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ORIGINAL ARTICLE

Isolation and characterization of *Achromobacter* sp. CX2 from symbiotic Cytophagales, a non-cellulolytic bacterium showing synergism with cellulolytic microbes by producing β-glucosidase

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Abstract A Gram-negative, obligately aerobic, non-cellulolytic bacterium was isolated from the cellulolytic association of Cytophagales. It exhibits biochemical properties that are consistent with its classification in the genus *Achromobacter*. Phylogenetic analysis together with the phenotypic characteristics suggest that the isolate could be a novel species of the genus *Achromobacter* and designated as CX2 (= CGMCC 1.12675=CICC 23807). The strain CX2 is the symbiotic microbe of Cytophagales and produces β-glucosidase. The results showed that the non-cellulolytic *Achromobacter* sp. CX2 has synergistic activity with cellulolytic microbes by producing β-glucosidase. To our knowledge, this is the first report on the synergetic effect of the combination of non-cellulolytic and cellulolytic microbes, which is significant to help understand the cellulolytic mechanism of cellulose-digesting Cytophagales.

Keywords *Achromobacter* · Cellulolytic · β-Glucosidase · Cytophagales · Isolation · Synergic

Introduction

Cellulose consists of only a linear structure of β-1,4-linked glucose residues, but is very difficult to be degraded, because it is highly crystalline and often tightly intertwined together with hemicelluloses and other polymers presenting a barrier to degradation by cellulase (Carpita and Gibeaut 1993). Therefore, efficient degradation is the result of multiple activities working synergistically to efficiently solubilize crystalline cellulose (Sánchez et al. 2004; Li et al. 2009). Most known cellulolytic organisms produce multiple cellulases that act synergistically on native cellulose (Wilson 2008) as well as produce some other proteins that enhance cellulose hydrolysis (Wang et al. 2011a, b). Synergistic cooperation of different enzymes is a prerequisite for the efficient degradation of cellulose (Jalak et al. 2012). Both *Trichoderma reesi* and *Aspergillus niger* were co-cultured to increase the levels of different enzymatic components (Kumar et al. 2008). However, the ability of major cellulolytic member of microbial strains identified so far produced a limiting level of one or other enzymatic component (Maki et al. 2009). Thus, search for the potential source of cellulolytic activity is continuing in the interest of successful bioconversion of lignocellulosic biomass.

In our study of cellulose degradation, it was found that the cellulolytic Cytophagales were always associated with some non-cellulolytic microbes in their process of cellulose digestion. Kato et al. (2008) also confirmed that the cellulose-degrading culture contains four organisms, but only one can hydrolyze cellulose, and the others are not directly linked to cellulose hydrolysis. To eliminate those non-cellulolytic contaminants, serial dilution and parallel streaking on the cellulose medium was repeatedly performed. However, even after the cellulolytic Cytophagales were grown as a single colony on the cellulose plate, non-cellulolytic strains were still associated with it, because such an association can develop upon transferring to the glucose agar plate. In order to understand the attribution of those associations on cellulose digestion, one such non-cellulolytic microbe was isolated. It was observed that the novel isolate could enhance the cellulolytic activity of its partner Cytophagales when co-cultured together, although it did not show any activity on cellulose in the pure culture. In

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this paper, we report on the isolation and some properties of this novel non-cellulolytic association, *Achromobacter* sp. CX2, and its synergic effect with cellulolytic microbes by the produced β-glucosidase. To our knowledge, it is the first report on non-cellulolytic microbes working synergistically with cellulolytic strains to hydrolyze cellulose.

Materials and methods

Strains and culture condition

Cytophaga sp. LX-7 was isolated previously in our lab (Li and Gao 1997) and cultured in the mineral medium in the presence of 0.5 % (*w/v*) cellulose CF11 (Whatman) and 0.2 % (*w/v*) peptone at 30 °C and 150 rpm. The mineral medium consists of (per liter) 0.2 g $MgSO_4 \cdot 7H_2O$, 0.75 g KNO_3, 0.5 g K_2HPO_4, 0.02 g $FeSO_4 \cdot 7H_2O$, and 0.04 g $CaCl_2 \cdot 2H_2O$, pH 7.0–7.2. *Penicillium decumbens* JU-A10 was obtained by physical and chemical mutagenesis (Sun et al. 2008) and cultured at 30 °C and 160 rpm in the medium containing (per liter) 30 g wheat bran, 6 g microcrystalline cellulose, 30 g corncob residue, 2 g $(NH_4)_2SO_4$, 1 g urea, 3 g KH_2PO_4, 0.5 g $MgSO_4$, 0.3 g $CaCl_2$, 4 g peptone, and 1 g Tween-80.

Isolation of non-cellulolytic strain

A soil sample was collected from the surrounding enrichment with lignocellulose at the Bangcuidao Garden of Dalian, and inoculated on the filter paper agar plate by stamping the soil sample on the filter paper with the flat end of glass rod. The inoculated plates were incubated at 37 °C and the transfers were made as early as possible from the margin of the decomposed filter paper to a fresh filter paper agar plate when cellulolytic bacteria grew. The transfer was performed by streaking on the filter paper agar plate, and the plates were incubated at 37 °C for 5–6 days. This transfer was carried out over ten times to set up a steady cellulolytic association. A 10-fold serial dilution of the bacterial suspension with sterile saline was spread on the glucose medium plate, in which the bacterial suspension was prepared by mixing cellulolytic bacteria picked out from the thoroughly digested filter paper with the sterile glucose medium. After incubation at 37 °C overnight, the individual single colonies were picked out from the glucose medium plates and incubated on the fresh glucose medium plate. To ensure the strain purity, the isolate was streaked on the glucose medium plate and the single colony was used for the further study.

Filter paper agar plate was prepared by placing a piece of filter paper (Whatman No. 1, 8.5 cm in diameter) on a mineral medium with 2 % (*w/v*) soaked agar. Glucose medium contains (per liter) 15 g glucose, 5 g beef extract, 10 g peptone, and 20 g soaked agar in the mineral medium. The basal medium used for the taxonomic assay is 0.05 g yeast extract in 100 mL of the mineral medium. Soaked agar was prepared by soaking and washing agar gel with distilled water to remove soluble contaminant after agar was melted in water by heating and cooled down to form agar gel.

Morphological properties

The unidentified strain was cultured on the glucose medium plate overnight at 37 °C and the cellular morphology was observed by light microscopy. The motility was checked in the hanging-drop mount as described previously (Murray et al. 1994). Flagella were examined with a transmission electron microscope (TEM) by negative staining. For TEM, a droplet of the diluted culture was fixed with 3 % potassium phosphotungstate (pH 7.0) and the observation was made on a model JEM-2000EX TEM.

Physiological and biochemical characteristics

The Hucker staining method was used for Gram staining (Murray et al. 1994). Oxidase activity was detected using 1 % tetramethyl-p-phenylene-diamine as substrate, and the result was read within 30 s. Catalase activity was determined by mixing 3 % hydrogen peroxide with colonies cultured for 24–48 h, and the formation of gas bubbles within 2 min indicated a positive result. Nitrate reduction was tested on five consecutive days of incubation in culture medium containing 0.1 % KNO_3 and a negative result was confirmed by adding zinc dust. Urea hydrolysis was observed on Christensen urea agar slant and the occurrence of a red-violet color was considered as positive. Hydrolysis of starch, casein, esculin and gelatin, methyl red (MR) reaction, Voges-Proskauer (VP) test, indole production, hydrogen sulfide production, and citrate usage were carried out as described elsewhere (Smibert and Krieg 1994). The assimilation of glucose, xylose, maltose, sucrose, and cellobiose was determined by incubating the unidentified isolate in the mineral solution supplemented with 0.2 % of each carbohydrate at 37 °C for 7 days. Acid and gas production were tested at the same time as described by Smibert and Krieg (1994). Resistance to sodium chloride was detected by growing on the glucose medium plate supplemented with 1–6 % NaCl at 0.5 % intervals.

Nitrogen and carbon source usage

Nitrogen sources for cell growth were evaluated by comparing the turbidity of the culture in mineral solution supplemented with 0.3 % of glucose and 0.2 % nitrogen sources including yeast extract, peptone, beef extract, urea, sodium nitrate, ammonium citrate, diammonium phosphate, and ammonium sulfate. To test the carbon sources for cell growth the unidentified strain was incubated in the basal medium supplied with 0.3 %

carbon sources including glucose, fructose, maltose, soluble starch, cellobiose, sucrose, lactose, carboxymethyl cellulose, mannose, and inulin.

Temperature and pH growth

The temperature range for cell growth was determined by incubating the unidentified isolate in the glucose medium at different temperatures ranging from 5 to 60 °C. The pH range for cell growth was evaluated by culturing the unidentified isolate in the same medium at pH 2.0–10.0. The optimum temperature or pH for the maximum cell growth was detected by comparing the absorbance value of the culture.

Extraction and guanine-plus-cytosine content of genomic DNA

Genomic DNA was extracted from the cells growing in the glucose medium at 37 °C overnight and purified using the protocol described previously (Moore and Dowhan 1997).

The guanine-plus-cytosine (G+C) content of DNA was determined by thermal denaturation (Marmur and Doty 1962). Sample DNA was dissolved in 0.1×SSC buffer (0.15 M sodium chloride ⁄ 0.015 M sodium citrate buffer, pH 7.0) to make a concentration of OD_{280} between 0.3 and 0.5. The prepared sample was placed in a thermo-cuvette and the temperature was increased at a rate of 1 °C/min. Melting temperature (Tm) was determined at the mid-point of the denaturing curve. G+C content was calculated according to the following equation: G+C content (%)=(Tm −69.3)× 2.44×100 %. DNA of *Escherichia coli* K12 was used as a standard (G+C=50.6 mol %) and was always assayed together with the tested DNA to confirm the reliability of the results.

Phylogenetic analysis based on 16S rDNA gene sequence

The amplification and sequencing of 16S rDNA gene from a single colony cultured on the glucose medium overnight was commissioned to Takara Co. (Dalian, China). To avoid misreading as a result of PCR error, the sequencing was repeated at least twice.

The 16S rDNA gene sequence of the unidentified strain was compared with those available in the GenBank/EMBL database and Ribosomal Database Project-II, and the sequences of the closely related strains were retrieved (Johnson et al. 2008; Cole et al. 2009). Those sequences were aligned with CLUSTAL X program and corrected manually (Thompson et al. 1997). Phylogenetic analysis was performed using the PHYLIP software package by bootstrapping (100 replicates) with programs SEQBOOT, DNADIST, NEIGHBOR, and CONSENSE (Felsenstein 2005). The percentage similarities of 16S rDNA gene sequence of the unidentified strain with other closely relative bacteria were calculated with MegAlin program in DNAStar package (DNAStar Inc., Madison, USA).

Cellulase preparation

Cytophaga sp. LX-7 was cultured in a 250-mL flask containing 60 mL of mineral medium in the presence of 0.5 % cellulose CF11 (Whatman) and 0.2 % peptone at 30 °C and 150 rpm for 6 days. The culture supernatant was obtained as cellulase by centrifugation at 4 °C and 10,000 *g* for 15 min. After *P. decumbens* JU-A10 was incubated at 30 °C and 160 rpm for 5 days, the culture supernatant was collected as fungal cellulase by centrifugation at 4 °C and 10,000 *g* for 15 min. The isolate CX2 was incubated in the glucose medium at 37 °C and 150 rpm for 48 h, and the culture supernatant was obtained by centrifugation at 4 °C and 10,000 *g* for 15 min.

Enzyme assay

Cellulase activity on Avicel was determined by incubating 1 mL of the culture supernatant with 1 mL of 2 % Avicel (Serva) in 100 mM phosphate-citrate buffer (pH 6.6) at 37 °C for 2 h. The soluble carbohydrate and reducing sugar was assayed respectively by phenol-sulfuric acid method (Dubois et al. 1956) and dinitrosalicylic acid method (Miller 1959) after the insoluble material was sedimented by centrifugation. One unit of cellulase was designated as the amount of enzyme that produced 1 μmol glucose equivalent per min under the condition as described above. Cellulase activity on filter paper was determined using a 1×6 cm strip of Whatman No. 1 paper as described previously (Mandels et al. 1976). β-Glucosidase was determined by measuring the release of p-nitrophenol as previously reported (Li and Gao 1997).

Cellulolytic synergism assay

To assay the synergy between isolate CX2 and *Cytophaga* sp. LX-7, cellulase activity on filter paper or Avicel in the culture supernatant of the isolate CX2 together with strain LX-7 was assayed by measuring the soluble carbohydrate (Dubois et al. 1956) and reducing sugar (Miller 1959), respectively. The synergistic reaction was performed at 37 °C for 2 h in the suspension of 1 % Whatman No. 1 filter paper or Avicel cellulose (Serva) in 50 mM phosphate-citrate buffer (pH 6.6). The culture of the isolate CX2 and strain LX-7 was heated at 100 °C for 15 min and used for control to remove the interference of soluble carbohydrate occurred in the culture supernatant.

To determine the synergy between isolate CX2 and *P. decumbens* JU-A10, cellulose hydrolysis was performed at 50 °C for 8 h and the release of reducing sugar was measured (Miller 1959). The synergistic test system consisted

of 7.5 mg Whatman No. 1 filter paper, 100 μL 50 mM acetate buffer (pH 4.8), 25 μL JU-A10 culture supernatant, and 25 μL CX2 culture supernatant. For the control test, the culture supernatant of strain CX2 and strain JU-A10 was replaced with 25 μL acetate buffer, respectively. The inactivated supernatant was heated to 100 °C for 15 min and used as a blank to exclude the interference of reducing sugar existed in the broth. All assays were performed in triplicate.

Production of β-glucosidase

To evaluate the optimum temperature for β-glucosidase production, isolate CX2 was incubated in the glucose medium for 36 h at different temperatures ranging from 25 to 45 °C, and β-glucosidase activity in the culture supernatant was determined. The optimal pH for β-glucosidase production was analyzed by culturing the isolate CX2 in the glucose medium with different pH. The effect of carbon source on β-glucosidase production was determined by assaying β-glucosidase activity in the culture supernatant after incubating in the mineral medium with 0.2 % (w/v) peptone and 0.5 % of different carbohydrates at 30 °C and 150 rpm for 36 h.

After incubating in the glucose medium overnight, a 2 mL culture of the isolate CX2 was inoculated in 200 mL of fresh glucose medium and incubated at 37 °C with shaking at 150 rpm. The culture was sampled at 4 h intervals over 36 h incubation. Cell concentration was determined by measuring the culture turbidity at 600 nm and β-glucosidase activity in the culture supernatant was evaluated using the above-mentioned method.

Statistical analysis

All of the assays and determinations described in this paper were performed in triplicate unless otherwise stated. The data were subjected to one-way analysis of variance (ANOVA) to detect statistical significance.

Results

Isolation of associated cellulolytic bacterium

After several transfers by streaking on the filter paper agar plates, a yellowish Cytophagales association was established. Such an association was streaked on the glucose medium plates and several single colonies were selected. The sequencing results of 16S rDNA gene indicated that all those sequences were identical. Thus, one single colony was purified further by streaking on the glucose medium plate, and the purified species was designated as CX2 for further study.

Phenotypic characterization

The strain CX2 was Gram-negative and had straight rods (1.0–2.0×0.5–1.0 μm) with rounded ends. It is motile with peritrichous flagella and obligate aerobe. Colonies on the glucose agar are circular, opaque, flat, or slightly convex with smooth margin and white in color.

Strain CX2 exhibited the enzyme activity in oxidase and catalase, but not in lysine decarboxylase, ornithine decarboxylase, arginine dihydrolase and urease. It produced acid from xylose, maltose, sucrose, and cellobiose, but not from glucose. The tests in gas production from glucose and nitrate reduction were positive, and both MR reaction and VP test were negative. The isolate CX2 failed to hydrolyze gelatin, starch, and casein, but it did hydrolyze esculin. The carbohydrates capable of being assimilated included D-glucose, D-xylose, maltose, and cellodextrin. There was no production of hydrogen sulfide and indole. Strain CX2 could grow in the mineral medium with glucose as the sole carbon and energy source, and use citrate for growth.

Phylogenetic analysis

The 16S rDNA gene from the isolate CX2 was sequenced and a 1475-base sequence was obtained (accession JX645344, GenBank), which was most similar to that of the species belonging to the genus *Achromobacter*. The level of sequence similarities among strain CX2 and species of the genus *Achromobacter* ranged from 98.1 to 99.5 %. Strain CX2 exhibited the highest sequence similarities to the sequence of *Achromobacter ruhlandii*. A dendrogram generated by the neighbor-joining method was shown in Fig. 1, in which the dataset used for the construction of the phylogenetic tree contained 1456 nucleotides. Our strain formed a phylogenetic cluster with *A. ruhlandii*.

The GenBank accession numbers were shown in Fig. 1.

DNA base composition

The G+C content of strain CX2 was 65.1 mol %, which was comparable to that of the genus *Achromobacter* ranging from 65 to 68 mol %.

Culture condition for cell growth

Good growth was supported by sugars including glucose, sucrose, maltose, mannose, fructose, and cellobiose, but poor growth was observed when cellulose, inulin, starch, lactose, and carboxymethyl cellulose were used. All the tested nitrogen sources including ammonium citrate, ammonium biphosphate, ammonium sulfate, sodium nitrate, urea, beef extract, peptone, and yeast extract supported the isolate CX2

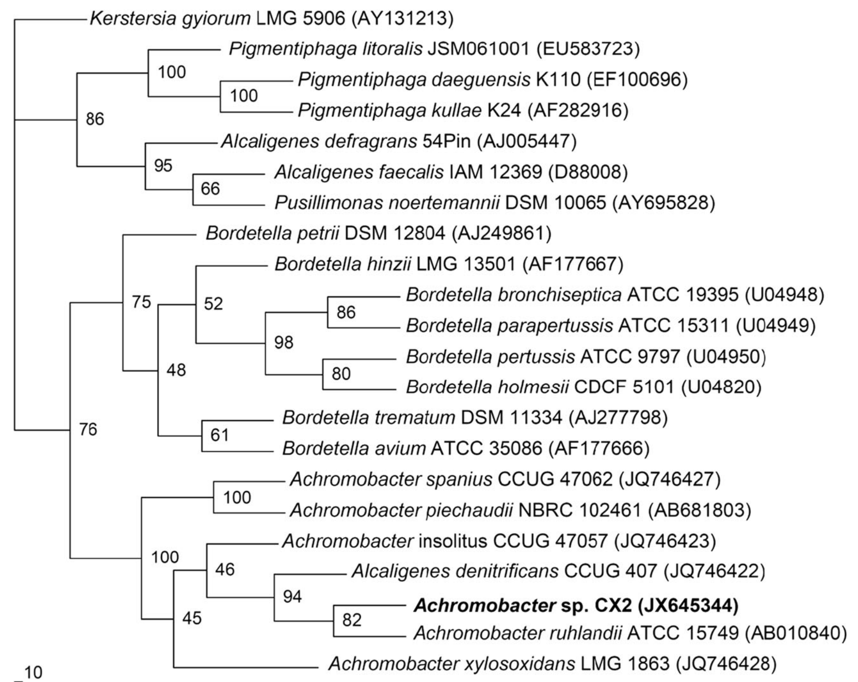

Fig. 1 Neighbour-joining phylogenetic tree showing the position of the isolate CX2T within the genus *Achromobacter* based on 16S rDNA gene sequence data. GenBank accession numbers were shown in parentheses. Bootstrap values expressed as a percentage of 100 replications were given at the branching points. The scale bar represents 1 % sequence dissimilarity

growth. There was no special growth factor requirement by the isolate CX2.

Strain CX2 could grow at the temperature range from 4 to 60 °C, and the optimum temperature was at 37 °C. The pH range for cell growth was from 5 to 11 and maximum growth occurred at pH 7.0–8.0. Strain CX2 was not halophilic, but was able to grow in the presence of NaCl concentrations up to 4 %.

Role of *Achromobacter* sp. CX2 in cellulose degradation of *Cytophaga* sp. LX-7

As shown in Fig. 2, there was no cellulase activity on filter paper or Avicel to be detected in the culture of the isolate CX2. However, the cellulase activity of *Cytophaga* sp. LX-7 was increased by 18–20 % when the culture of *Achromobacter* sp. CX2 was combined in the culture supernatant of *Cytophaga* sp. LX-7 and cellulose degradation was detected by measuring the released soluble carbohydrate. It suggested that a synergism between cellulolytic *Cytophaga* sp. LX-7 and non-cellulolytic strain CX2 was involved in the degradation of filter paper or Avicel. To confirm such a synergistic degradation in the combination of strain CX2 and *Cytophaga* sp. LX-7 further, cellulase activity was also assayed by the released reducing sugar. Almost the same cellulase level as that by measuring the released soluble carbohydrate was observed (Fig. 2), whereas only a little cellulase activity on filter paper or Avicel was detected in the culture supernatant of *Cytophaga* sp. LX-7 by measuring the released reducing sugar.

Production of β-glucosidase by *Achromobacter* sp. CX2

The β-glucosidase activity could be detected in the culture supernatant of the isolate CX2 in the presence of glucose, sucrose, maltose, mannose, fructose, or cellobiose, which was capable of hydrolyzing cellodextrin into glucose. As shown in Fig. 3, the maximal β-glucosidase activity was obtained when the isolate CX2 was cultured at 35 °C and pH 7.0–8.0. The time course of the isolate CX2 growing in the glucose medium

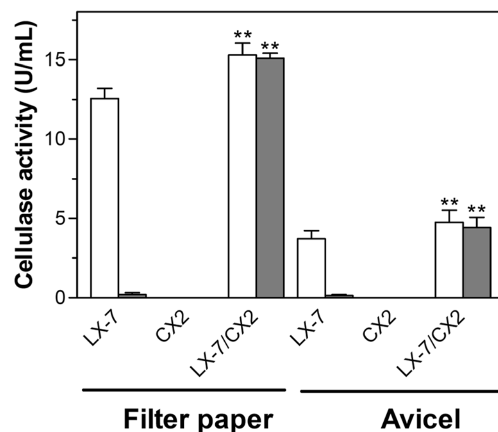

Fig. 2 Synergistic interaction occurred between *Achromobacter* sp. CX2 and *Cytophaga* sp. LX-7. The culture supernatant of the isolate CX2 was incubated with strain LX-7 together in filter paper or Avicel suspension for 2 h, in which the cellulase was determined by assaying soluble carbohydrate (clear column) or reducing sugar (filled column). X-labels LX-7/CX2 is for the synergistic results between LX-7 and CX2; LX-7 and CX2 is for the cellulolytic result by LX-7 and CX2 individually. Symbol ** at the top of the bars indicates significant differences between the co-culture of LX7/CX2 and the individual LX7

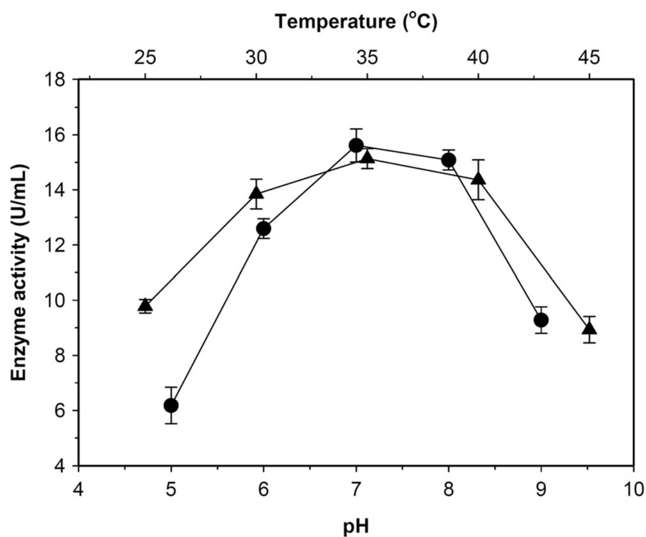

Fig. 3 Effect of temperature (▲) and pH (●) on β-glucosidase production by *Achromobacter* sp. CX2 cultured in the glucose medium

for cell growth and β-glucosidase production is shown in Fig. 4. β-Glucosidase activity increased progressively with the cell growth, and the maximal activity was obtained when cell growth approached a plateau.

Cellulolytic synergism between isolate CX2 and *P. decumbens* JU-A10

The release of reducing sugar from cellulose degradation was measured when incubated the culture supernatant of *P. decumbens* JU-A10 or the combined fungal culture filtrate with the isolate CX2 culture in filter paper suspension. As shown in Fig. 5, the reducing sugar released by fungal culture filtrate mixed with the cell-free culture of strain CX2 was greater than the sum of the amounts released by the individual fractions. The cellulolytic activity of *P. decumbens* JU-A10 was enhanced by *Achromobacter* sp. CX2.

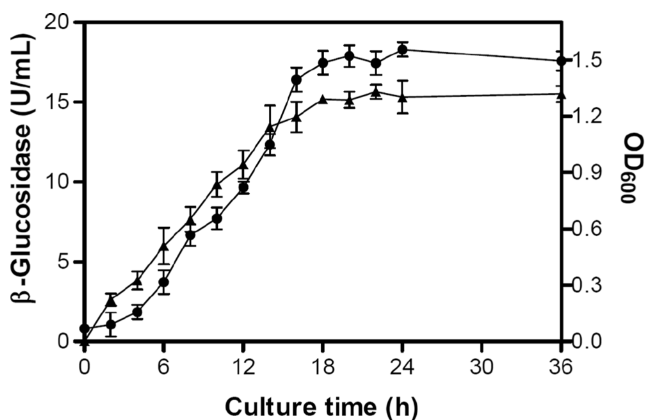

Fig. 4 Cell growth (●) of *Achromobacter* sp. CX2 and process of β-glucosidase production (▲) in the glucose medium at 37 °C with shaking at 150 rpm

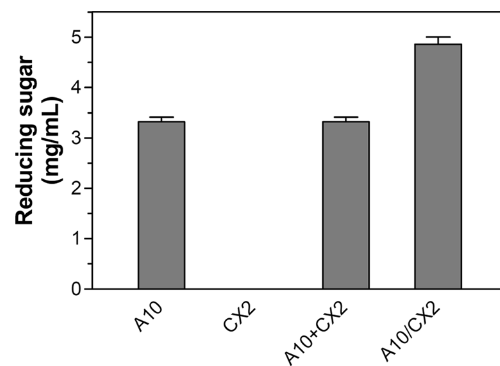

Fig. 5 Synergistic interaction occurred between *Achromobacter* sp. CX2 and *Penicillium decumbens* JU-A10. The culture supernatant of strain CX2 was incubated with strain JU-A10 culture filtrate in filter paper reaction system for 8 h. X-labels A10/CX2 is for the synergistic results between A10 and CX2; A10 and CX2 is for the cellulolytic result by A10 and CX2 individually, and A10+CX2 is the sum of the combined A10 and CX2

Discussion

Although *Achromobacter* spp. is commonly isolated from the human gastrointestinal tract as a causative pathogen of various infections (Park et al. 2012), some *Achromobacter* species are also confirmed to exist in the cellulose-degrading bacterial community by means of 16S rDNA comparison or fatty acid analysis (Dumova and Kruglov 2009). However, there was no single colony to be isolated from the cellulolytic coenosis so far, as well as the function of those strains was not clarified (Dumova and Kruglov 2009; Yang et al. 2011; Talia et al. 2012). Recently an *Achromobacter* species was found to be always associated with the cellulolytic Cytophagales in our searching for cellulose-degrading microbes. In order to understand its roles in the cellulose digestion, one such *Achromobacter* strain was isolated from the cellulolytic association and designed as CX2.

The taxonomic properties of isolate CX2 are consistent with the key characteristics of the genus *Achromobacter* including Gram-negative, straight rod, non-spore forming, motile with peritrichous flagella, obligately aerobic, oxidase- and catalase-positive, reduces nitrate to nitrite (Busse and Auling 2005). Therefore, strain CX2 should be phenotypically classified in the genus *Achromobacter*.

Phylogenetic analysis based on 16S rDNA gene sequence also supported that strain CX2 was a member of the genus *Achromobacter*. Sequence search on the database revealed that the newly determined sequence was closely related to the sequences from strains belonging to the genus *Achromobacter*. The levels of sequence similarity between the isolate CX2 and species in the genus *Achromobacter* were higher than 98.0 %, which were in accordance with the proposal of 95 % 16S rDNA gene sequence similarity as a cut-off value for delineating genera (Wagner-Döbler et al. 2004). Moreover, the G+C content of the genomic DNA also

fell into the range of 65–68 mol %, which is just the range of G+C content for the genus *Achromobacter*.

As shown in the neighbour-joining phylogenetic tree constructed based on 16S rDNA sequence (Fig. 1), the isolate CX2 was clearly clustered into the clade of the genus *Achromobacter*, in which the most closely related species was *A. ruhlandii* with a similarity of 99.5 %. *Alcaligenes denitrificans* also was clustered with isolate CX2 and showed higher homology (99.2 % similarity), whereas it has been formally transferred to the genus *Achromobacter* as *Achromobacter denitrificans* (Coenye et al. 2003a). Strain CX2 exhibited over 98 % 16S rDNA sequence similarity with the other species in the genus *Achromobacter*, whereas with the species in the other related genera, such as *Bordetella* and *Pigmentiphaga,* the similarities of 16S rDNA sequence was all lower than 98 % except *Bordetella hinzii* and *B. avium* having 98.2 % similarity. Such a differentiation at the genus level was further supported by biochemical characteristics.

Although the phenotypic characteristics of strain CX2 were consistent with its classification in the genus *Achromobacter*, the isolate CX2 could be distinguished from its nearest apparent phylogenetic neighbor *Achromobacter* spp. in some notable phenotypic traits (Table 1), such as denitrification, assimilation of D-glucose and D-xylose, hydrolysis of esculin, H_2S production, acid production. On the basis of the differential phenotypic and phylogenetic characteristics, it was confirmed that the isolate CX2 could not be assigned to any previously recognized bacterial species. Thus, we propose that strain CX2 represents a new member of the genus *Achromobacter*, and named *Achromobacter* sp. CX2.

Table 1 Phenotypic characteristics that distinguish *Achromobacter* sp. CX2 from other related *Achromobacter* species

Characteristic	1	2	3	4	5	6	7
Denitrification	+	+	+	−	−	−	−
Assimilation of							
D-Glucose	+	+	−	−	−	−	+
D-Xylose	+	+	−	−	−	−	+
Esculin hydrolysis	+	−	+	−	−	−	−
H_2S production	−	−	−	−	+	−	−
Acid production from							
Glucose	−	+	−	−	−	−	−
Xylose	+	+	−	−	−	−	−
Sucrose	+	−	−	ND	−	ND	−
Citrate utilization	+	+	+	+	−	+	+

References (Busse and Auling 2005; Coenye et al. 2003a, b; Gomila et al. 2011; Papalia et al. 2013)

Species: 1, *Achromobacter* sp. CX2; 2, *A. xylosoxidans*; 3, *A. denitrificans*; 4, *A. spanius*; 5, *A. piechaudii*; 6. *A. insolitus*; 7, *A. ruhlandii*

Symbol: +, 90 % or more of strains are positive; −, 90 % or more of strains are negative; d, 11–89 % of strains are positive; ND, no data available

No matter how many times the yellowish Cytophagales were purified by serial dilution or parallel streaking on cellulose plates, some identical white colonies develop when transferred to the glucose medium plate. A deliberate search was made for synergic microbes in the Cytophagales association, and *Achromobacter* sp. CX2 was obtained. However, the strain CX2 is a non-cellulolytic microbe, because it could not grow on the cellulose medium and there was no detectable cellulolytic activity in the culture of strain CX2 (Fig. 2). This suggested that the cellulose-digesting Cytophagales were associated with free-living non-cellulolytic microbes.

To ascertain the role of *Achromobacter* sp. CX2 in the cellulose degradation of Cytophagales, the cellulose-degrading activity of *Cytophaga* sp. LX-7 was assayed in the presence of *Achromobacter* sp. CX2. As shown in Fig. 2, the cellulase activity of *Cytophaga* sp. LX-7 increased by *Achromobacter* sp. CX2 when cellulose degradation was evaluated by phenol-sulfuric acid (Dubois et al. 1956), suggesting that non-cellulolytic strain CX2 had a synergism with cellulolytic *Cytophaga* sp. LX-7 in cellulose degradation. It is interesting that the significantly increased cellulase activity was observed in the combination of strain CX2 and *Cytophaga* sp. LX-7 when cellulase activity was determined by measuring reducing sugar (Fig. 2), although only detectable cellulase activity could be detected in the culture of *Cytophaga* sp. LX-7 by assaying reducing sugar. In fact, the main cellulolytic products by *Cytophaga* sp. LX-7 were cellodextrin, which was supported by the reverse cellulase activity of *Cytophaga* sp. LX-7 by assaying the soluble carbohydrate and reducing sugar (Li and Gao 1997). It was presumed that the oligosaccharide produced by *Cytophaga* sp. LX-7 was hydrolyzed subsequently by *Achromobacter* enzymes, leading to the increased reducing sugar in the synergistic degradation of the combination of *Cytophaga* sp. LX-7 and *Achromobacter* sp. CX2. Therefore, β-glucosidase was presumably produced by *Achromobacter* sp. CX2.

In the following experiments, such β-glucosidase activity was detected in the culture supernatant of *Achromobacter* sp. CX2, which can hydrolyze cellodextrin into glucose. The β-glucosidase activity could be detected in the culture supernatant with all tested carbohydrates as carbon sources, suggesting that β-glucosidase was produced constituently by *Achromobacter* sp. CX2. The optimum temperature for β-glucosidase production was at 35 °C and the maximal β-glucosidase activity was detected in the culture supernatant when the isolate CX2 was incubated at pH 7.0 and 8.0 (Fig. 3).

To confirm such synergism of β-glucosidase in cellulose degradation, the cellulose-degrading activity of *P. decumbens* JU-A10 was determined in the presence of *Achromobacter* sp. CX2. It has been reported that the low secretion of β-glucosidase results in cellobiose accumulation acting as a feedback-inhibitor of cellulose depolymerisation (Mfombep et al. 2013). As shown in Fig. 5, the isolate CX2 could

increase the cellulase activity on filter paper of *P. decumbens* JU-A10 when mixed with the fungal culture filtrate, although the pure culture of strain CX2 did not have any effect on filter paper. This result suggested a synergistic interaction occurred between *P. decumbens* JU-A10 and strain CX2. Thus, the culture supplement of the strain CX2 presumably is a complement to β-glucosidase activity in *P. decumbens* JU-A10 to allow cellulolytic enzymes to function more efficiently by scavenging end product cellobiose (Sun et al. 2008; Liu et al. 2012).

Conclusion

Achromobacter sp. CX2 is a non-celllulolytic microbe associated with the cellulolytic Cytophagales and produces β-glucosidase capable of hydrolyzing cellodextrin into glucose. It shows synergism with the cellulolytic microbes as the complement of β-glucosidase to relieve the feedback inhibition of end product cellobiose. This is the first report on the synergistic effect of the combination of the non-cellulolytic and cellulolytic microbes. Such an obtained association of *Achromobacter* sp. CX2 is significant to help understand the cellulolytic mechanism of cellulose-digesting Cytophagales.

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Conflict of interest The authors declare that they have no conflict of interest.

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