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Short Report

Cellobiose Dehydrogenase Production by the Genus *Cladosporium*

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ABSTRACT

Cellobiose dehydrogenase (CDH; EC.1.1.5.1) is an extracellular enzyme that mainly produced by wood-degrading fungi. It oxidizes cellobiose to cellobionolactone using a wide spectrum of electron acceptors. The key roles of CDH in growth, metabolism, and some other important cellular processes such as cellulose degradation in fungi have been noted. Since the demands for finding new sources of CDH among different organisms have been dramatically increased, this study was focused on the presence of CDH in the genus *Cladosporium* as a well-known cellulolytic fungus. Twenty strains of *Cladosporium* isolated from soil samples from different geographical origin were evaluated for CDH-producing ability. The early screening of the fungus by zymogram method revealed the presence of CDH as an extracellular form in all of the examined isolates. Submerged cultivation of the best producer of CDH (selected from initial screening) on a specific medium showed the maximum amounts of enzyme produced in shaking cultures with pH 4.5 at 28ºC for a 14-day period. The enzyme activity was determined in the range of 27.83 to 1284.84 unit/mg protein among the isolates. Our observations show that *Cladosporium* isolates with high CDH producing ability i.e. isolates No. 10 and No. 18 can be used as selective candidates for large-scale production of this industrially important enzyme in further research programs. This is the first documented report on the presence of CDH in the fungus *Cladosporium*. *Iran. Biomed. J. 8 (2): 107-111, 2004*

Keywords: Cladosporium, Cellobiose dehydrogenase, Production, Screening

INTRODUCTION

The genus *Cladosporium* is one of the most important group of fungi which includes many saprophytic and some pathogenic species. This fungus is involved in the etiology of mycoses such as chromoblastomycosis and different types of fungal allergies in human and animals [1, 2]. Also, its ability to biodegradation of some aromatic compounds in industry has been well established [3]. In spite of the importance of *Cladosporium* in medicine, agriculture and industry, very little documented data have been presented about cellular and molecular aspects of this cosmopolitan fungus. Because an interesting metabolic aspect of the genus *Cladosporium* is found to be related to its cellulolytic activity, this work designed for study of the presence of an important cellulose-oxidizing enzyme, cellobiose dehydrogenase (CDH) in this fungus. CDH is an extracellular enzyme which first discovered in fungi *Sporotrichum pulverulentum* and *Polyporus versicolor* [4, 5] and has subsequently been found in several other white-rot fungi as well as some ascomycetes and the fungi imperfecti [6-9]. Although the major importance of CDH is related to its cellulolytic activity, the key roles of this enzyme in iron uptake, oxidative phosphorylation and killing the competing organisms by fungi have been well established [9, 10]. Also, this interesting enzyme has several applications in measuring cellobiose and lactose in foods and clarification processes in pulp and paper industry [9, 11]. In spite of the presence of CDH in different fungal groups

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such as soft rot, white rot and the others [6-9], the majority of these fungi are not attractive for large scale applications because of their low CDH yields in fermentative culture systems. Thus, at present, identification of organisms capable of producing larger amounts of CDH on cheap and simple media would certainly further increase the applied interest in this enzyme. To our knowledge, there is not any documented data regarding CDH production by the fungus *Cladosporium*. In this study, we reported the presence of the enzyme and optimized some important parameters as pH, temperature, incubation time and aeration for its production at submerged cultures of *Cladosporium*. This is the first documented report on the presence of CDH in *Cladosporium* as an interesting cosmopolitan fungus.

MATERIALS AND METHODS

Zymogram method for screening of CDH. CDH producing ability of *Cladosporium* isolates obtained from soil samples of different geographical areas was first screened by the method of Dekker [8] on blue colored agar plates. The Czapek-dox agar medium containing 1 mM dichlorophenolindophenol (DCPIP), cellulose (1%, w/v), 20 mM cellobiose and chloramphenicol (0.005%, w/v) was inoculated with 20 µl spore suspension (10^6 spores/ml) and incubated at 25ºC for 5 days. CDH activity was monitored as colorless zones in the agar around the fungal colonies as a consequence of DCPIP reduction during oxidation of cellobiose by the enzyme. The diameter of clear zone was measured to quantify activity.

Submerged culture conditions. *Cladosporium* isolate No.10, the best producer of extracellular CDH on agar plates (zymogram method) was further examined in submerged cultivation. Liquid cultures were grown on a synthetic culture medium containing microcrystalline cellulose (Avicel Type 123), 10 g; KH_2PO_4, 2 g; $(NH_4)_2SO_4$, 1.4 g; peptone, 0.25 g; $CaCl_2$, 0.3 g; urea, 0.3 g; $MgSO_4.7H_2O$, 0.3 g; yeast extract, 0.1 g; $FeSO_4.7H_2O$, 5 mg; $ZnSO_4.7H_2O$, 3.34 mg; $CoCl_2$, 2 mg; $MnSO_4.7H_2O$, 1.56 mg and distilled water, 1,000 ml according to Sadena and Patil [12]. Medium pH was adjusted accordingly. The prepared medium was divided as 50-ml aliquots in 250 ml Erlenmeyer flasks and sterilized at 121ºC for 15 min. Fungal spores were prepared from one-week old cultures grown on Sabouraud dextrose agar by gently washing the culture surface with distilled water containing 0.2% (v/v) Tween 80. Each flask was inoculated with 5×10^6 spores. The fungal isolates were separately grown at stationary and shaking (120 rpm) conditions at 25, 28, 32 and 35ºC for 6, 8, 10, 12, 14 and 16 days. The cultures were harvested by filtration through filter papers and the culture media were used as a source of enzyme after clarification through centrifugation at 4,000 ×g for 30 min.

Enzyme assay. CDH activity was measured by monitoring the decrease in absorbance of an electron acceptor, 2,6-DCPIP spectro-photometrically at 600 nm ($\varepsilon 600 = 1.85 \times 10^4\ M^{-1}cm^{-1}$) according to Sadena and Patil [13] by some modifications. The reaction mixture consisted of DCPIP (0.05 ml, 2 mM in 10 mM phosphate buffer, pH 6.3), cellobiose (0.85 ml, 2.5 mM in the same buffer), sodium fluoride (0.05 ml, 4 mM in the same buffer as an inhibitor for laccases) and 0.05 ml of appropriately diluted enzyme solution in a 1 ml glass microcuvette. The blank was contained all above materials except enzyme solution that substituted with equal amount of phosphate buffer. The reaction was started by addition of enzyme solution and the decrease in 600 nm absorbance was monitored during the first 5 min at 37ºC. One unit of CDH activity is defined as the amount of enzyme reducing 1 µmol DCPIP/min/ml under the assay conditions. The specific activity was presented as unit/mg protein. Protein concentration was measured by the dye-binding method of Bradford [14] using bovine serum albumin as standard.

RESULTS

Screening of CDH production by zymogram method. *Cladosporium* isolates were screened on agar plates for CDH-producing ability. All of the tested isolates produced extracellular CDH as a strain-dependent manner. The diameter of colorless zones (resulted from CDH-mediated DCPIP reduction) around the colonies was determined in a range from 12.5 to 47.5 mm among the isolates (Table 1). There were significant differences in CDH production between some tested isolates ($P<0.05$). The maximum enzyme activity was obtained for *Cladosporium* isolate No.10 and this isolate was selected for further steps.

Determining of optimal conditions for CDH production at submerged cultures. The time curve of CDH production by *Cladosporium* isolate No.10,

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Table 1. Cellobiose dehydrogenase (CDH) activity on agar plates and in submerged cultures of 20 *Cladosporium* isolates.

Fungal isolate	Gelatinase activity*	Agar plate screening** (clear zone diameter) (mm)	Submerged culture** (CDH activity) (u/mg protein)
1	-	32.5	48.30
2	-	31.5	27.83
3	+	24.0	519.50
4	-	32.6	481.74
5	-	31.5	124.70
6	+	31.0	285.21
7	-	42.5	62.84
8	-	32.5	398.56
9	-	32.7	334.35
10	-	47.5	1284.84
11	-	37.3	307.79
12	+	25.0	252.98
13	+	27.5	460.83
14	+	12.5	571.62
15	+	22.5	650.24
16	+	24.0	202.27
17	-	32.5	398.01
18	-	34.0	906.02
19	+	27.5	280.95
20	+	25.0	33.28

*+, activity present; -, activity not-detected, **, results are the means of three experiments with 3 replicate each.

the best-screened producer of enzyme, at submerged cultures on mineral liquid medium, is shown in Figure 1. The fungus produced the highest extracellular CDH in both stationary and shaking cultures after 14 days cultivation at 28ºC as 1100. 42 and 1284.84 unit/mg protein, respectively. There was no significant differences in enzyme activity between stationary and shaking cultures at similar periods of time (P>0.05). The optimal pH and temperature values for enzyme production at 14 days shaking cultures were determined as 4.5 and 28ºC, respectively (Fig. 2). Overall, the optimum CDH production was obtained in shaking cultures of the fungus on mineral medium with pH 4.5 after a 14-day incubation at 28ºC.

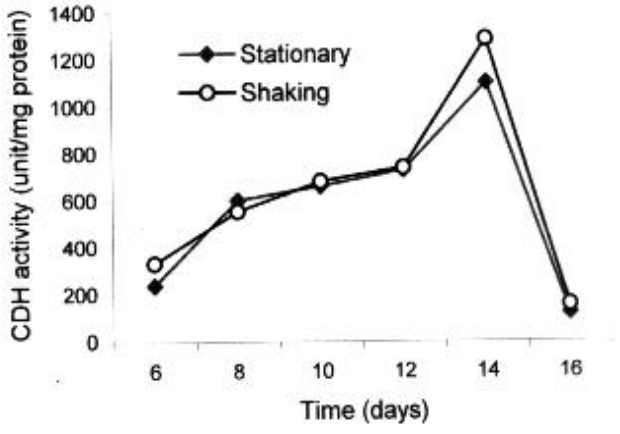

Fig. 1. Time curve of CDH production by *Cladosporium* isolate No.10 in submerged cultures at 28ºC.

Assay of CDH activity in Cladosporium isolates at submerged cultures. CDH production by 20 isolates of *Cladosporium* was assessed on mineral liquid medium at predetermined optimal conditions (Table 1). The obtained results showed that similar to solid medium, the isolates had different abilities for enzyme production at submerged cultures. The enzyme activity was determined in the range from 28.73 to 1284.84 unit/mg protein among the isolates (Table 1). There were some significant differences in CDH production in this regard (P<0.05). Although CDH activity was detected in all tested *Cladosporium* isolates, the results obtained for agar plate screening and submerged cultivation were not parallel (Table 1).

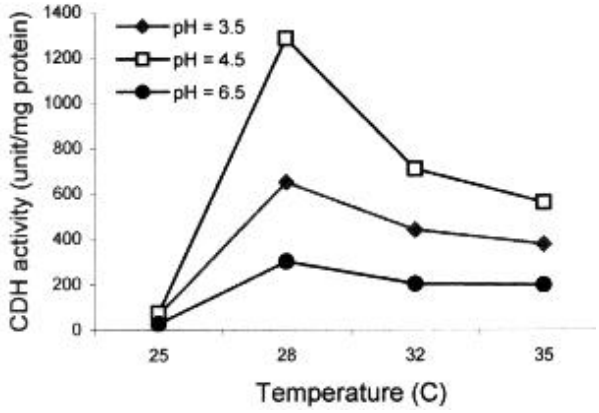

Fig. 2. Effect of pH and temperature on CDH production by *Cladosporium* isolate No. 10 at 14th days of submerged shaking cultures.

DISCUSSION

The fungal CDH appeared to be an extracellular enzyme released into the culture medium. Although the exact *in vivo* and *in vitro* functions of CDH have still not been completely resolved, study of this interesting enzyme is continuously growing because of its unique specificity and structure and also its application in the pulp and paper industry [9, 11]. In spite of the presence of CDH in different fungal groups such as soft rot, white rot and the others [6, 9], most investigations were focused on the enzyme from *Phanerochaete chrysosporium* [6, 9]. However, as fermentation yield of CDH in *P. chrysosporium* is low [15], this fungus is not attractive for large-scale applications. Thus, at present, identification of organisms producing larger amounts of CDH on cheap and simple media would certainly further increase the applied interest in this enzyme. In this investigation, we studied the presence of CDH and its optimal culture conditions in *Cladosporium* as an important well-known

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cellulolytic organism. Initial screening of 20 *Cladosporium* isolates by zymogram method revealed that CDH is probably an extracellular enzyme with strain-specific production pattern. Also, we showed that DCPIP-based assay for detection of CDH activity could be especially useful for screening a large number of organisms. The only report upon the probable presence of CDH activity in the genus *Cladosporium* is related to the work of Dekker in 1988 [8]. He screened some fungi for CDH-producing ability by zymogram method using DCPIP as an electron acceptor and concluded that it was an extracellular enzyme produced by *Monilia* sp., *Cladosporium* and also some other fungal species [8]. However, because of the reaction of a functionally related group of fungal enzymes i.e. laccases toward DCPIP [9], the screening of CDH activity on media contained this indicator as reported by Dekker [8] should be further confirmed by complementary tests on the enzyme production in the presence of specific inhibitors of interfering laccases. In this study, we used 4 mM sodium fluoride in reaction mixture of the measuring CDH activity, which has been previously shown to inhibit laccases completely [16]. On the basis of Figure 1, maximum activity of extracellular CDH occurred after 14 days of growth on shaking cultures (120 rpm) at 28ºC. Other researchers showed that the highest amount of CDH in major fungal producers *Phanerochaete chrysosporium* [17], *Monilia* [8], *Sclerotium rolfsii* [13], and *Schyzophyllum commune* [18] produced after 12-15 days of incubation at 120-150 rpm. These results indicate that although CDH production in fungi is generally correlated with fungal strain and applied culture media, maximum amounts of enzyme production occur after 10^{th} day of growth at shaking cultures. Production of CDH by *Cladosporium* was much dependent on pH and temperature (Fig. 2). The enzyme production had a similar pattern in all applied pH values with an increasing from 25ºC to 28ºC and then decreasing toward 30ºC and 32ºC (Fig. 2). The highest amount of enzyme activity was occurred in pH 4.5 at 28ºC (P<0.05). Optimal pH and temperature for CDH production on submerged cultures have been determined in the range of 4 to 7 and 25-30ºC by different fungi [5, 9, 13, 17, 18]. Comparison of CDH activity in determined optimal conditions showed obvious and significant differences among some *Cladosporium* isolates. The enzyme activity was recorded between 27.83 to 1284.84 unit/mg protein in this regard. This activity has been reported in a range from 19.5 to 1960 unit/mg protein for various cellulolytic fungi [7, 13, 15, 18]. It is seemed that CDH production by fungi is largely dependent on fungal strain (enzyme source), media composition (i.e. the type of enzyme substrate), and culture conditions. Also, there was not any obvious correlation between gelatin hydrolysis as a probable index of fungal pathogenesis and CDH activity in both solid and liquid cultures. According to our results, the fungus *Cladosporium* should also be added to the list of documented CDH producer organisms. The results indicate that *Cladosporium* isolates, No. 10 and No.18, with high CDH-producing ability can be used as selective candidates for commercially enzyme production in further research programs.

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