PURIFICATION AND CHARACTERIZATION OF CELLULOSE DEGRADING ENZYME FROM NEWLY ISOLATED *CELLULOMONAS* SP.

HONGZHI BAI, MUHAMMAD IRFAN,[*] YAN WANG,[**] HUI WANG[***] and XIAORI HAN

National Engineering Laboratory for Efficient Utilization of Soil and Fertilizer Resources, College of Land and Environment, Shenyang Agricultural University, Shenyang, Liaoning 110866, China
[*]*Department of Biotechnology, University of Sargodha, Sargodha 40100, Pakistan*
[**]*English Teaching Department, Shenyang Agricultural University, Shenyang 110866 Liaoning, China*
[***]*Bioscience and Biotechnology College, Shenyang Agricultural University, Shenyang 110866 Liaoning, China*
✉ *Corresponding authors: Hui Wang, wanghuisyau@sina.com*
Xiaori Han, hanxiaori@163.com

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In this study, a cellulolytic bacterium was isolated from soil and was identified as *Cellulomonas* sp. Maximum endoglucanase production was observed with an inoculum size of 1.5% (v/v), initial medium pH of 7, and substrate concentration of 3% for 48 h of incubation at 33 °C. Further supplementation of CMC and tryptone as carbon and nitrogen sources improved endoglucanase production, respectively. The enzyme had a molecular weight of 53.55 kDa. The enzyme had pH and temperature optima of 7.0 and 50 °C, respectively. The stability of the enzyme was in the pH range of 6.0 to 8.0 and at temperatures up to 50 °C. The metal profile showed that Co^{2+} and Mn^{2+} were activators, while Hg^{2+} and Fe^{2+} were inhibitors. The enzyme was highly stable toward alcohols. The enzyme had K_m and V_{max} values of 1.481 mg/mL and 13.64 mM/mL/min against CMC as substrate, respectively.

Keywords: purification, characterization, endoglucanase, *Cellulomonas* sp.

INTRODUCTION

The most abundant renewable resource on Earth is cellulose, which is the primary product of photosynthesis.[1] Cellulose is hydrolyzed by cellulase enzymes. There are three kinds of cellulase enzyme: endo-β-1, 4-glucanase (EC 3.2.1.4, EG; cleaves inner linkages randomly), exocellobiohydrolase (EC 3.2.1.74; hydrolyzes cellobiosyl entities from non-reducing ends), and β-D-glucosidase (EC 3.2.1.21; hydrolyzes glucosyl entities from cello-oligosaccharides).[2] Cellulases can be produced from fungi and bacteria. Among bacteria, *Bacillus*, *Thermomonospora*, *Bacteriodes*, *Ruminococcus*, *Cellulomonas*, *Clostridium*, *Erwinia*, *Acetovibrio*, *Streptomyces* and *Microbispora* can effectively produce cellulases.[3] Most commonly, commercial cellulases are produced from *Aspergillus niger* and *Trichoderma reesei*[4] using the submerged fermentation process.[5] Sometimes bacterial cultures are preferred over fungal culture due to faster growth, synergistic enzyme complex system and ease of genetic engineering.[6]

To reduce the cost of enzyme production, cheap media and agricultural wastes are used for cellulase production in both submerged and solid state fermentation. Previous studies have suggested that agricultural wastes are effective substrates for cellulase production.[7] These agricultural wastes contain low lignin contents, as compared to woody wastes.[8]

Due to potential uses of cellulases in industries, scientists have mainly investigated the thermophilic nature of cellulases. Currently, cellulases are widely used in fruit juice processing, animal feed, textile industries, laundry detergents, baking processes, the paper and pulp industry, and bioconversion of cellulosic waste materials into biofuels.[9-11] This study aimed to

isolate cellulolytic bacteria from soil, optimize its production and characterize the cellulase produced by submerged fermentation.

EXPERIMENTAL

Isolation and identification of bacteria

A cellulase producing bacterium was isolated from soil samples. A pour plate and the serial dilution technique were used for isolation. The cellulolytic activity of the bacterium was checked by culturing on CMC medium, followed by Congo red staining. Bacterial colonies were purified by repeated streaking and identified by morphological and physiological tests.[12,13]

Inoculum preparation

The inoculum was prepared in a medium comprising 0.2% K_2HPO_4, 1% glucose, 1% peptone, 0.03% $MgSO_4$ and 0.25% $(NH_4)_2SO_4$, with the pH adjusted to 7 and sterilized. After cooling down at room temperature, the bacterial isolate was inoculated and incubated in a shaking incubator for 48 h at 35 °C with a shaking speed of 120 rpm.[14]

Production of endoglucanase

The medium used for production of endoglucanase was composed of 1% rice straw, 0.2% K_2HPO_4, 1% peptone, 0.03% $MgSO_4$ and 0.25% $(NH_4)_2SO_4$. The medium was autoclaved, inoculated with vegetative cells of *Cellumonas* sp., and incubated at 35 °C for a fermentation period of 48 h. After completion of the fermentation, the broth was cleared by centrifugation at 10,000 x g for 10 min at 4 °C. The clear supernatant obtained was used for endoglucanase estimation.[14]

Estimation of endoglucanase activity

Endoglucanase activity was determined by the DNS method.[15] The reaction volume containing 500 µL of the appropriate enzyme solution and 500 µL of 1% CMC (prepared in 0.05 M citrate buffer at pH 5) was incubated for 30 min at 50 °C. After incubation, 1.5 mL of DNS solution was added to stop the reaction and the reaction product was boiled in a water bath for 10 min. After that, the solution was allowed to cool down at ambient temperature and optical density was recorded at 550 nm. One unit (U) of enzyme activity is enzyme required to liberate 1 µmol of glucose *per* min.

Protein determination

Total protein content was measured by Lowery *et al.*[16]

Optimization of process parameters

Different production parameters, such as fermentation period (12, 24, 36, 48, 60, 72, 84 and 96 h), inoculum size (0.5, 1.0, 1.5, 2.0 and 2.5%), initial medium pH (4.0 to 8.0), incubation temperature (27, 30, 33, 35, 37 and 40 °C), additional carbon sources (0.5% (w/v) CMC, maltose, starch, sucrose, glucose and cellulose), and nitrogen sources (0.25% $(NH_4)_2SO_4$, ammonium citrate, KNO_3, NH_4Cl, $NaNO_3$, peptone, tryptone, yeast extract and urea), were optimized for maximum production of endoglucanase from *Cellulomonas* sp. in submerged fermentation.

Purification of CMCase

The whole purification process was done as described in earlier report.[17]

Characterization of endoglucanase

Effect of pH on activity and stability

The optimum pH of purified enzyme activity was determined by incubating a crude enzyme mixture in appropriate buffers; pH 3.0 to 6.0 (0.05 M citrate buffer), pH 6.0 to 8.0 (0.05 M sodium phosphate buffer), pH 8.0 to 9.0 (0.05 M Tris-HCl), and pH 9.0 to 11.0 (0.05 M glycine-NaOH). The enzyme solution was incubated for 30 min in pH buffers at 50 °C. Using the DNS method, endoglucanase activity was assayed. The pH stability was determined by incubating the enzyme mixture in the above-mentioned buffers containing 1% CMC at room temperature for half an hour and then incubating for another half an hour at 50 °C. Enzyme stability was determined using the DNS method.

Effect of temperature on activity and stability

Temperature activity and stability of endoglucanase was measured by incubating a purified enzyme mixture in 1% CMC in 10 mM phosphate buffer (6.0 to 8.0) at temperatures ranging from 20 to 90 °C. Half-lives ($T_{1/2}$) of the enzyme at various temperatures were calculated as follows:

$$T_{1/2} = \frac{\ln 2}{k_d} = \frac{0.693}{k_d} \quad (1)$$

Effect of metal ions and alcohols

The effect of various metal ions, including Co^{2+}, Ni^{2+}, EDTA, Hg^{2+}, Ca^{2+}, Mn^{2+}, Fe^{2+}, Mg^{2+}, and alcohols, such as ethanol, methanol, isopropanol and butanol, was determined. The concentration of each metal ion was 0.05 M. Enzyme activity was assayed by the DNS method as described above.

Enzyme kinetics

The K_m and V_{max} values for endoglucanase were determined by linear regression analysis by Lineweaver-Burk plot (double reciprocal plot) with different concentration of CMC (5, 10, 15, 20, 25 and 30 mg/ml). Triplicate readings were taken and the activity was measured according to the standard assay conditions.

Statistical analysis

One-way analysis of variance (ANOVA) was performed using SPSS for significant differences at P

< 0.05 within different conditions; the Tukey test was also applied.

RESULTS AND DISCUSSION

Isolation and identification of *Cellulomonas* sp.

In this study, different soil samples were collected for screening of cellulolytic bacteria. From three different locations of a peanut field, nearly sixty bacterial colonies were found. From these sixty colonies, only ten bacterial colonies produced cellulase. The bacterial strain producing the highest amount of cellulase was further purified and identified by biochemical and morphological tests (Table 1). From the identification procedure, the bacterial strain was identified as *Cellulomonas* sp.

Table 1
Morphological and physiological characteristics of the isolate

Characteristics		Results
Morphology	Colony pigment	Off-white
	Colony size	1 mm
	Colony margin	Round
	Colony elevation	Convex
	Colony surface texture	Smooth
	Cell shape	Short bacilli
	Spore formation	-
	Motility	+
	Gram's stain	-
Biochemical	Catalase test	+
	Nitrate reduction test	+
	Indole test	-
	Methyl Red test	-
	Voges-Proskuar test	-
Growth on	CMC	+
	Avicel	+
Fermentation of	Glucose	+
	Sucrose	+
	Fructose	+
	Maltose	+

Optimization of cellulase production

To check the optimum fermentation period for endoglucanase production, experiments were conducted by varying fermentation time, ranging from 12 to 96 h. Results (Fig. 1) stated that increased enzyme production was observed with the passage of time, and maximum enzyme production was observed at 48 h of fermentation. Further increased fermentation period caused reduced production of enzyme. These findings are in agreement with those of Safdar *et al*.,[18] who also reported that 48 h fermentation was the most suitable for endoglucanase production. Sangkharak *et al*.[19] reported optimum CMCase production at 60 h of fermentation period from *Cellulomonas* sp. TSU-03.

Inoculum size affects enzyme production in a fermentation system. Different inoculum sizes were tested for maximum production, and the results (Fig. 1) showed that a 1.5% (v/v) inoculum size resulted in significantly ($P < 0.05$) higher enzyme production. Safdar *et al*.[18] reported that 2.0% inoculum size was best for CMCase production. Inoculum concentrations other than this resulted in reduced enzyme production. An insufficient number of bacteria leads to reduced cellulase production.[20]

The effect of initial medium pH was tested by changing the pH of the initial medium from 4.0 to 8.0. The highest titer of the enzyme was recorded at pH 7.0, as shown in Figure 1. Acidic and alkaline pH had negative effects on enzyme production. Previous studies indicated that 7.0 medium pH was optimum for cellulase production from *Cellulomonas* sp.[19,21,22] Sugumaran *et al.*[23] reported that an initial medium pH of 6.0 was optimum for cellulase production from *Cellulomonas* sp. in solid state fermentation.

Different incubation temperatures were used for maximum production of endoglucanase from *Cellulomonas* sp. under submerged fermentation. It was noted that the maximum yield of enzyme

was found at 33 °C, as shown in Figure 1. Further increased incubation temperatures decreased enzyme production. These findings suggested that the newly isolated bacterial strain was not thermophilic. Previous studies suggested that *Cellulomonas* sp. produced maximum cellulase production at an incubation temperature of 35 °C.[19,24]

Different concentrations ranging from 1 to 6% rice straw were employed for maximum endoglucanase production in submerged fermentation.

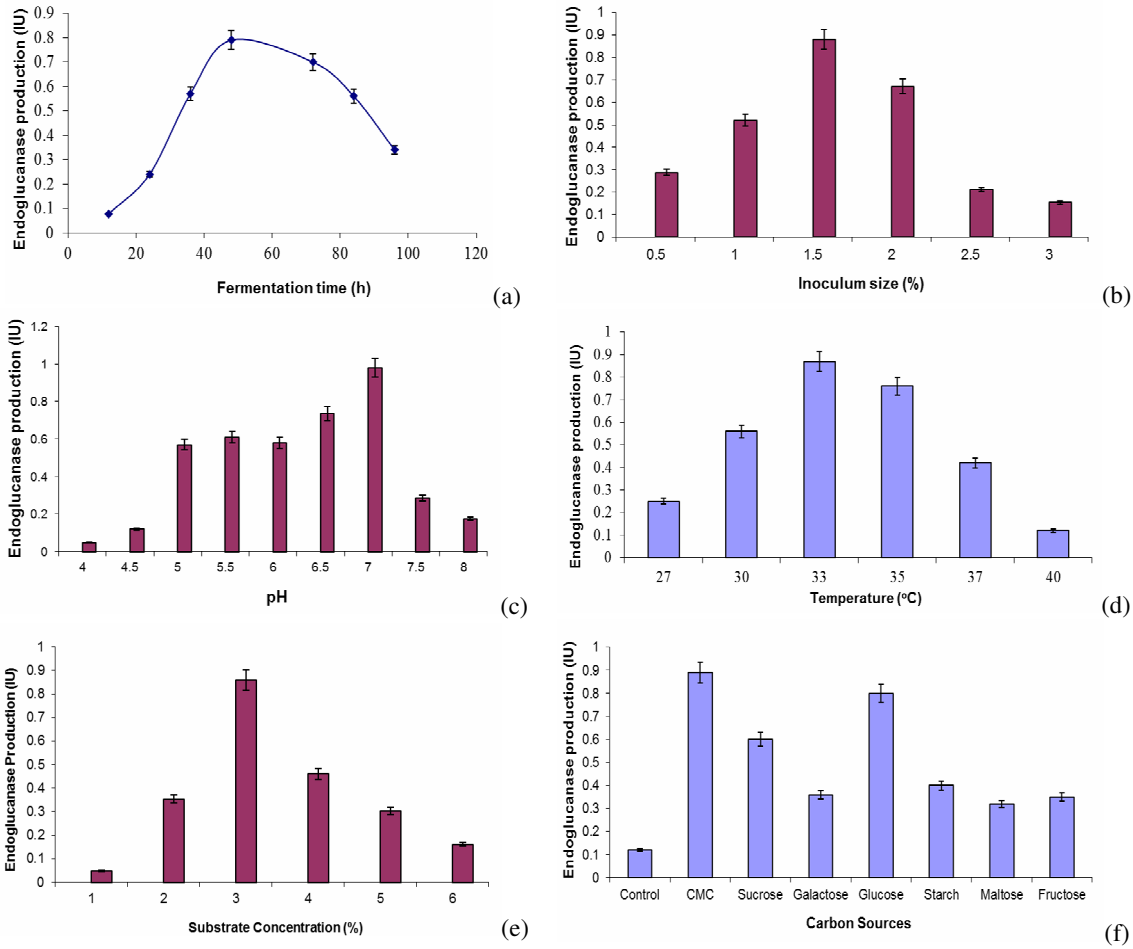

Figure 1: Effect of incubation time (a), inoculum size (b), initial medium pH (c), incubation temperature (d), substrate concentration (e) and various carbon sources (f) on endoglucanase production

Table 2
Effect of nitrogen sources on endoglucanase production by *Cellulomonas sp.*

Inorganic nitrogen sources	Endoglucanase production (IU)	Organic nitrogen sources	Endoglucanase production (IU)
Control	0.286 ± 0.011^{a}	Control	0.286 ± 0.011^{a}
KNO_3	0.786 ± 0.003^{c}	Peptone	0.763 ± 0.016^{d}
$NaNO_3$	0.798 ± 0.014^{c}	Urea	0.432 ± 0.011^{b}
$(NH_4)_2SO_4$	0.794 ± 0.011^{c}	Yeast extract	0.674 ± 0.017^{c}
NH_4Cl	0.818 ± 0.01^{d}	Tryptone	0.827 ± 0.019^{e}
Ammonium citrate	0.643 ± 0.004^{b}	Meat extract	0.472 ± 0.011^{b}

Superscript letters represent significant difference ($P < 0.05$)

Table 3
Purification profile of endoglucanase from *Cellulomonas sp*.

Purification steps	Total volume (mL)	Total activity (IU)	Total protein (mg/mL)	Specific activity ($U \cdot mg^{-1}$)	Purification folds	Yield (%)
Crude enzyme	1000	1.034	0.65	1.59	1.00	100.0
Amm. Sulfate ppt.	5.0	0.767	0.31	2.47	1.55	74.1
Sephadex G-100	1.5	0.516	0.11	4.69	2.94	49.9

Results (Fig. 1) showed that 3% substrate concentration yielded maximum enzyme production. Sangkharak *et al.*[23] reported optimum cellulase production from *Cellulomonas* sp. TSU-03 in a medium consisting of 4% wastepaper. Verma *et al.*[25] reported that 1.5% CMC yielded maximum cellulase production from *Bacillus* sp. in submerged fermentation.

Various additional carbon sources were supplemented for maximum endoglucanase production. The best enzyme production was observed with CMC, followed by glucose (Fig. 1). Other sugars did not significantly affect enzyme production. The findings of this study are in line with Das *et al*., who also suggested CMC as a suitable carbon source for cellulase production by *Bacillus* sp.[26] In another study, glucose was found to be a good supplement for cellulase induction by *Cellulomonas* sp. ASN2.[14]

Nitrogen plays an important role in protein formation. In this study, different nitrogen (organic and inorganic) sources were used for maximum production of endoglucanase. Results (Table 2) revealed that the addition of NH_4Cl and tryptone to the medium significantly enhanced endoglucanase production in submerged fermentation. Vyas *et al.*[27] reported that $(NH_4)_2SO_4$ was a potential source of inorganic nitrogen for cellulase production. Some studies have suggested that peptone is a remarkable source of nitrogen (organic) for cellulase production.[14,27,28] Yeast extract is also a good nitrogen source for cellulase production by *Cellulomonas cellulans.* [23]

Purification and characterization of endoglucanase

Purification of endoglucanase

The crude endoglucanase enzyme was subjected to purification, involving $(NH_4)_2SO_4$ fractionation and dialysis, followed by Sephadex G-100 chromatography. In the whole purification process, 2.94 fold purification and 49.9% yield were achieved (Table 3). To check the purity and molecular weight, SDS-PAGE was performed, which gave a single band with molecular weight of 53.55 kDa (approximately), as shown in Figure 2. Earlier reports stated that different chromatography methods, such as Sephadex column,[29] DEAE column,[30] and affinity chromatography,[31] have been applied for cellulase purification. Purification results differed with respect to the method applied. *Bacillus* sp. had a wide range of molecular weights for cellulases, such as 58 kDa,[29] 97 kDa[30] and 51.3 kDa.[32] *Paenibacillus polymyxa* secreted CMCase with a molecular weight of 72 kDa.[31]

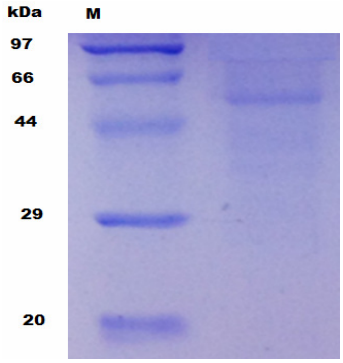

Figure 2: SDS-PAGE of purified endoglucanase from *Cellulomonas* sp.

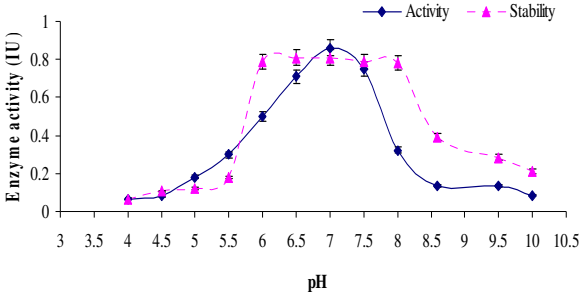

Figure 3: Effect of pH on activity and stability of endoglucanase

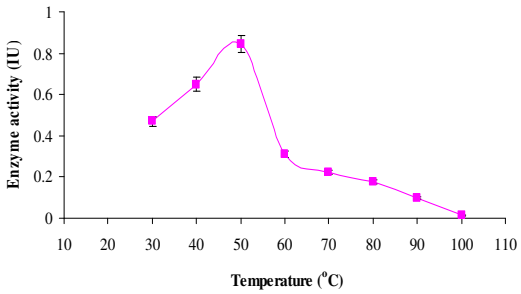

Figure 4: Effect of temperature on activity of endoglucanase

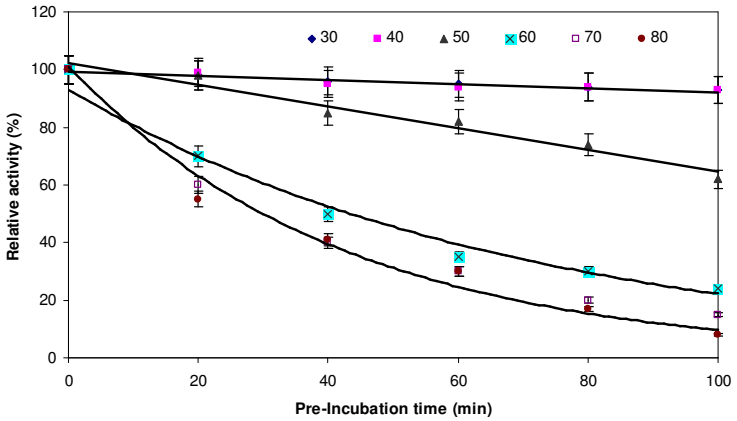

Figure 5: Activity and stability of endoglucanase as a function of pre-incubation time

Effect of pH on activity and stability

Different pH ranges (4.0 to 10.0) were tested to check the optimum pH of endoglucanase. The optimum pH of endoglucanase activity was 7.0, and it was stable in a pH range from 6.0 to 8.0 (Fig. 3). Previous studies reported that cellulase had an optimum pH in the range from 5.0 to 7.5[14,33-35] and was stable in the range from 4.0 to 7.0.[36,37]

Effect of temperature on activity and stability

Temperature also plays an important role in enzymatic reactions. To test the optimum temperature of endoglucanase, the enzyme substrate mixture was incubated at different temperature ranging from 30 to 100 °C. The enzyme showed optimum activity at 50 °C (Fig. 4). The thermostability study showed that the enzyme retains 50% activity at 60 °C for 40 min of pre-incubation time (Fig. 5). Cellulases produced from various microorganisms have been shown to have optimum temperatures of 30 °C,[38] 40 °C,[39] 50 °C,[40] 55 °C,[37] 60 °C,[14] 65 °C and 70 °C.[34] Half-lives of the enzyme were also measured, which showed (Table 4) that the maximum half-life was 1296 min, followed by 1175 min, at 30 and 40 °C, respectively. The half-life of this enzyme at various temperatures was comparatively lower than that reported by Singh *et al.*[41]

Effect of various metal ions on activity of endoglucanase

In this study, different metal ions were tested to determine their effect on endoglucanase activity. Results revealed that Co^{2+} and Mn^{2+} activated endoglucanase activity, while Hg^{2+} and Fe^{2+} inhibited enzyme activity (Fig. 6). The findings of this study are in accordance with earlier reports.[14,37] Previous studies suggested that Hg^{2+} and Fe^{2+} strongly inhibit cellulase activity.[42,43]

Table 4
Half-lives ($T_{1/2}$) of endoglucanase at various temperatures

Temperature (°C)	$T_{1/2}$ (min)
30	1296
40	1175
50	129
60	48
70	39.2
80	33.5

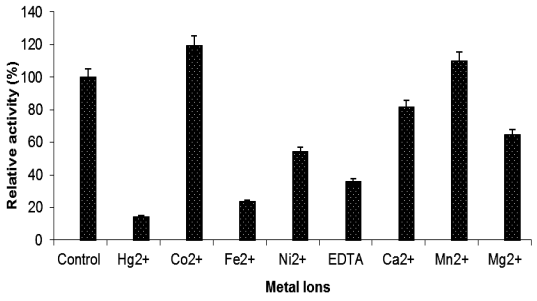

Figure 6: Effect of different metal ions on activity of *Cellulomonas* sp. cellulase

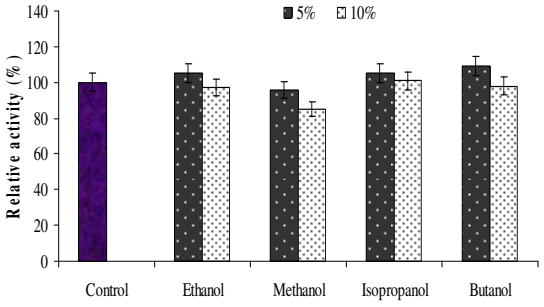

Figure 7: Effect of alcohols on endoglucanase activity

Effect of various alcohols on activity of endoglucanase

Different alcohols (ethanol, methanol, isopropanol and butanol) were tested to study their effect on endoglucanase activity. Results (Fig. 7) showed that the activity of purified endoglucanase was not substantially inhibited by alcohols. Alcohols slightly reduced the activity at a concentration of 10%. The purified enzyme was alcohol resistant. Our findings were in accordance with earlier reports,[44] Li and Yu[45] reported that purified endoglucanase from *Haloarcula* sp. G10 showed residual activities of 38.2%, 35.2% and 56.3% toward methanol, ethanol, and 1-butanol, respectively. Annamalai *et al.*[46] reported endoglucanase from *B. halodurans* CAS-1 having residual activities of 83.2%, 68.3%, 80.2% and 86.4% for methanol, ethanol, isopropanol and n-butanol, respectively. In another study, an endoglucanase residual activity of more than 70% was reported for methanol, ethanol and 1-butanol.[47] These results suggest that the endoglucanase enzyme could be used for the transglycosylation process.

Enzyme kinetics

The kinetic constants K_m and V_{max} of the enzyme were determined by a Lineweaver-Burk plot using various concentrations of carboxymethyl cellulose as a substrate. The enzyme exhibited K_m and V_{max} values of 1.481 mg/mL and 13.64 mM/mL/min, respectively. The K_m value from this study was lower than those found in earlier reports by Yan *et al.*,[32] Kumar *et al.*[31] and Zhang *et al.*[48] V_{max} values of 5.37 μg/mL·min, 17.805 mM $mL^{-1}min^{-1}$ and 333.33 U/mg were reported for endoglucanase from different microbial sources.[31,32,48]

CONCLUSION

A cellulolytic strain was isolated from soil and identified as *Cellulomonas* sp. This strain has the potential to utilize lignocellulosic waste, such as sugarcane bagasse, as a carbon source to produce valuable enzymes, thus reducing enzyme production costs. Enzyme production was enhanced by optimizing process parameters. The enzyme produced by this strain was thermostable and alcohol-stable. Thus, it could be concluded that it can be used in industrial processes, especially for saccharification in ethanol production.

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