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Cellulolytic activity of *Cellvibrio japonicus* and complete cellulase system

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Eighteen cellulytic bacterial isolates were isolated from different sites of agriculture soils in Hilla city. The purification and identification of isolates were performed, and the results showed that it belonged to gram negative bacteria species of *Cellvibrio jopanicus*. The cellulase activity of these isolates was investigated on two cellulosic agricultural waste compounds: rice straw and wheat brand as sole carbon sources. The activity of produced Carboxymethylcellulase (CMCase) and cellobiase enzymes produced by these isolates were studied. The high activity of CMCase enzymes was observed with isolate C.J.N 16 on CMC media which gave 0.152 unit/ml and the least one was isolate C.J.N 13 which gave 0.047 unit/ml. On wheat brand broth, isolate C.J.N 16 was the most active with 0.1 unit/ml, while isolate C.J.N 14 was the lowest with 0.052 unit/ml. On rice straw media, the highest activity was given by isolate C.J.A 6 that reached 0.333 unit/ml, while the least one was C.J.N 13 with 0.104 unit/ml. The cellobiase was produced on all the used media by the four isolates. On CMC broth, the enzyme produced by isolate C.J.N 13 was 0.723 unit/ml, whereas isolate C.J.A 6 gave the minimum activity at 0.333 unit/ml. On the other hand, isolate C.J.A 6 gave the highest activity on wheat brand broth of 0.752 unit/ml, and the least one was isolate C.J.N 16 which gave 0.266 unit/ml. Finally, on rice straw broth, isolate C.J.N 16 was the most active one, since it gave 0.928 unit/ml, and the lowest one was isolate C.J.N 14 which gave 0.214 unit/ml. The cellulase enzymes were partially purified and concentrated using ammonium sulfate precipitation and dialysis. HPLC analysis revealed that *C. jopanicus* has complete cellulase system by giving three separated peaks.

Key words: Cellulytic bacteria, cellulases, *Cellvibrio japanicus*, high performance liquid chromatography (HPLC).

INTRODUCTION

Cellulose is the most abundant organic compound on earth. Every year plants make more than a thousand metric ton of cellulose. A cellulose polymer is a linear chain of thousands of glucose molecules linked by B-(1:4) - glycosidic bounds and the basic repetition of unit cellubiose. The cellulose polymer chain is a flat ribbon like structure stabilized by internal hydrogen bounds between adjacent chains that are strongly linked with one another in a parallel arrangement and they all have the same polarity (Glazer et al., 2007). Cellulose is found in nature as pure cellulose in cotton or as lingocellulosic biomass which is a great potential resource because it is largely abundant and inexpensive, and production of each resource is environmentally sound. Agriculture residues are a great source of lingocellulotic biomass which is renewable, but mainly an unexploited resource and may include leaves, stems and stalks from sources such as corn fiber, corn stoves, sugar cane, rice hulls, woody crops and forest wastes. Also, there are multiple sources of lignocellulosic wastes from industrial activities, municipal solid waste and paper sludge (Maki et al., 2009).

Lignocelluloses are composed of cellulose, hemicelluloses, lignin, extractives and several inorganic

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materials. Hemicelluloses have lower molecular weight than cellulose and it is composed of mainly pentose (like xylose and arabinose) and hexose (like mannose, glucose and galactose), whereas lignin is an amorphous polymer whose attributes include providing rigidity to plant cell wall and resistance against microbial attack. The cellulose microfibrils which are present in hemicelluloses - lignin matrix are often associated in the form of bundle or macrofibrils. The structure of these naturally occurring cellulosefibrils is mostly crystalline in nature and highly resistant to attack by enzymes (limited accessibility of cellulose chain). The presence of lignin also impedes enzymatic hydrolysis, as enzymes bind onto the surface of lignin and hence do not act on cellulose chains (Palonen et al., 2004). If enzymatic hydrolysis of biomass is to proceed in typical processes, the crystalline structure of cellulose in lingocelluloses needs to be disrupted and the accessible area increased, after which the lignin and hemicellulose are separated from cellulose before treatment with enzymes. The process of pretreatment of lignocelluloses is considered to be one of the expensive steps in the conversion of lignocelluloses feed stocks to ethanol. Microbial degradation of plant cell wall is the primary mechanism utilized in the biosphere and is an important biological and industrial process. The factors affecting the hydrolysis of cellulose include porosity (accessible surface area) of waste materials, crystallinity of cellulose fibers, lignin and hemicelluloses content (Lo et al., 2009). Many bacteria and fungi produced enzymes that hydrolyzed the cellulose bio polymers. Cellulolytic bacteria can be observed to comprise several diverse physiological groups:

1) Fermentative anaerobic are typically gram positive bacteria (*Clostridium*, *Ruminococcus* and cladicellulosruptor) but contain a few gram negative species most of which are phylogenetically related to *Closteidium* assemblage (*Butyrivibrio*, *Acetovibrio*), but some of which are not (*pseudomonas*, *Cellvibrio*, *Fibrobacter*).
2) Aerobic gram - positive bacteria (*Cellulomanas*, *Thermobifida*).
3) Aerobic gliding bacteria (*Cytophage*, *Sporocytophaga*) (Lynd et al., 2002).

There are three major types of cellulases: Endoglucanase, cellobiohydrolases and B-glucosidases. These enzymes hydrolyzed the B (1:4) glucosidic bounds in cellulose but differ in their specifications based on the macroscopic features of substrates. *Cellvibrio* are aerobic gram-negative bacteria, with slender curved rods motile and mixed flagellation. Its cells have up to 11 lateral flagella. *Cellvibrio* are known to have the ability to hydrolyze cellulose and dextrin. Researchers have strong interest in cellulases because of their application in several industries. In recent years, the interest in cellulases has increased due to their applications in production of bioenergy and biofuel (Ahmed and Vermette, 2008; Zhou et al., 2008).

MATERIALS AND METHODS

Sample collection

Eighteen agriculture soil samples were collected from different agricultural lands in Hilla city, Babil province. These samples were brought to the laboratory in dry clean containers for microbial isolation and cultivation.

Isolation of cellulolytic bacteria

Ten grams of each agriculture soil samples were soluted in 10 ml of sterile distilled water using vortex mixer. Then 1 ml of soil suspension was added to 50 ml of minimum salt medium in 250 ml conical flasks [the minimum salt medium was M9 which consist of 12 g of Na_2Hpo_4, 6 g of KH_2PO_4, 1 g of NaCl, 2 g of NH_4Cl and supplemented with 2 ml of $MgSo_4$ (1 M) and 1 ml of $CaCl_2$ (1M) and carboxymethylcellulose (CMC) 1% (wt/vo1) which is used as a sole carbon and energy source[(Garder and Keating, 2010). After 7 days of incubation at 28°C, a loopful of each flask was cultured on blood agar. It was sub-cultured on blood agar for several times, and the pure isolated colonies of each isolate were obtained.

Enzyme production condition

The bacterial isolates were cultivated aerobically at 37°C for 7 days in production medium (1% CMC, 0.5% yeast extract, 0.5% NH_4H_2P04, 1% $MgS0_4$, $7H_20$, 0.02%KCl) (Aboul-Enien et al., 2010).

Pretreatment of carbon sources

The agriculture residues (wheat brand and rice straw) were pretreated and queried mechanically to convert these residues to small particulates to increase the surface area for hydrolyzed action (Alhasani, 2002).

Physico-chemical treatment

The milled carbon sources were treated by NaOH sodium (1.5%) with heating at 80°C for 30 min (Nadeem, 2009).

Determination of enzymatic activities

The enzymatic activities measurement depend on the amount of reducing sugar released throughout cellulose hydrolysis and one unit of activity was expressed as 1 µl of glucose liberated per ml of enzyme per minute.

Standard curve preparation

Glucose standard curve was prepared from 10-100 µg/ml

and measured using Nelson and Somogyi method at wavelength of 500 nm to determine the contraction of reducing sugars (Somogyi, 1951).

Enzymes assays

Endo-B-(1:4) gluconase (cmcase) activity

The enzymatic activity of endoglucanase was measured according to Tajaldeen (1993). 0.1 ml of enzyme solution was incubated with 0.9 ml of substrate (CMC, 0.5%) and dissolved in 50 mM of citrate buffer pH 4.8 at 50°C for 30 min. After the incubating time, the reducing sugar (glucose) was measured.

Cellobiohydrolase activity

The enzymatic activity of cellobiohydrolase was measured according to the study of Mandels (1976) in which 1 ml of enzyme solution was added to 1 ml of citrate buffer pH 4.8, and then added to a 50 mg Whitman filter paper no. 1. The mixture was incubated for 2 h at 50°C and after incubation time, the reducing sugar (glucose) was measured.

Cellulases purification and concentration

Crude enzyme production

C. japonicus isolates were cultured on CMC, wheat and rice broths (1% each of wheat and rice) and incubated in a shaking incubator for 7 days. After the incubation period, 10 ml of each conical flask were taken and centrifuged at cooling temperature (4°C) for 20 min at 10000 rpm/min, after which the supernatant was collected and the pellet was discarded. Finally, the supernatant was sterilized by filtration through 0.45 Millipore fitter.

Ammonium sulfate precipitation

Ammonium sulfate was added to cell-free culture firstly to attain 30% saturation. The precipitated protein was collected by centrifugation at 7000 rpm / min at 40°C for hours. Solid ammonium sulfate was added under stirring to a final saturation of 80% saturation. The suspension was stirred for 1 h and kept overnight. The precipitated protein was collected by centrifugation at 7000 rpm / min for 1 h, after which the pellet was dissolved in 50 mM of citrate buffer pH 4.8 (Harris, 1989).

Dialysis

Desalting of enzyme solution was carried out by putting it in a dialysis tube visiking 18/32 and in a baker. Dialysis was carried out against citeate buffer pH 9.8, 0.005 M on stirrer at 42°C for 24 h.

High performance liquid chromatography (HPLC)

The cellulases from the concentrated supernatant were applied to HPLC (Shimazu, Japan) equipped with C 18 column (1.2 × 150 ml) at 50°C using Aceternitril (70%) as a mobile phase at a flow rate of 0.5 ml / min on 280 nm wavelength and by using uv-detecdor. This test was used to detect the types of cellulases which were produced by bacterial isolates.

RESULTS AND DISCUSSION

Eighteen cellulytic bacterial isolates were collected from different agricultural sites in Hilla city. These isolates were purified and identified according to cultural properties and biochemical tests. The results showed that these isolates belonged to gram negative species of *C. japonicus*. The Cmcase and cellobiase activity of the isolates were studied (Table 1).

Total activity of each enzyme was variable from one media to another production media (CMC, wheat brand and rice straw) and this could be illustrated by many factors which affected the ability of the bacteria to hydrolyze cellulose in these lingocellulosic materials. Berlin et al. (2005) indicated that pretreatment of lingocellulosic materials (wheat brand and rice straw) allowed the separation of the major lingocellulosic compounds (cellulose, hemicelluloses and lignin) on each other and this made the cellulose more accessible for hydrolyzation of the cellulases. Also, the chemical structure of lignocellulosic materials played an important role in efficacy of bacteria to hydrolyze these materials. Lee et al. (2007) illustrated that the chemical structure and composition of lingocellulosic materials are highly different in genetic factors like: plants species, plant environment and the interaction between them. The results of the current study are in agreement with those of Gautam et al. (2010) who showed that the bacteria were isolated from municipal solid waste and found that these bacteria have high enzymatic activity against cellulose. They also observed that the cellulases enzymes produced from these bacteria were better than the enzymes produced from other bacteria types which appeared very low due to the absence of the cellulose activity in the lignocelluloses materials. These results were not in agreement with those of Wierzba and Latata (2007) in Poland who proposed that there is no sign of cellulytic activity of these bacteria when they grow in the medium with cellulose. These results were also in disagreement with those of Otajevwo and Aluji (2010) who showed that these bacteria were isolated from cow dung samples. They found that the CMCase activity reached 3.61 unit/ml, whereas the highest enzyme activity for CMCase in the current study was 0.333 unit/ml on rice straw broth. Because the previous classification of *Cellvibrio* included *Pseudomonas*, many research papers are being published on the cellulytic activity of

Table 1. Total activity of Cmcase and cellobiase of isolates were studied.

Isolate no.	Production medium	Total activity of CMCase (unit/ml)	Total activity of cellobiase (unit/ml)
	CMC	0.057	0.333
C.J.A 6	Wheat brand	0.076	0.752
	Rice straw	0.333	0.590
	CMC	0.047	0.723
C.J.N 13	Wheat brand	0.066	0.504
	Rice straw	0.104	0.314
	CMC	0.071	0.666
C.J.N 14	Wheat brand	0.052	0.509
	Rice straw	0.119	0.214
	CMC	0.152	1.380
C.J.N 16	Wheat brand	0.1	0.266
	Rice straw	0.171	0.928

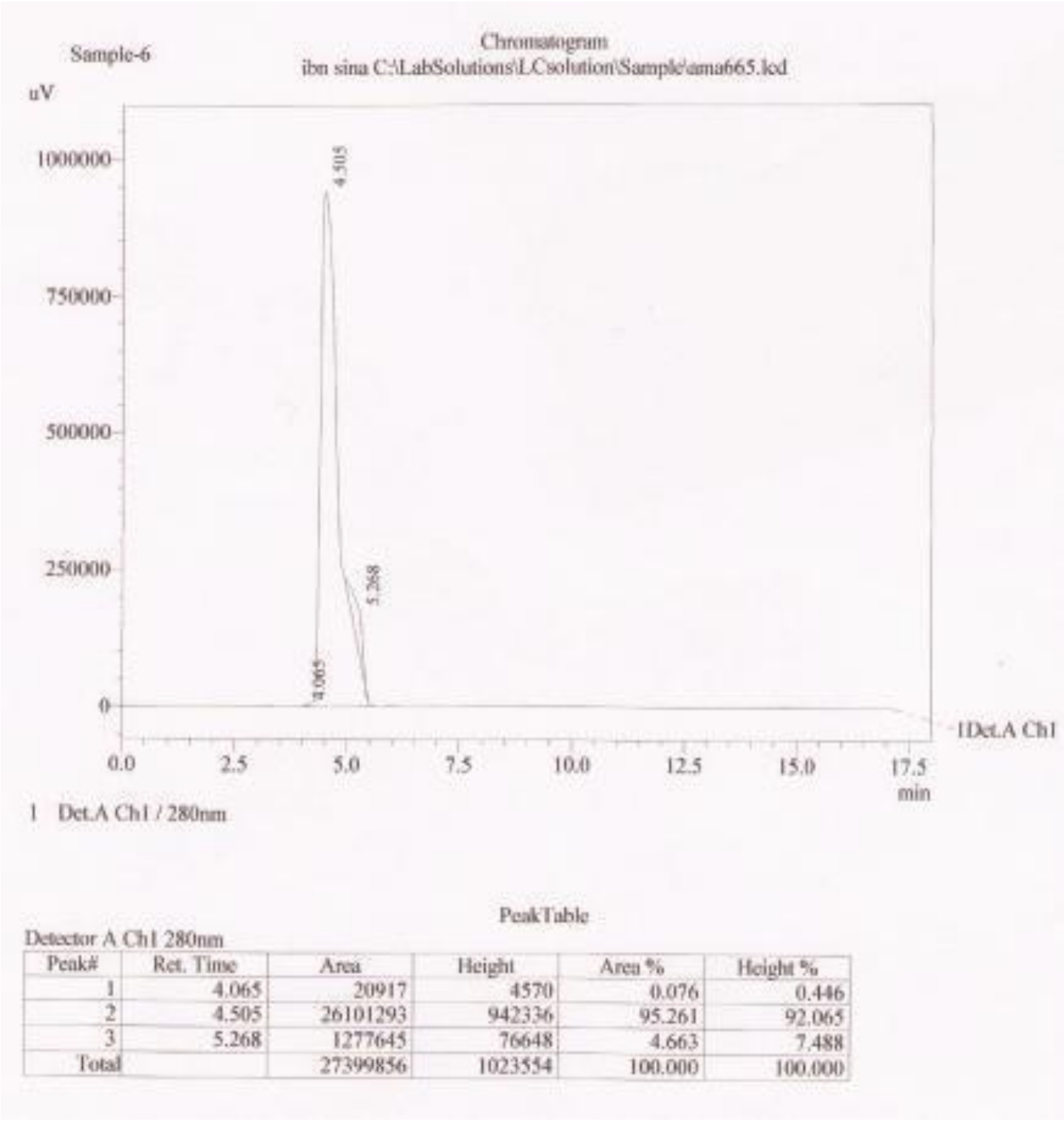
Table 2. Refered to Enzymatic activities after ammonium sulfate precipitation and dialysis.

Isolate no.	Production medium	Activity of CMCase (unit/ml)	Activity of cellobiase (unit/ml)
	CMC	0.180	0.380
C.J.N 6	Wheat brand	0.142	0.957
	Rice straw	0.404	0.666
	CMC	0.061	0.809
C.J.N 13	Wheat brand	0.080	0.966
	Rice straw	0.133	0.485
	CMC	0.095	0.761
C.J.N 14	Wheat brand	0.061	0.880
	Rice straw	0.142	0.452
	CMC	0.190	1.404
C.J.N 16	Wheat brand	0.119	0.476
	Rice straw	0.180	1.357

Pseudomonas return to *Cellvibrio*. Table 2 shows the enzymatic activity after ammonium sulfate precipitation and dialysis.

The addition of ammonium sulfa allows the concentration of enzymes to increase and the amount of water to decrease, after which the concentrated enzymes were obtained. The optimum salt concentration for enzymes precipitation depends on the number and distribution of charges and the hydrophobic groups. In addition to the size and shape, other compounds found affected protein solubility (Koolman and Roehm, 2005). After that, dialysis was used to increase the purity of cellulases. Nelson et al. (2004) referred to the fact that dialysis led to discard of the ammonium salts and other attached compounds which lead to increased enzyme activity. Also, the enzymes types which were produced by these bacteria were deleted. This was achieved by using HPLC and its results showed that *C. japonicus* have complete cellulase system. This appeared from three peaks in HPLC which indicate that these three enzyme types were produced by these bacteria (Figures 1, 2 and 3).

The complete hydrolysis for cellulosic material requires synergism action to be performed for the three enzymes which are Endoglacanase (cmcase), Exoglucanase (cellobiase) and B-gluconasidase (Gao et al., 2010).



PeakTable

Detector A Ch1 280nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	4.065	20917	4570	0.076	0.446
2	4.505	26101293	942336	95.261	92.065
3	5.268	1277645	76648	4.663	7.488
Total		27399856	1023554	100.000	100.000

Figure 1. HPLC analysis for *Cellvibrio japonicus* C.J.A 6 isolate.

Some microorganisms have been known to have incomplete cellulase system in that one of these enzymes is absent and for this reason, it is required to add one enzyme from external sources to complete the hydrolysis

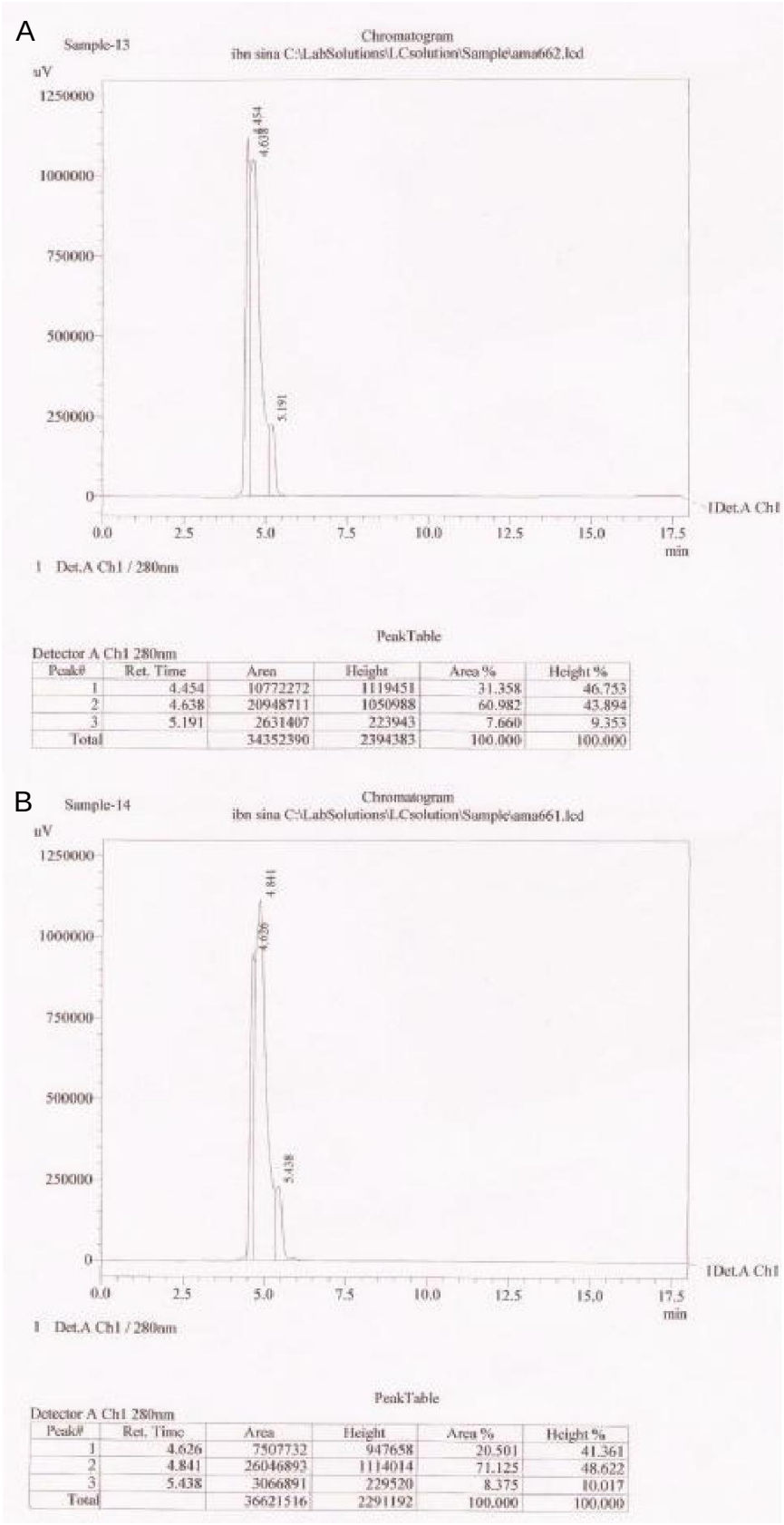
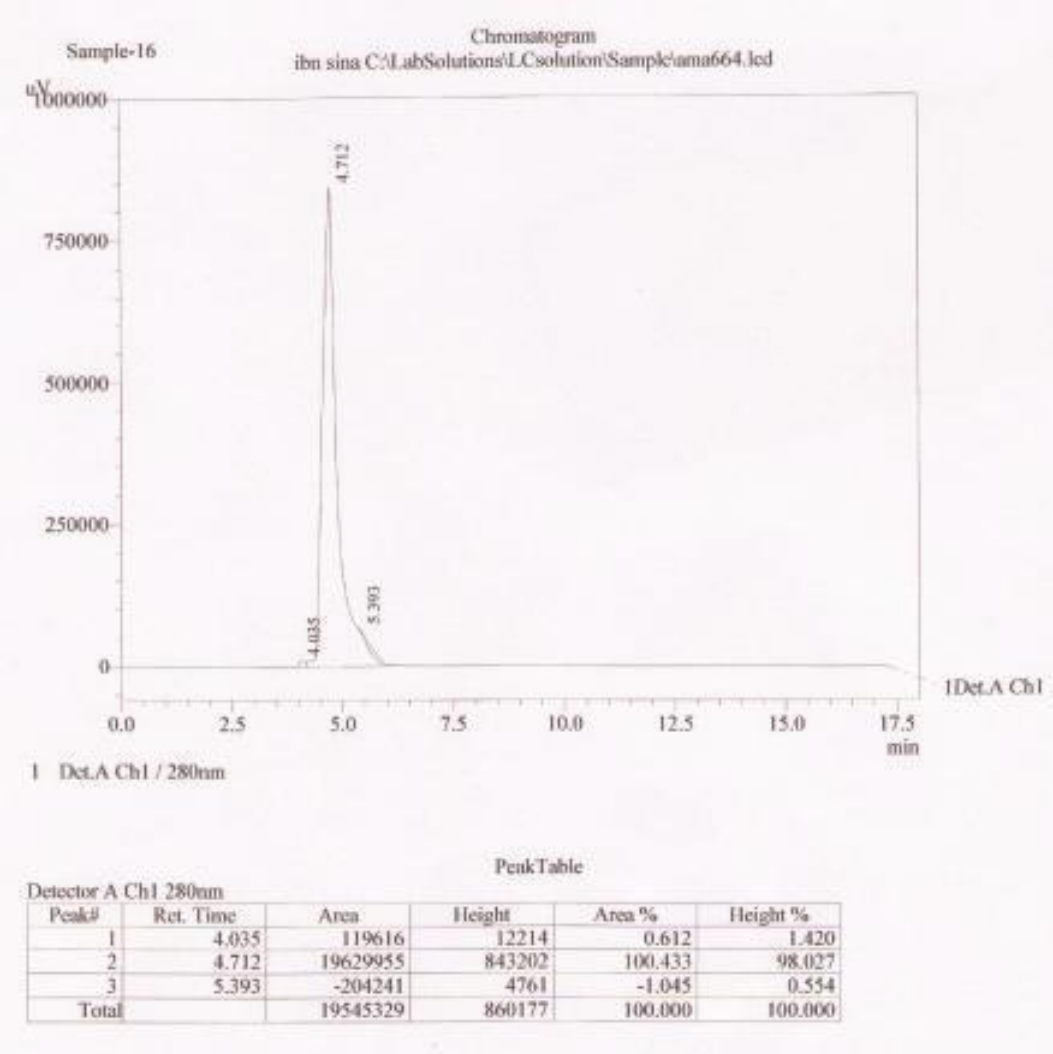


Figure 2. (A) HPLC analysis for *Cellvibrio japonicus* C.J.N 13 isolate. (B) HPLC analysis for *Cellvibrio japonicus* C.J.N 14 isolate.



PeakTable

Detector A Ch1 280nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	4.035	119616	12214	0.612	1.420
2	4.712	19629955	843202	100.433	98.027
3	5.393	-204241	4761	-1.045	0.554
Total		19545329	860177	100.000	100.000

Figure 3. HPLC analysis for *Cellvibrio jopanicus* C.J.N 16 isolate.

of cellulose to glucose (Gautam et al., 2010). In this study, it was observed that *C. japonicus* have a complete cellulase system. These results are in agreement with those of Jabber et al. (2004) who found out that the activity and molecular weight of CMCase are produced from different *Archniotus citrinus* isolates taken from different regions in Pakistan. In this study, there are three different peaks in HPLC which referred to the three enzyme types produced from these bacteria. However, the CMCase and cellobiase activity was measured and predicted by the B-glucosidase activity produced in the final setup of this process.

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