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Characteristics of cellulase in cellulose-degrading bacterium strain *Clostridium straminisolvens* (CSK1)

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Microbial degradation of biomass was considered as a clean and highly efficient approach to produce bioenergy, as it could mitigate the urgent demand for limited petroleum and natural gas. A thermophilic microbial consortium (MC1) was proved a high efficiency cellulose-degrading bacterial community in previous studies. A novel anaerobic, thermophilic, and cellulolytic bacterium (strain CSK1) was isolated from MC1. The cellulase activity characteristics of CSK1 were analyzed and evaluated by exploring new measuring conditions via the 3,5-dinitrosalicylic acid (DNS) spectrophotometry and the carboxymethyl cellulose (CMC) saccharogenic power method. The results indicated that the optimal measuring wavelength, reaction temperature, and pH value, were 530 nm, 60°C and 6.0, respectively. The ideal reaction time to achieve stable and significant measuring cellulase activity was about 10 min. Cellulase of CSK1 remained stable when the temperature was below 70°C, and the pH between 5.0 and 10.0, and its activity was quickly reduced when the temperature and pH exceeded such ranges. The cellulase activity of CSK1 reached the highest level on culturing day 8, and high correlations were found among cellulase activity variation, pH and CSK1 biomass change.

Key words: Strain CSK1, cellulose degradation, composite microbial system microbial consortium (MC1), 3,5-dinitrosalicylic acid (DNS) spectrophotometry, enzyme activity.

INTRODUCTION

With the intensified crisis of global petroleum supply and climate change, the urgent demand in the production of sustainable and renewable energy becomes an important issue all over the world (Stephanopoulos, 2007; Kerr, 2007). As one of the most widely used renewable energy, bioenergy has high potential in alleviating the energy crisis. Among all the possible materials for production of bioenergy, cellulosic resources have their distinct advantages compared to others, such as widespread distribution, no competition with food production, low pollution, and sustainability (Bugg et al., 2011). However, the natural structure of cellulosic resources is a big obstacle for utilizing them to produce bioenergy and renewable chemicals: Cellulose is embedded in a lignin matrix, which has an insoluble high crystal structure framework, and is difficult to hydrolyze. Such structure has largely limited the full utilization of the available cellulosic resources.

Microorganisms provide many advantages as potential sources of cellulosic material degradation: They can

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degrade various types of materials, pose little threat to the environment, are highly efficient at degradation and are reusable. Numerous studies have been conducted on the degradation of cellulosic materials by using different types of microorganisms (Anand et al., 2010; Bayer et al., 1998; Pérez et al., 2002; Wang and Gao, 2003; Xu and Goodell, 2001). However, without pre-treatment and/or sterilization of cellulosic materials, effective utilization of these microorganisms for such causes remains difficult. Cellulosic materials are naturally degraded by the cooperation of many microorganisms working in tandem. Previous studies have shown that an ideal culture for cellulose degradation was a mixed culture comprised of a cellulolytic bacterium and a non-cellulolytic bacterium (Guo et al., 2010; Lewis et al., 1988; Lü et al., 2013; Odom and Wall, 1983).

In our past research, a stable bacterial community, MC1 (thermophilic microbial consortium), was constructed that was capable of effectively degrading various cellulosic materials (for example, Filter paper, cotton and rice straw) under aerobic static conditions at 50°C (Cui et al., 2002). It was capable of completely degrading 0.5 g/100 mL filter paper within 48 h, and of completely degrading 2 g/100 mL rice straw within 8 days (Wang et al., 2005). The cellulose-degradation efficiency and the composition of this bacterial community have remained unchanged for over 10 years. To fully understand the mechanisms responsible for the effective cellulose degradation, the characteristics of each individual bacterium must be clarified (and especially those of the cellulose-degrading bacterium). CSK1 (*Clostridium straminisolvens*) is the only cellulose-degrading bacterium that has been successfully isolated from MC1 (Kato et al., 2004a, b). Researchers have studied the relationship between CSK1 and other bacterium strains in MC1 (Kato et al., 2008), and the loss of the substrate weight of CSK1 (Kato et al., 2004b). However, no research has been done by using cellulase characteristics to evaluate the cellulase production and the cellulose degradation ability of CSK1. Cellulase is a multicomponent enzyme, for which composition and proportion can be significantly different. Also, the substrates with which that cellulase works are themselves complex. Therefore, many methodological studies have been conducted for cellulase activity determination and evaluation (Bailey et al., 1975; Ghose 1987; Goksyr and Eriksen, 1980; Singh et al., 2009), and reported that different types of cellulase require different reaction temperatures, pH, reaction times, and other specific conditions. Characteristics of MC1 cellulase activity have been studied previously (Cui et al., 2004; Zhe et al., 2003). However, no research has been done on the determination conditions, the production mechanisms, and the evaluation of the cellulase of CSK1.

In the current study, the measuring wavelength, reaction time, temperature and pH were taken into consideration for optimizing the determination parameters of cellulase in CSK1. The stability of callulase at different pH and temperature conditions were evaluated. CSK1 was cultured for 16 days; its cellulase activitiy, pH, and protein quantity throughout the growth cycle were measured to evaluate the production mechanisms and characteristics of cellulase.

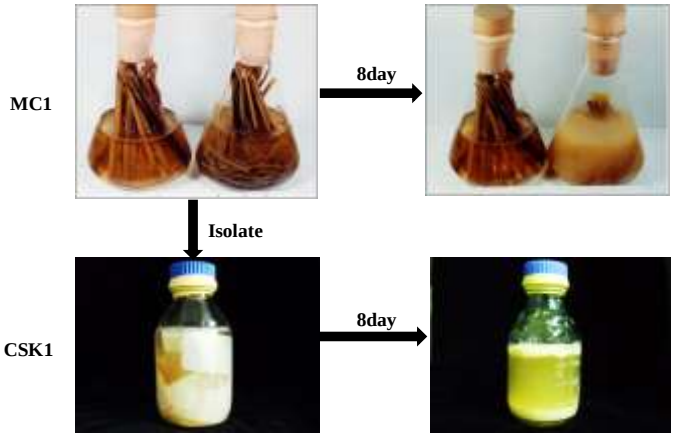

Figure 1. 2g/100mL rice straw was completely degraded within 8 days by MC1. 1g/100mL filter paper was completely degraded within 8 days by CSK1.

MATERIALS AND METHODS

Bacterial strain

CSK1 is a novel anaerobic, thermophilic and cellulolytic bacterium. The 16S rRNA gene sequence of the CSK1 stain was mapped to cluster III of the genus *Clostridium*. Strain CSK1 is closely related to *Clostridium thermocellum* (96.2%) and *Clostridium aldrichii* (95.1%) (Kato et al., 2004a). In the current research, the CSK1 strain which was anaerobically isolated from the cellulose-degrading bacterial community MC1, and stored in our lab was used for the production of cellulase for the following research (Figure 1).

Culture conditions

The inoculation and culturing were conducted under stringent anaerobic conditions. The culturing media were prepared by dissolving and thoroughly mixing 1 g yeast extract, 5 g peptone, 2 g $CaCO_3$, 5 g NaCl, 1 mg resazurin and 10 g filter paper with 1 L of water (pH 7.0). Reducing agent resazurin (7-Hydroxy-3H-phenoxazin-3-one 10-oxide) (Sangon Biotech, Shanghai, China) was used as an oxidation-reduction indicator in culturing media. The fully reduced anaerobic culturing media, which indicated as colorless state of resazurin was used for the cultivation of CSK1 strain. The culturing media were sterilized and cooled down in an anaerobic chamber operated by an anaerobic gas mixture (H_2/N_2: 10/90, v/v). The CSK1 was inoculated and cultured in the culturing media under anaerobic conditions at 50°C for 8 days.

Preparation of crude enzyme

To estimate enzyme activity, culture samples were taken out in anaerobic condition from the previous shook cultural flask. 15 mL

centrifuge tubes, each contained 7 mL culture sample was centrifuged at 12,000 rpm/min for 10 min at 4°C. The supernatants and pellets were separated, and the supernatants were used as extracellular crude enzyme samples to determine cellulase activity.

Determination of cellulase activity

The enzyme activity of CSK1 was determined using the CMC saccharogenic power and the DNS spectrophotometry method. The CMC is water soluble, thus can be easily hydrolyzed. It was used as the substrates to determine the activity of CMCase. Basically, the CMC was degraded by the cellulase to produce free reducing sugars, which quantity was then determined by the DNS method to evaluate the cellulase activity (Aoyama et al., 2015; Eveleigh et al., 2009; Wang et al., 2014). In operation, the substrate was suspended in 1/15 mol/L disodium hydrogen phosphate-potassium-dihydrogen phosphate buffer (pH 6.24) at 1% (w/v). 0.5 mL substrate solution was pre-heated at 60°C for 5 min prior to the addition of 0.5 mL culture supernatant. The mixture, including buffer, crude enzyme solution, and substrate, was incubated for 10 min at 60°C. The DNS method was used for the measurement of enzyme activity (Ghose, 1987). All samples were analyzed in triplicate. One unit (IU) of enzyme activity was defined as the amount of enzyme releasing 1 µg of reducing sugars during a 1 min reaction.

Wavelength for cellulase activity measurement

The ultraviolet spectrophotometer (Model UV-2550, SHIMADZU, Japan) was used to scan the samples, and the measurement wavelength range from 490 to 580 nm was selected for the scanning. The absorption of DNS and its reaction products with crude cellulase extract produced by CSK1 were measured by the spectrophotometer.

Effect of reaction temperatures on cellulase activity

To determine cellulase activity at different temperatures, the cellulase reaction temperature was set as 30, 40, 50, 60, 70 and 80°C. Cellulase activity measurements at each respective temperature were conducted as described in the determination of cellulase activity section.

Effect of pH on cellulase activity

To determine cellulase activity at different pH levels, we used sodium phosphate buffer solution to modulate the reacting substrate CMC solution into different pH levels: 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0 and 11.0. Cellulase activity measurements were conducted as described in the determination of cellulase activity section.

Effect of reaction time on cellulase activity

To determine cellulase activity at different time periods, the reaction time was set as 5, 10, 20, 30, and 40 min. The quantity of reducing sugar produced during the reaction process, as well as the cellulase activities were measured for evaluation of the influence of the reaction time.

Effect of treatment pH on cellulase activity

Buffer solutions with different pH levels from 2 to 12 were prepared of 0.2 mol·L^{-1} sodium phosphate. 25% crude enzyme samples were added into the buffer solutions respectively, and allowed each to sit for 1 h at 30°C. Cellulase activity measurements were conducted as described in the determination of cellulase activity section. The cellulase activity of crude enzyme samples (pH 6.0) without any added sodium phosphate (possessing 100% cellulase activity) was used as the control for the overall measurement. The cellulase activities of different processed samples were compared to the control to estimate the stability of cellulase at different pH values.

Effect of treatment temperature on cellulase activity

The CSK1 crude enzyme samples were placed at constant temperatures of 30, 40, 50, 60, 70, 80, 85 and 90°C for 30 min. Then the cellulase activity of the samples was measured as described in the determination of cellulase activity section. The cellulase activity of crude enzyme samples was set as 100% cellulase activity in order to estimate the relative stability of samples processed at the above temperatures.

Characteristics of cellulase activity throughout the growth cycle

During the culturing period of CSK1, a 5 mL culture sample was extracted each day, and centrifuged at 12,000 rpm/min for 10min at 4°C to obtain the separated the supernatants and pellets. The supernatants were used to determine the cellulase activity and the protein quantity. Meanwhile, the pH of the culture solution in the growth cycle was determined and recorded each day.

RESULTS AND DISCUSSION

Optimization of measuring the wavelength for cellulase activity

There are many methods for the determination of cellulase activity, and they are diverse and complicated (Dashtban et al., 2010). DNS spectrophotometry is a widely accepted and applied method in research (Aoyama et al., 2015; Miller, 1959; Wood and Bhat, 1988; Xiao et al., 2004; Zhao et al., 2016), which determined the ability of DNS to saccharify CMC via the analysis of its hydrolysis products, such as glucose and cellobiose (reducing sugars).

The absorbance of DNS was scanned with wavelengths between 490 and 580 nm, and found that the OD values decreased from 1 to 0 with the scanning wavelengths from 490 to 530 nm (Figure 2). No absorbance was found in the DNS with scanning wavelength over 530 nm. The results indicated that the influence of DNS decreased with the increasing measuring wavelength from 490 to 530 nm. The scanning results of the reaction products from the crude cellulase extract and the chromogenic reagent DNS showed thatthe OD values increased from 490 to 500 nm, and reached the peak at 500 nm, then slowly decreased with the increasing scanning wavelength from 500 to 580 nm. Compared to the decreased rate in OD values of the DNS, the changing rates of reducing sugar were much lower, and no significant differences were found among

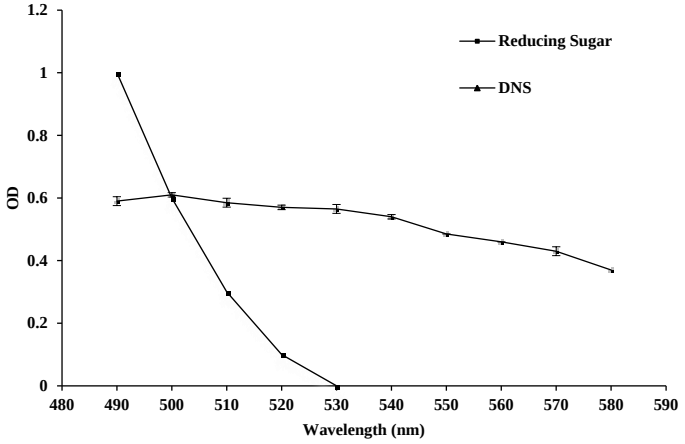

Figure 2. The absorption of DNS and reducing sugar at different wavelengths.

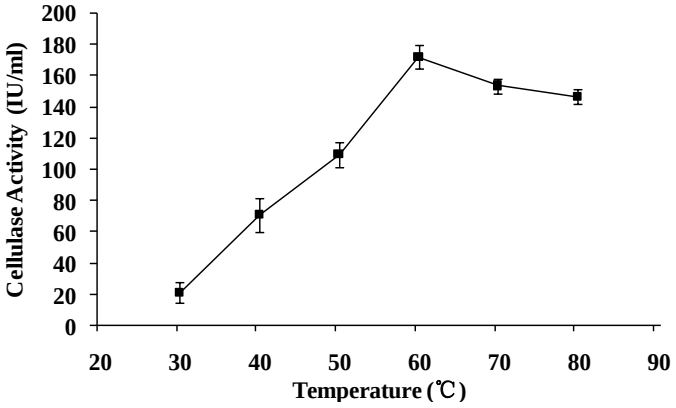

Figure 3. Cellulase activity at different reaction temperatures.

scanning wavelengths from 490 to 530 nm. Therefore, any wavelength of 490 to 530 nm was good for the measurement of OD values in the reaction products of cellulase and reagent DNS. In order to eliminate the influence of DNS and obtain more repeatable results, the measuring wavelength at 530 nm was selected for the determination of cellulose activity in CSK1.

A wide range of wavelengths has been used to determine the reducing sugars using the broadly defined DNS method (Stellmach, 1992; Zhang et al., 2009). Different measurement wavelengths resulted in different OD values, and the OD value was used to evaluate the cellulase activity, thus, as OD values shift, so will cellulase activity results.

In the current study, the absorption peak of the reducing sugar was under wavelength at 500 nm, but DNS showed obvious absorption in the 450 to 530 nm wavelength range and had an amplitude change of absorbency with an apparent declining tendency. Thus, the reducing sugar measuring results with a wavelength of 500 nm was not stable due to the influence of the high absorption values of the DNS.

In a previous study, the optimal wavelength for the cellulase activity measurement of bacterial community MC1 is at 490 nm (Piao et al., 2002); however, researchers did not take the scanning interference of DNS into consideration. Although the absorption of crude cellulase reacting to DNS of CSK1 under 530 nm declined in comparison to 500 nm, DNS interference of DNS was relatively lower. So we chose 530 nm as the optimal measurement wavelength.

Optimal temperature and pH of the cellulase reaction

Cellulase degradation has a wide application value in industrial fields concerning food, feed, medicine, textiles, detergent, and papermaking. However, it is required to fulfill special conditions in terms of pH, temperature and stability to achieve targeted activities. The growing conditions of the microorganisms are various depending on the process configurations. Thus, to study the characteristics of cellulase, especially the optimal reaction pH, temperature and its stability are critical to further understand the degradation mechanism of cellulases.

The measured cellulase activity of CSK1 increased quickly with the temperature increased from 30 to 60°C, and then gradually decreased with the increased temperature up to 80°C (Figure 3). Significant differences were found between the temperatures 60°C and 50 and 70°C, which were the two temperatures that have closer cellulase activity to 60°C. Thus, the measuring temperature at 60°C was selected for the future determination of cellulase activity. The cellulase activity of CSK1 maintained a high and consistent value around 150 IU/mL under pH 4.0 to 7.0, and reached the highest at pH 6.0 (Figure 4). The cellulase activity decreased with the increasing pH from 6 to 11.

Consequently, the optimal pH value for the determination of cellulase activity was 6.0. Similar results were found by other research: the optimal pH and temperature to achieve maximum CMCase activity of a *Bacillus amyoliquefaciens* were 7.0 and 50°C (Lee et al., 2008), of a *Clostridium thermocellum* were 5.7 to 6.1 and 70°C (Johnson et al., 1982). In these studies, the CMCase activities were close to each other with pH from 6.0 to 7.0 and temperature from 50 to 70°C, which were comparable to the results in this study. However, different optimal pH and temperature results were also found by others: Kalogeris et al. (2003) reported that the CMCase activity of a *Thermoascus aurantiacus* reached the highest at pH 3.5 and 4, and temperature 75°C; while Coral et al. (2002) reported pH 4.5 and 7.5, and temperature 40°C for obtaining maximum CMCase activity in an *Aspergillus niger.* Therefore, the current optimal pH and temperature were good for the measurement of cellulase in CSK1. Other cellulases from

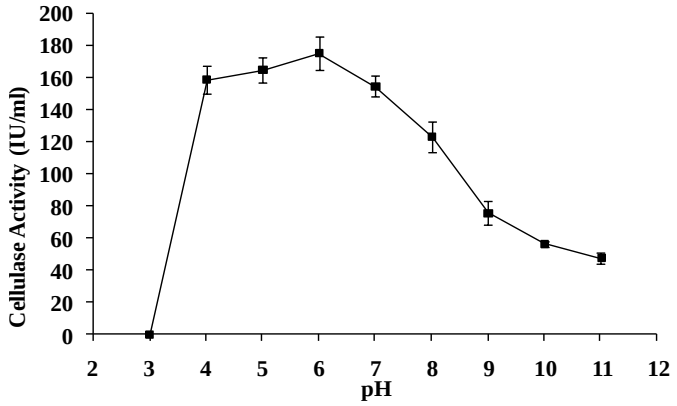

Figure 4. Cellulase activity at different reaction pH.

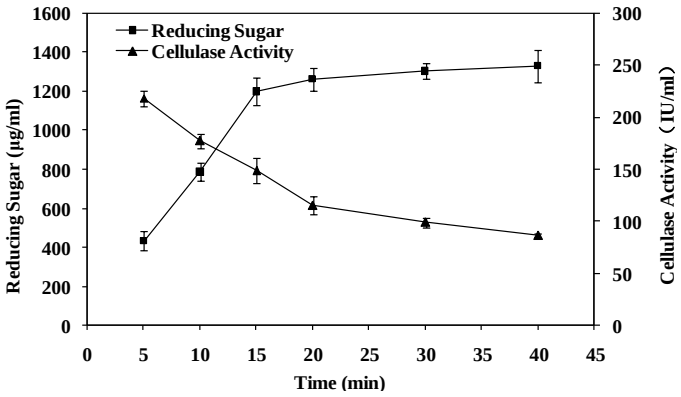

Figure 5. Cellulase activity at different reaction times.

different microbes need their own optimal pH and temperature for determining the cellulase activity.

Optimal time for the cellulase reaction

The amount of reducing sugar produced by the cellulase decomposing substrate increased from 430 to 1198 μg /mL as reaction time increased from 5 to 15 min. The change of the rate of reducing sugar slowed after 15 min, and there was no significant difference from 20 to 40 min, when the amount of reducing sugar reached around 1300 μg/mL (Figure 5). Cellulase activity on the other hand, declined gradually as time extended, and the highest value was observed when the reaction time was 5 min. However, the total amount of reducing sugar was too limited to achieve a stable result at reaction 5 min.

When the reaction time was 10 min, the amount of reducing sugar produced was in a rapid augmentation stage, and it had significantly increased as compared with the value achieved in 5 min. The cellulose activity at 15 min far exceeded that at 5 min. At a reaction time of 10 min, the cellulase activity could be more accurately provided, so the optimal time selected for the cellulase reaction was 10 min.

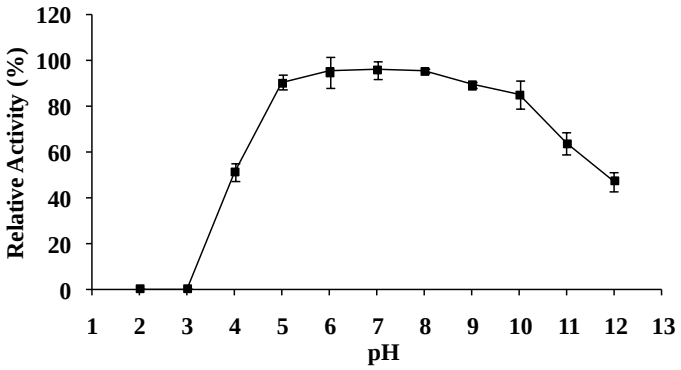

Figure 6. Cellulase stability under different pH treatments

Stability of cellulase

The cellulase stability of CSK1 was evaluated by mixing the crude cellulase extract with different pH media, and the results indicated that there was no cellulase activity determined at pH ranging from 2.0 to 3.0, and the relative activity increased first and then decreased with the increasing pH (Figure 6). The relative CSK1 cellulase activity was higher than 90% when the pH ranged from 5 to 10, which indicated that the cellulase was relatively stable with a pH close to neutral, both too low pH (<5) and too high pH (>10) lead to a decline of the cellulase activity.

The influence of temperature on the cellulase stability was determined by measuring the relative cellulase activity of the crude cellulase extract of CSK1 that processed in 30 to 90°C for 30 min. The results indicated that the cellulase activity was basically stable and relative enzyme activity was maintained above 70% between processing temperature 30 and 70°C. However, when the processing temperature exceeded 70°C, relative enzyme activities quickly declined to 32% at 80°C, and the cellulase activity was completely lost when the temperature reached 85°C (Figure 6). Therefore, the cellulase of CSK1 could maintain a higher enzyme activity for certain times (30 min in the current study) with temperature below 70°C, which agree with its relatively high culturing temperature environment (50°C).

Different cellulases (that is, different types or from different sources) require different conditions to maintain their functional ability. Temperature and pH are the two important ambient factors that can directly influence the cellulase stability. In the production of cellulose enzyme bacteria, such as *A. niger* (Ikeda et al., 1973; Sohail et

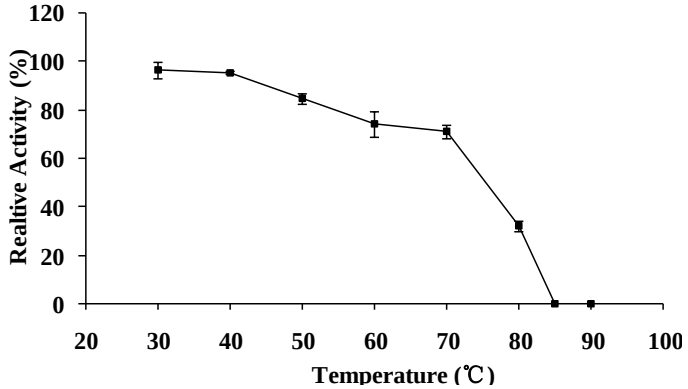

Figure 7. Cellulase stability under different temperature treatments.

al., 2009) and *Trichoderma reesei* (Sprey and Lambert, 1983; Szengyel and Zacchi 2000), the acid enzyme is commonly produced, for which the optimal pH has typically been between 4.0 and 6.0. Some microbes also exhibit basophil and alkali resistance (Ariffin et al., 2006; Singh et al., 2004).

Therefore, the stable conditions of cellulase from different microbes might quite different. Farinas et al. (2010) studied the stability of cellulase and xylanase of *A. niger* by using response surface methodology, and reported that the optimum pH and temperature for maintaining high activities of endoglucanase, β-glucosidase and xylanase ranges from 4.0 to 5.5 and 35 to 60°C, respectively.

Lee et al. (2008) studied the influence of pH and temperature on CMCase produced by *B. amyoliquefaciens* DL-3, and found the CMCase was relatively stable ranging from pH 4.0 to 9.0 and temperature 50 to 70°C. In this study, the CSK1 cellulase activity remained high and stable below 70°C and in the pH range of 5.0 to 10.0 (Figure 7). Thus, the CMCase stable conditions of CSK1 were different to CMCase from other microorganism, also different to other types of cellulase.

Cellulase application could be more extensive if its stable ranges were larger. The large stable ranges of CSK1 CMCase were meaningful for cellulose decomposition that includes large pH changes before and after the reaction process, and in some high temperature reaction environments. These conditions are especially appropriate for composting fermentation and silage processing.

Cellulase activity, pH, and protein quantity during culture of CSK1

The cellulase activity increased with the increasing culturing time from 0 to 8 days, and reached its highest value of 182 IU/mL on day 8. Then the cellulase activity

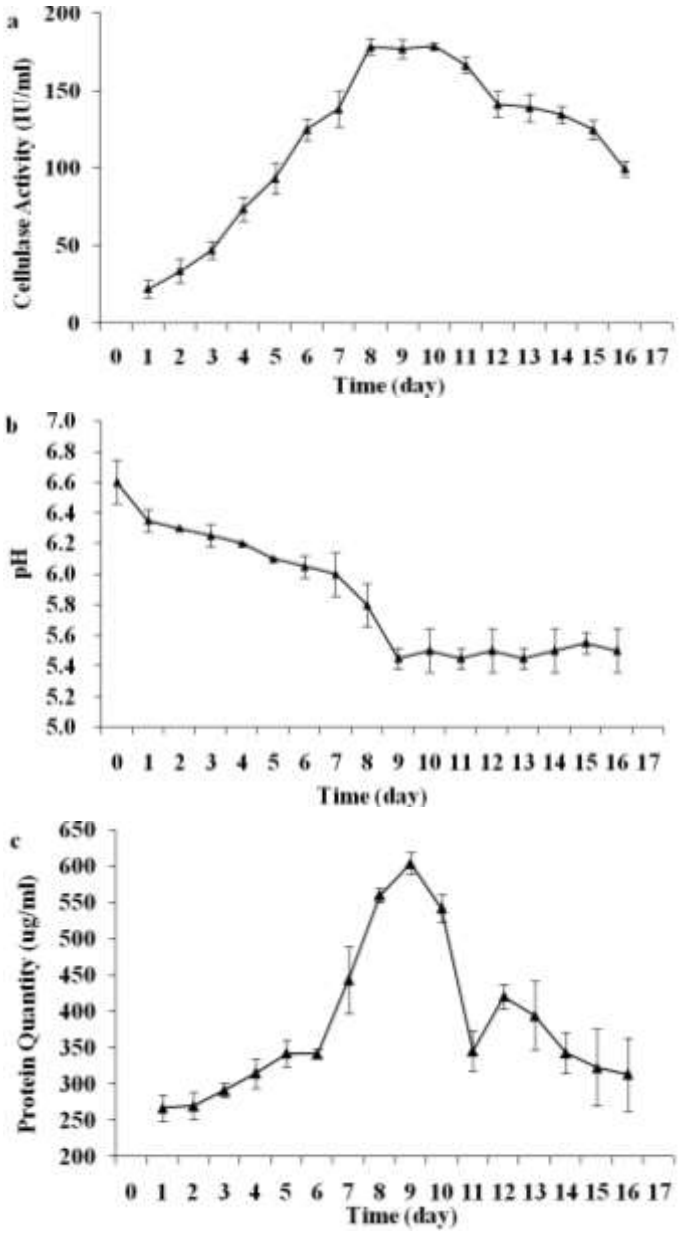

Figure 8. Cellulase activity (a), pH (b), and protein quantity (c) in the CSK1 growth cycle.

keeps high values during culturing days from 8 to 10. After day 10, the cellulase activity declined gradually with the extension of culturing time, and reached 96 IU/mL at day 16 (Figure 8a). The pH values declined gradually from 6.7 to 6.1 with culturing time from 0 to 7 days; then declined rapidly from 6.1 to 5.4 with culturing time from 7 to 9 days, and then remained nearly stable (at around 5.4) from 10 to 16 days (Figure 8b). Protein quantity also changed correspondingly, which increased gradually from 267.09 to 341.54 μg/mL with culturing time from 0 to 7 days, and then increased rapidly from 443.19-603.85 μg/mL with culturing time from 7 to 9 days. In the last 7

days (10 - 16 day), the protein quantity generally declined from 542.04 to 313.53 µg/mL (Figure 8c).

The rate of enzymatic cellulose hydrolysis was influenced by the changes of pH of the suspension, which resulted from the accumulation of acetate during cellulose degrading process. Romsaiyud et al. (2009) reported that the accumulation of acetate resulted in a decrease in pH, which resulted in the decrease of both cellulase production and cellulose hydrolysis.

In the early stage of culturing of CSK1, cellulase activity increased with the improvement of CSK1 growth, and achieved its highest value around day 8, and the protein quantity also increased to the peak correspondingly. Meanwhile, the pH of the culture solution declined gradually with the degradation of filter paper, and reach around 5.8 at day 8.

Kato et al. (2004b) reported that with the degradation of the cellulose substrate, large amounts of acid (acetic acid primarily) were produced and led to a decline of pH. In the later culturing stage, the low culture solution pH inhibited the growth CSK1, which resulted in a gradual decline in the cellulase activity and also the total amount of protein in solution.

The determination of the culturing time when the enzyme activity reaches the highest is a key procedure to evaluate the degradation efficiency of cellulase producer bacteria and cellulase itself. It took considerably longer time for CSK1 to reach the highest cellulase activity compared to MC1. This may be because the bacterial community MC1 has a vigorous metabolism, and the other bacteria of the community have a stimulative effect on CSK1 cellulase production. Thus, MC1 achieved the highest cellulase activity in a relatively short time.

Conclusion

A systematic cellulase activity determination method based on the DNS spectrophotometry was developed. Following this step, the cellulase characteristics of CSK1 were determined and evaluated. The optimal conditions for determination of CSK1 cellulase activity were obtained in terms of the measuring wavelength, the reaction temperature and pH, and the ideal reaction time. Cellulase of CSK1 was relatively stable at temperatures below 70°C, and pH between 5.0 and 10.0, which indicated high application value and development potential in practical process. Through the determination of the cellulase characteristics and activity of CSK1 in 16 days, we found significant influences with each other among cellulase activity variation, pH and CSK1 biomass changes. Corresponding adjustment measures could be applied based on such information to obtain more efficient and higher-yield processes.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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