin, 1999). It was difficult to use a general induction mechanism to explain diversified inductions, though several inducers have been found. For example, Nochure et al. (1993) demonstrated fructose as the best inducer of avicellase in *Clostridium thermocellum*. Bagga et al. (1989) identified lactose as the best inducer of endo-glucanase and cellobiohydolase. Thirumale has been reported

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Figure 1. Morphology of cells of *Bacillus* sp. X18 observed under light microscope (100×).

Figure 2. Morphology of cells of *Bacillus* sp. X10-1-2 observed under light microscope (100×).

Figure 3. Colony and clear halo formed by *Bacillus* sp. X18 on the screening plate stained with Congo red.

Figure 4. Colony and clear halo formed by *Bacillus* sp. X10-1-2 on the screening plate stained with Congo red.

as the best inducer of cellulases in a *Clostridium* sp. (Thirumale et al., 2001). Fructose and cellobiose, among the monomeric and saccharides and disaccharides examined, respectively were found to be the best inducer of cellulase in *Aspergillus niger* (Hanif et al., 2004). Recently, with the rapid development in both biochemistry and molecular biology, along with the intensive application of cellulase in some new fields, many people are interested in the regulating mechanism of cellulase synthesis, being a new focus in the study of cellulase. At the same time, because of the demand of bacteria cellulase, the mechanism of cellulase production will be a research pivot. Some studies showed that cellulases in bacterial and fungal systems are different. Incomparison with the cellulases of the fungi, very little is known about the mechanisms by which bacteria produce cellulose (Wood and Carcía-Campayo, 1990). There is no report on the inducer of *Bacillus* species at present.

The main purpose of this study was to investigate the induction mechanism of cellulase production in two *Bacillus* spp. Preparation and assay of endocellular enzyme and cell wall/envelope enzyme were determined. In addition, the reactions of endocellular enzyme and cell wall/envelope enzyme with inducers were also evaluated.

MATERIALS AND METHODS

Organism and chemicals

Bacillus sp. X18 and *Bacillus* sp. X10-1-2, isolated and screened by the laboratory of Green Chemical Technology of College of Heilongjiang Province, were used in this study. After isolation, the organisms were purified through repeated plating in beef extract-peptone agar media. For the identification of selected isolates, different morphological and cultural characteristics such as size, shape, arrangement, colour, growth on agar plate, agar slants, in liquid or in deep agar media, etc., were observed. The morphology of cells of *Bacillus* sp. X18 and *Bacillus* sp. X10-1-2 are shown in Figures 1 and 2. There were few significant differences among the morphology images of the two strains. As show in Figures 3 and 4, colony and clear halo were formed by *Bacillus* sp. X18 and *Bacillus* sp. X10-1-2 on the screening plate stained with Congo red. Finally, the characteristics were compared with Buchanan and Gibbons (1974) and provisionally identified as *Bacillus licheniformis* and *Bacillus cereus*, respectively. Two bacterial strains were maintained

on beef extract-peptone slants. All chemicals were of analytical grade except acetonitrile which was of chromatographicgrade.

Enzyme production

The medium for enzyme production was prepared by mixing 3.0 g Na_2HPO_4, 2.0 g $(NH_4)2SO_4$, 0.5 g urea, 0.5 g $MgSO_4•7H_2O$, 0.5 g $CaCl_2$, 7.5 mg $FeSO_4•7H_2O$, 2.5 mg $MnSO_4•H_2O$, 2.0 mg $ZnSO_4$ and 3.0 mg $CoCl_2$ in 1 L distilled water, autoclaved for 20 min at 121°C, adding specific carbon source. The concentration of carbon source: cellulose and sugar was 0.5 % (W/V) and 0.1 % (W/V), respectively. The seed culture was developed on beef extract-peptone slants for 48 h at 30°C. Slant culture was washed with 4 ml of sterile water to obtain spore suspension. In order to produce cellulase, 0.1 ml of the spore suspension was inoculated into 150-ml Erlenmeyer flasks containing 30 ml fermentation medium to give a spore concentration of 106 spores per millilitre. The flasks were incubated at 30°C on a gyratory shaker at 170 rpm. Sample flasks in one time replicate were removed for assaying enzyme activities present in the culture every 24 h intervals. The culture medium after fermentation was centrifuged at 3000 rpm for 15 min to remove the insoluble substrates and cells. Supernatants were used as crude enzyme solution.

Enzyme assays

Carboxymethyl cellulase (CMC) activity (endo-1,4glucanase activity) was assayed by incubating (at 50°C for 30 min) 1 ml of 0.5% (W/V) CMC diluted in 0.2 mol/L sodium acetate (NaAc) buffer at pH 4.8 mixed with 1 ml of the enzyme solution (suitably diluted). The reducing sugars produced during incubation were determined following the DNS method by measuring the absorbance at 530 nm after adding 2 ml of the DNS reagent, heating in a boiling water bath for 5 min and cooling immediately.

The β-glucosidase activity was assayed by incubating (at 50°C for 30 min) 1 ml of 0.5 % (W/V) salicin diluted in 0.02 mol/L NaAc buffer at pH 4.8 mixed with 1 ml of the enzyme solution (suitably diluted). The reducing sugars produced during incubation were assayed as in the method previously described.

For enzyme activity on filter paper, 50 mg Whatman No.1 filter paper, 1 ml of 0.02 mol/L NaAc buffer (pH 4.8) and 1 ml of the enzyme solution (suitably diluted) were mixed together into a tube. The samples were incubated for 1 h at 50°C by the method of Eriksson et al. (1990); reducing sugars produced during incubation were assayed as in the method previously described.

For enzyme activity on cotton, 50 mg absorbent cotton, 1 ml of 0.02 mol/L NaAc buffer (pH 4.8) and 1 ml of the enzyme solution (suitably diluted) were mixed together into a tube. The samples were incubated for 24 h at 50°C according to the method of Vallander and Eriksson (1985) reducing sugars produced during incubation were assayed as in the method previously described.

The microcrystalline cellulase activity was assayed by incubating (at 50 °C for 2 h) 1 ml of 2% (W/V) microcrystalline cellulose diluted in 0.02 mol/L NaAc buffer at pH 4.8 mixed with 1mL of enzyme solution (suitably diluted) according to the method of Coudray et al. (1982). The samples were centrifuged at 5000 rpm for 10 min to remove insoluble substrates. Reducing sugar in the supernatant was assayed following the DNS method. One unit of enzyme activity is defined as the amounts of enzyme that liberated 1 µg of glucose in 1 min at 50°C.

Preparation of endocellular enzyme and cell wall/envelope enzyme

A loopful of slant culture was inoculated in 150-ml Erlenmeyer flask containing 30 ml beef extract-peptone liquid medium (3 g beef extract, 10 g peptone and 5 g NaCl per liter) at pH 7.0 to 7.2. The flasks were incubated at 30°C on a gyratory shaker at 170 rpm for 10 h. The culture was used as inoculum. Medium with the following composition: 3 g beef extract, 10 g peptone, 5 g NaCl, 5 g glucose, 2 g $NaNO_3$ at pH 7.0 to 7.2 was utilized for endocellular enzyme and cell wall/envelope enzyme. Two millliter (2 ml) of inoculum was inoculated in a 500-mL Erlenmeyer flask containing 200 ml cultivation medium. The flasks were incubated at 30°C on a gyratory shaker at 170 rpm for 10 h (*Bacillus* sp. X10-1-2) and 18 h (*Bacillus* sp. X18), respectively. The culture medium after fermentation was centrifuged at 1000 g for 10 min to collect cells. The cells were washed once, cooled with 0.05 mol/L phosphoric acid buffer (pH 7.2) and centrifuged. The pellets were re-suspended in chilled 0.05 mol/L phosphoric acid buffer (pH 7.2) at a ratio of 1:10 (10 ml buffer per gram wet cell weight). The suspension was disrupted by probe sonication 30 times for 30 s at 500 won ice. The sonicated suspension was centrifuged at 1000 g for 10 min at 4°C to remove undisrupted cells. The particulate fraction called cell wall enzyme (or cell envelope enzyme associated) was obtained as a pellet by centrifugation at 20000 g for 20 min at 4°C. The supernatant formed cytosolic fraction called endocellular enzyme. The pellet was suspended in 0.05 mol/L phosphoric acid buffer (pH 7.2). The cell wall enzyme (or cell envelope enzyme associated) and endocellular enzyme were dialyzed against deionized water at 4°C for 12 h. To prevent the contamination with bacteria, 0.02% sodium azide was added to the deionized water. The dialyzate was frozen for the following assays.

The reactions of endocellular enzyme and cell wall/envelope enzyme with inducers

The enzyme solution and sugar solution were prepared with deionized water. The initial enzyme solution and sugar solution were 20 mg/ml. The reaction mixtures, mixed at a ratio of 1:1 of the initial enzyme solution and sugar solution in 1.0 ml Eppendorf tubes, were incubated at 37°C for 24 h, and subsequently were centrifuged at 16000 rpm for 30 min at 0°C. The supernatants were collected for HPLC analysis. The experiment was repeated three times for each condition and the average of the three trials was calculated. The relative standard deviation (RSD) using this method was approximately 5%.

HPLC analysis

Products in reaction of endocellular enzyme and cell wall/envelope enzyme on inducers were analyzed on a Waters analytical HPLC instrument with refractive index detector. A 15 µl of the samples were injected into a carbohydrate column (4.6 ×250 mm). Acetonitrile and water (75/25; V/V) were pre-mixed, degassed, filtered and used as the mobile phase for analyses at a flow rate of 1.4 ml/min.

RESULTS

The results in Table 1 indicate that the species of cellulases (CMCase, β-glucosidase, Fpase, microcrystalline cellulose, activities on cotton) produced by the two strains of *Bacillus* sp. were same, though their activities differed from each other. For example, CMCase, β-glucosidase, Fpase, microcrystalline cellulose, and activity on cotton were 2.71, 3.83, 12.93, 1.50 and 17.99 IU/ml produced by *Bacillus* sp. X10-1-2 using sorbose as carbon source, respectively while cellulase activities of *Bacillus* sp. X18 were 1.17, 4.35, 2.62, 0 and 9.43 IU/ml, respectively. All

Table 1. Cellulase activities produced by *Bacillus* sp. X10-1-2 and *Bacillus* sp. X18 in the presence of different carbon sources (IU/ml).

Carbon source	Cellulase activity of *Bacillus* sp. X10-1-2					Cellulase activity of *Bacillus* sp. X18				
	CMCase	β-Glucosidase	Fpase	Microcrystalline cellulase	Activity on cotton	CMCase	β-Glucosidase	Fpase	Microcrystalline cellulase	Activity on cotton
No carbon source	nd	0.01	nd	nd	nd	nd	0.09	2.52	nd	2.11
CMC	nd	0.02	nd	nd	nd	nd	nd	nd	nd	nd
A	nd	0.04	nd	nd	nd	nd	nd	nd	nd	nd
B	nd	0.04	nd	nd	nd	nd	0.01	nd	nd	nd
C	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
D	nd	0.00	nd	nd	nd	nd	0.06	nd	nd	nd
CMC+E	2.34	6.07	12.15	nd	10.60	3.93	3.05	9.51	nd	5.70
A+E	nd	2.63	11.43	0.02	13.06	3.28	3.70	9.64	nd	5.12
B+E	0.56	3.90	11.09	nd	14.84	2.38	1.25	11.23	nd	3.50
C+E	0.52	5.49	10.83	nd	15.45	2.74	1.48	9.48	nd	4.11
D+E	5.14	2.87	13.93	nd	20.33	3.77	4.73	4.00	nd	12.42
CMC+F	nd	2.90	14.49	0.36	14.07	1.34	2.02	3.49	nd	8.18
A+F	nd	2.53	14.20	nd	13.84	1.73	3.19	4.11	nd	7.16
B+F	nd	2.42	12.73	nd	12.27	2.89	4.87	4.20	nd	8.95
C+F	1.64	3.29	14.83	nd	16.49	2.81	2.82	2.73	nd	9.17
D+F	2.52	2.49	14.18	nd	16.89	2.57	3.76	4.00	nd	14.84
Glucose	nd	1.85	15.14	nd	16.51	2.18	21.46	4.56	nd	5.59
Cellobiose	nd	0.88	13.38	nd	14.03	2.04	3.56	4.28	nd	11.20
Fructose	4.02	3.61	13.79	0.18	16.41	3.88	6.37	4.10	nd	6.42
Maltose	1.61	2.19	12.66	0.82	11.07	2.94	4.62	4.05	nd	9.75
Sorbose	2.71	3.83	12.93	1.50	17.99	1.17	4.35	2.62	nd	9.43
Lactose	nd	1.19	12.75	0.08	14.41	2.24	3.15	4.06	nd	11.03
Xylose	1.81	2.75	11.91	0.89	20.98	1.13	2.91	3.27	nd	15.84
Galactose	1.66	2.14	12.53	nd	16.26	2.30	3.13	4.08	nd	12.75
Arabinose	1.22	0.75	13.29	nd	16.21	0.78	0.90	3.74	nd	21.04
Mannose	5.34	5.92	10.96	0.07	24.12	6.47	7.76	7.34	nd	27.66
Rhamnose	3.41	4.26	6.89	0.01	19.48	5.83	8.00	8.10	0.49	32.36
Soluble starch	nd	1.05	0.37	nd	0.01	nd	0.51	3.44	1.08	4.55
Sucrose	nd	nd	nd	nd	nd	nd	0.05	0.26	1.01	1.57
Reffinose	nd	0.00	nd	nd	nd	nd	0.05	nd	nd	nd
Xylan	nd	0.07	nd	nd	nd	nd	0.07	nd	nd	nd
Mannitol	nd	0.01	nd	nd	nd	nd	0.10	nd	nd	nd
Sorbitol	nd	0.03	nd	nd	nd	nd	0.03	nd	nd	nd
Xylitol	nd	0.00	nd	nd	nd	nd	0.03	nd	nd	nd

A: Microcrystalline cellulose; B: Filter paper; C: Cotton; D: Sigmacell; E: glucose; F: Cellobiose. nd: Not detected.

cellulases showed activities on salicin, filter paper and cotton, no matter which inducer or which strain of *Bacillus* sp. was utilized. The crude enzyme could react on salicin, which means that there was β-glucosidase activity in it, while there were also exo- and endo- synergetic effect in fermented broth, as crude enzyme could degrade filter paper and cotton. When exo-cellulase solely reacted on microcrystalline cellulose, only a little reducing sugar was released, while in this study, the activity of cellulase on microcrystalline cellulose was defined by using the quantity of reducing sugar, thus this activity was very hard to be detected (Desphande et al., 1984). It can be concluded that all the conditions that could induce cellulase production may accelerate main cellulases com-ponents synthesizing at a certain rate. This showed that the synthesis and expression of all cellulase components were synergic. This conclusion supported the work of Wang (2002).

The data in Table1 (cellulase activities produced by *Bacillus* sp. X10-1-2 and *Bacillus* sp. X18 under the condition of different carbon sources) were both the results of 24 h, as all cellulase activities appeared to reach maximum value in 24 h. Results (Table 1) show that bacteria would quickly produce cellulase by easy metabolizable carbon sources as inducer.

As we know, cellulase is an inducible enzyme (Hohn and Sahm, 1983). In the process of its production by means of microorganism fermentation, the substrates are not only used as carbon sources, but also the main sources of inducer. In order to make sure that there is a real inducer for the two strains of *Bacillus* sp., several common cellulose materials (such as CMC, microcrystalline cellulose, filter paper, cotton, sigmacell) and soluble saccharide (glucose, cellobiose, fructose, maltose, sorbose, lactose, xylose, galactose, arabinose, mannose, rhamnose, soluble starch, reffinose, xylan, mannitol, sorbitol, xylitol) were chosen as carbon sources. Catabolite repression plays an important role in the regulation and secretion of inducible enzyme. Such repression effect has been observed in other organisms (Magnelli and Forchiassin, 1999; Beguin, 1990; Hrmova et al., 1991). To prevent catabolite repression, 0.1% (W/V) of lower initial concentration of soluble saccharide in the fermentation medium was used. The results in Table 1 indicate that when pure cellulose, whether crystal (microcrystalline cellulose, filter paper, cotton, sigmacell) or non-crystal (CMC), soluble (CMC) or insoluble (microcrystalline cellulose, filter paper, cotton, sigmacell), was used as the sole carbon source, except for faint β-glucosidase (0.01~0.06 IU/ml) these two strains of *Bacillus* sp. could not be induced to produce cellulase. Induction mechanism for their production of cellulase is quite different from that of fungi. Natural cellulose materials are always good inducible carbon sources for fungi. In this research, only soluble carbon sources with reducing group (glucose, cellobiose, fructose, maltose, sorbose, xylose, galactose, arabinose, mannose, and rhamnose) could induce them to produce cellulase (highest cellulase activity was reached at 32.36 IU/ml, this was activity on cotton produced by *Bacillus* sp. X18 using rhamnose as carbon source). When soluble non-reducing sugars such as xylan, sucrose, etc, were utilized as sole carbon sources, cellulase activity could not be detected in fermented broth. However, some reducing sugars would not be induced to produce cellulase after their reducing groups were substituted by hydroxyl groups to become sugar alcohol like mannitol, sorbitol, and xylitol.

The results of Figure 5 and Table 2 show that the retention times of products formed in the reactions of endocellular enzyme and cell wall/envelope enzyme with inducers were almost the same as standards (for example, retention time of xylose standard on HPLC was 4.07 min. Retention time of products formed in the reaction of endoenzyme and cell wall/envelope enzyme of *Bacillus* sp. X10-1-2 and *Bacillus* sp. X18 were 4.07 and 4.08 min, respectively). This illuminated that enzyme located on cell wall/envelop enzyme and endoenzyme was inactive on inducers that could induce *Bacillus* sp. to produce cellulases. Thus, these small molecules inducers might be regarded to enter cells directly and serve the function of induction. Induction mechanism of cellulase production in two *Bacillus* sp. was quite different from that of fungi.

DISCUSSION

According to the results, a conclusion might be drawn that these two strains of *Bacillus* sp. have similar inductive chart. It means that their induction mechanisms seem alike. Eberhart and Beck (1973) have suggested a quasi-regulatory role of cell wall bound β-glucosidase in enzyme regulation by alteration or destruction inducer molecules. However, it is not clear if the products formed in the action of cell wall bound enzyme and endoenzyme after these small molecules saccharide that can induce *Bacillus* species to produce cellulase enter cells is same or not, that is, real inducer is the same or not, or these inducer enter cells directly to serve function of induction without any transformation. So, the endoenzyme and cell wall/envelope enzyme from two *Bacillus* sp. were prepared, which were reacted with inducer for cellulase, then qualitative analysis of the products was developed to confirm the accurate one. These two *Bacillus* spp. may be simultaneously affirmative and can only distinguish molecules with certain structural groups. These molecules were transformed to induce cellulase by some alteration or destruction on cell envelope or in cells. On the other hand, all the small molecular soluble sugar might enter into cells directly, but only a kind of molecule with specific structural groups can bind with certain protein, then, this protein directly or indirectly affects some kind of protein bound with DNA; the transcription and expression of cellulase gene is then activated.

Since cellulose and the cellulosic components in lingo-cellulosic biomass substrates are essential for the forma-

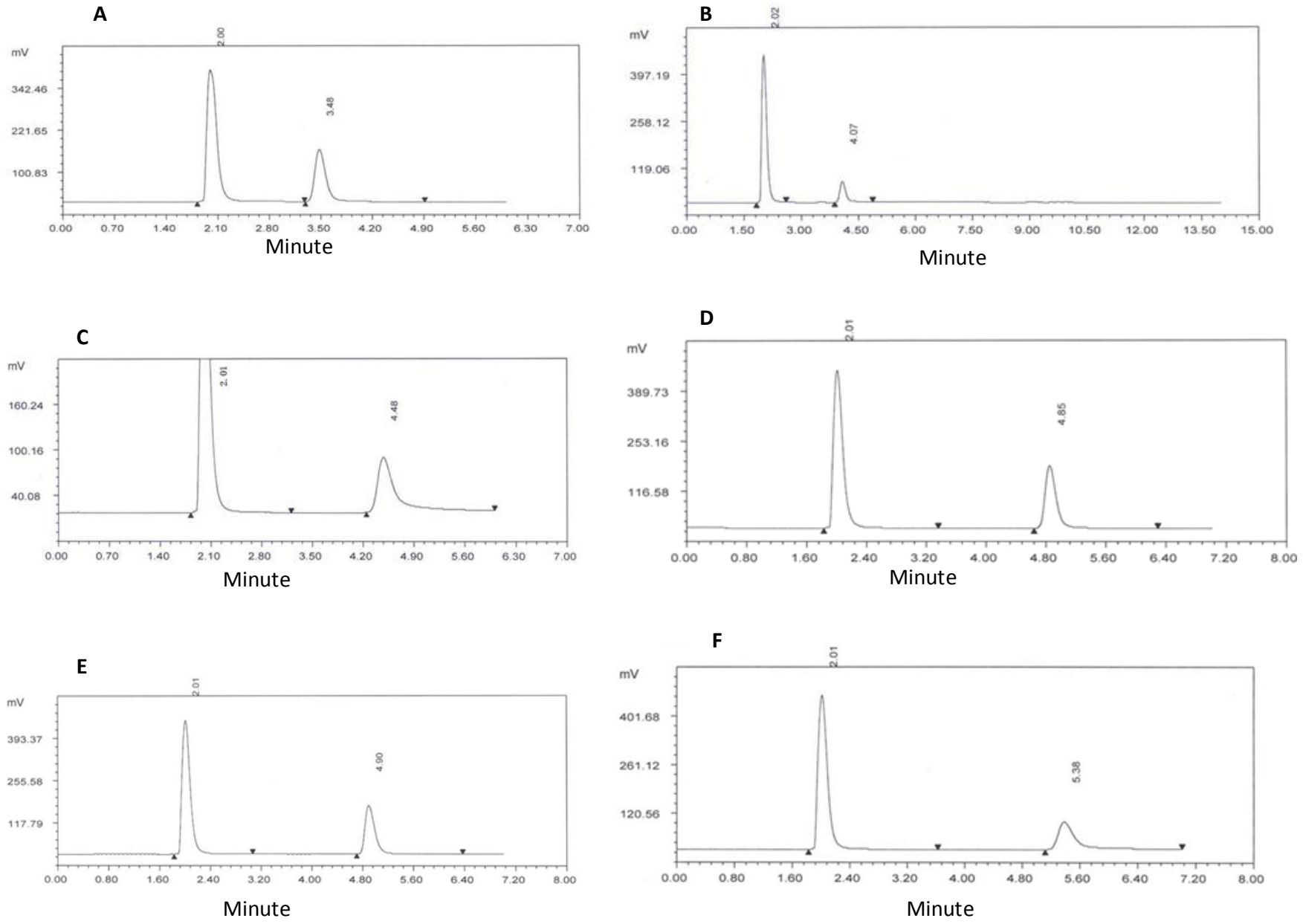

Figure 5. HPLC graph of sugar standards: (a) rhamnose (b) xylose (c) arabinose (d) sorbose (e) fructose (f) mannose (g) glucose (h) galactose (i) cellobiose (j) maltose (k) lactose.

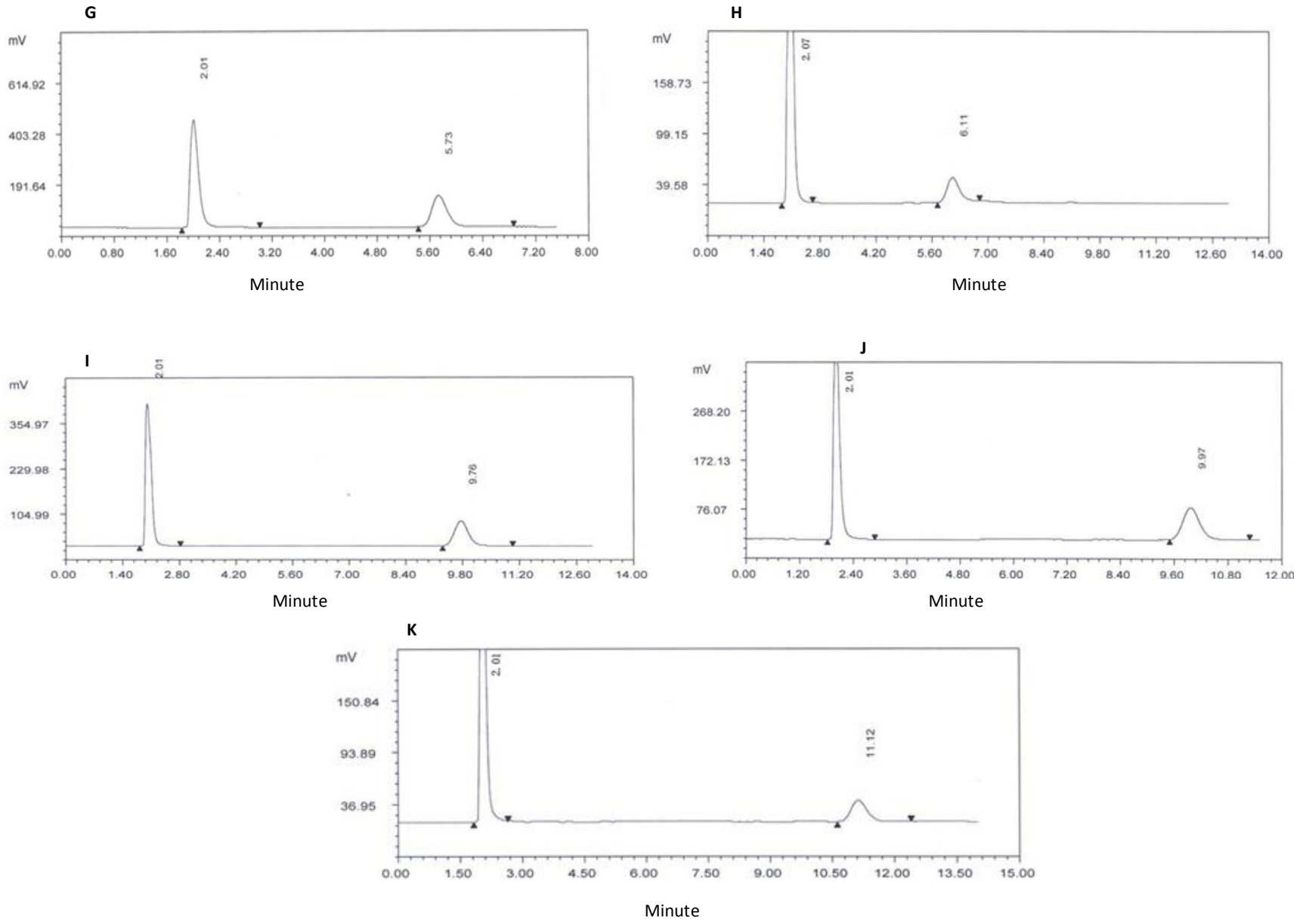

Figure 5. Contd.

Table 2. Retention time of samples on HPLC (min).

Number	Sugar standard	A	B	C	D
1	3.48	3.50	3.50	3.50	3.50
2	4.07	4.07	4.08	4.07	4.08
3	4.48	4.51	4.51	4.49	4.49
4	4.85	4.87	4.86	4.84	4.84
5	4.90	4.95	4.93	4.93	4.91
6	5.38	5.43	5.39	5.38	5.38
7	5.73	5.77	5.73	5.73	5.71
8	6.11	6.17	6.11	6.10	6.10
9	9.76	9.78	9.78	9.74	9.74
10	9.97	9.95	9.95	9.95	9.95
11	11.12	11.12	11.12	11.12	11.12

A: Retention time of products formed in the reaction of endoenzyme of *Bacillus* sp. X10-1-2 with different sugar; B: Retention time of products formed in the reaction of cell wall/ envelope enzyme of X10-1-2 with different sugar; C: Retention time of products formed in the reaction of endoenzyme of *Bacillus* sp. X18 with different sugar; D: Retention time of products formed in the reaction of cell wall/ envelope enzyme of *Bacillus* sp. X18 with different sugar.

tion of mRNA to support maximum formation of cellulases at the transcription level (Gutierrez-Nova et al., 2003), according to the stated analysis, a hypothesis might be drawn upon the mechanism of the two *Bacillus* spp. producing cellulases. Natural cellulose castoff, like straw, wheat bran, and so on, most possibly contained a small quantity of reducing sugar which could induce *Bacillus* sp. to produce cellulases. Therefore, when *Bacillus* species was used to degrade natural cellulose materials under the natural conditions, the reducing sugar entered the cells of these bacteria and induced them to produce cellulases, and then the cellulases were released out of cells to degrade cellulose. In fact, several reports in the literature suggested that wheat bran was demonstrated as the best carbon source to cellulase production in many organisms (Jiang and Liu, 1999; Chen and Qu, 2000; Hanif et al., 2004). On the other hand, natural cellulose often included some associated components like lignin and hemicellulose; hemicellullose is a kind of in homogeneous polymer whose degree of polymerization is less than 200, composed of xylose, galactose, mannose, arabinose, glucose and glycuronate, and they physically combine with cellulose. *Bacillus* sp. might produce a basic level or a constitutive amount of cellulase or other substances that could degrade hemicellulose to soluble hydrolysis products like xylose, galactose, mannose, etc., that could function as an inducer; these products entered the cells inducing *Bacillus* sp. to produce cellulases. Furthermore, cellulolytic microorganisms can be found in all biota where cellulosic waste accumulates. They usually occur in mixed populations comprising cellulolytic and non-cellulolytic species, which often interact synergistically. Non-cellulolytic species might provide cellulolytic species with inducers formed in the reaction of non-cellulolytic species with non-cellulose components in cellulosic waste accumulates. These interactions lead to the complete degradation of cellulose.

Conclusions

The study presents that the induction of cellulase production in two *Bacillus* sp. were investigated by means of measuring cellulase activities under the condition of different carbon sources. The results indicate that cellulase could not be induced by cellulose material as a sole carbon source. Instead, they could be induced by monosaccharide or disaccharide with reducing group. Moreover, the expression of cellulase components was synergistic.

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