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Full Length Research Paper

Production and characterization of cellulolytic activities produced by *Trichoderma longibrachiatum* (GHL)

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The indigenous cellulolytic fungus *Trichoderma longibrachiatum* (GHL) isolated from soil near an Algerian hot spring was used for the production of cellulases by submerged fermentation on Mandels medium with cellulose Avicel (1%) as the sole carbon source. Endoglucanase and filter paper activities of the wild-type strain of *Trichoderma* were compared to the hypercellulolytic mutated *Trichoderma reesei* Rut C-30, in shake flask cultures at 35°C. After seven days of fermentation, *T. longibrachiatum* show equivalent activities than *T. reesei* (10.61 IU/ml of endoglucanase (CMCase) and 2.04 IU/ml of filter paper activity (FPA)). On the other hand, the ß-glucosidase activity of *Trichoderma* GHL was twice more important than that of *T. reseei*. The influence of inoculum size on cellulase activities did not prove significant differences in enzymatic activities for spore concentrations between 10^5 and 10^8 spores/ml. The cellulases produced by the isolated strain were also characterized. The optimum temperatures were 55 and 60°C for endoglucanase and FPA, respectively. The endoglucanase was thermostable at 70°C after 5 h incubation, and it preserved 80% of the original activity. The half-life of the FPA appeared to be 3 h at 60°C. The endoglucanase was optimally active at pH 4.0, and the FPA was optimal at pH 4.0 and 5.0. These activities were stable at 50°C after 5 h incubation in a pH range of 3.0 to 6.0 and 4.0 to 6.0, respectively. These results suggest that the non-mutated strain *T. longibrachiatum* (GHL) should be an attractive producer for cellulases production.

Key words: Cellulase, *Trichoderma reesei*, *Trichoderma longibrachiatum*, submerged fermentation, characterization.

INTRODUCTION

β-(1,4)-linked glucose polymer cellulose is a product of the utilization of solar energy and carbon dioxide by plants and it exhibits an annual production of about 1.3 $\times 10^{10}$ tons (Duff and Murray, 1996). It is the major polysaccharide compound in plants and is the most abundant organic compound on earth (Coughlan, 1985). Accordingly, its turnover in the carbon cycle is of prime importance for all living organisms (Beguin and Aubert, 1994; Tomme et al., 1995). Degradation of cellulolytic materials can be achieved chemically, enzymatically, or by the combination of both chemical and enzymatic methods. Although today, the enzymatic hydrolysis of cellulose by the enzyme cellulase is preferred over chemical methods for environmental reasons. When subjected to enzymatic hydrolysis, these polysaccharides are transformed into glucose and other fermentable carbohydrates, which might further be converted to liquid fuels and many other useful chemicals (Gusakov, 2011).

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Abbreviations: CMCase, Carboxymethyl cellulase; **FPase,** filter paper activity; **FPA,** filter paper activity; **T,** *Trichoderma*.

The cellulase enzyme complex consists of three types of enzymes (that is, endoglucanases (EG) (EC 3.2.1.4), exoglucanases/cellobiohydrolases (CBH) (EC 3.2.1.91), and β-glucosidase (BG) (EC 3.2.1.21) that act synergistically in cellulose hydrolysis (Bhat and Bhat, 1997). Synergistic reaction occurs as a result of sequential, cooperative action between the three enzyme components in a complex in which the product of one enzyme reaction becomes the substrate for another (Roussos and Raimbolt, 1982). Endoglucanases randomly attack internal glycosidic bonds of cellulose chains and release cello-oligosaccharides, exoglucanases (cellobiohydrolases) cleave cellobiose units from the non reducing ends of cellulose chains and β-glucosidase converts the resulting cellobiose to glucose (Bhat and Bhat, 1997). In many microorganisms, we found several enzymes for the same activity (e.g. there are at least five endoglucanases (EGI to EGV) in *Trichoderma reesei* and three endoglucanases (EGI to EGIII) in *Trichoderma longibrachiatum* (Andri et al., 2010). A broad range of bacteria and filamentous fungi can produce cellulases. One of the most extensively studied cellulolytic microorganisms is genus *Trichoderma* species (*T. reesei* and *Trichoderma viride*) and especially soft rot fungi *T*. *reesei* and *T. longibrachiatum*, which are also used industrially for enzyme production (Kubicek, 1992). The extracellular cellulolytic system of *T. reesei* is composed of 60 to 80% cellobiohydrolases or exoglucanases, 20 to 36% of endoglucanases, and 1% of β-glucosidases (Gritzali and Brown, 1979). *T. reesei* has been improved throughout the years by random mutagenesis, which has resulted in substantially improved enzyme productivity (Roussos and Raimbolt, 1982; Tolan and Foody, 1999). *T. reesei* Rut C-30 is a high yielding parent strain of many commercially strains used today. With the advent of new frontiers in the field of biotechnology, cellulases have attracted much interest, because of the diversity of their applications, and also for facilitating the understanding of mechanism of enzymic hydrolysis of plant carbohydrate polymers (Bhat and Bhat, 1997). The major industrial applications of cellulases are in textile industry for “biopolishing” of fabrics and producing stone washed look of denims, as well as in house hold laundry detergents for improving fabric softness and brightness (Cavaco-Paulo, 1998). Besides, they are used in animal feeds for improving the nutritional quality and digestibility, in processing fruit juices, and in baking, while de-inking of paper is yet another emerging application (Tolan and Foody, 1999). A potential challenging area where cellulases would have a central role is the bioconversion of renewable cellulosic biomass to commodity chemicals (Lark et al., 1997; Himmel et al., 1999). Among them, monomeric sugars, since sugars can serve as raw materials in a number of biotechnological production processes. For instance, sugars produced can be converted to ethanol (Lawford and Rousseau, 2003), lactic acid (El Hawary et al., 2003), and hydrogen (Taguchi et al., 1996).

In addition, the growing concerns about the shortage of fossil fuels and air pollution caused by incomplete combustion of fossil fuel have also led to an increasing focus on production of ethanol from lignocellulosics and especially the possibility to use cellulases to perform enzymatic hydrolysis of the lignocellulosic materials (Zaldivar et al., 2001; Sun and Cheng, 2002). Currently, the high market price of cellulases prohibits commercialization of the lignocellulosics to fuel ethanol process. Novozymes Company describes the production of biofuel from agricultural waste and announced that the enzymes can produce ethanol at a price below $2 per gallon (www.bioenergy.novozymes.com). For this reason, research aimed at understanding and improving cellulase production is still a hot topic in cellulase research. In order to make those large-scale applications economically feasible, the cost of the cellulolytic enzymes needs to be reduced (Pothiraj et al., 2006; Adsul et al., 2007). Thus, the conversion of waste cellulose to glucose is still not commercially feasible. To resolve these problems, one of the key issues is the generation of improved producer strains with higher specific activity and greater efficiency. This has traditionally been reached by classical mutagenesis and selection procedures. Thus, the expanding application of the enzyme calls for an urgent need for the exploration of microorganisms from pristine environments as valuable source of this commercial enzyme. In this study, we report a wild type strain of *T. longibrachiatum* (GHL) from soil near the hot spring of Guelma (North-east of Algeria). Here, we first describe the comparison of cellulolytic activities of the local isolated and the hyper-producing cellulolytic fungus *T. reesei* Rut C-30. The characterization of cellulases produced by *T. longibrachiatum* is also described.

MATERIALS AND METHODS

Microorganisms and inoculum preparation

Trichoderma (GHL) was isolated at our laboratory (Microbiological Engineering and Applications Laboratory, Mentouri University, Constantine, Algeria), from soil samples collected near the hot spring of Guelma located in the North-east of Algeria. This strain was identified as *T. longibrachiatum* Rifai by German Collection of Microorganisms and Cell Cultures (DSMZ, GmbH laboratory). *T. reesei* Rut C-30 VTT-D-86271 was obtained from the National Center of Technical Research, Finland. The strains were maintained on potato dextrose agar (PDA, Biokar Diagnostics). PDA Petri plates were incubated at 30°C for *T. reesei* Rut C-30 and 35°C for the isolated strain until good sporulation occurred, and then they were stored at 4°C. The spores were harvested by washing the Petri plate with 10 ml of sterile water containing 0.1% of tween 80 (Sigma-Aldrich). The spore concentration was determined in a counting chamber after appropriate dilution.

Production medium

The seed medium for submerged fermentation for cellulase production was the same as that proposed by Mandels and Weber (1969) with the

following composition (per liter): 1.4 g $(NH_4)_2SO_4$, 2.0 g KH_2PO_4, 0.3 g urea, 0.3 g $CaCl_2$, 0.3 g $MgSO_4.7H_2O$, 1 ml trace metal stock solution (495 ml distilled water, 5 ml concentrated HCl, 4.6 g $FeSO_4.7H_2O$, 0.89 g $MnCl_2.4H_2O$, 1.78 g $ZnSO_4.H_2O$, 1.83 g $CoCl_2.6H_2O$), 2 ml Tween 80, 0.25 g yeast extract, 1 g proteose peptone, and 10 g cellulose Avicel PH-101. The initial pH value of this medium was 5.5. The medium was sterilized by autoclaving at 120°C for 20 min before inoculation.

Cellulase production in shake flasks

The enzyme production was carried out in 500 ml Erlenmeyer flasks with 100 ml of production medium (initial culture pH 5.5). For cellulases production, the flasks were inoculated with spores at 10^7 spores/ml and incubated at 30 and 35°C, in order to compare the enzymatic activities produced by the two strains, with shaking at 150 rpm. To elucidate the influence of the inoculum size on enzyme production by the isolated strain at optimal growth temperature of 35°C using Mandel's medium, the inoculum sizes were varied from 10^5 to 10^8 spores/ml. After inoculation, Erlenmeyer flasks were incubated in a rotary shaker for 10 days. Samples were removed every day, centrifuged at 4000 g for 10 min and the supernatants (used as the enzyme sources) were collected for pH and enzyme activities measurements. Every experiment was performed in triplicate.

Production in stirred fermenter

Cellulolytic enzyme production was performed in batch culture conditions in a 4 L fermenter (SETRIC Genie Industriel) with a working volume of 2.7 L. The inoculum was a spore suspension of the isolated strain at 10^6 spores/ml. Temperature was kept at 35°C, agitation at 300 rpm, and the inoculum was later aerated. Under these conditions, dissolved O_2 did not drop below 20% of air saturation in culture media. The pH was set at 5.5, and thereafter, it was not controlled during cell cultivation. Foam was controlled by the addition of colza oil whenever it was necessary. Enzyme production was continued for ten days, and two samples were withdrawn daily for analysis of cellulolytic activities and pH measurements.

Analytical techniques

Determination of enzyme activities

Filter paper activity (FPA) was used to determine the overall activity of cellulase complex according to the method of Ghose (1987). This method measures the release of reducing sugar produced in 60 min from a mixture of enzyme solution (0.5 ml) and of citrate buffer (0.1 M, pH 4.8, 1 ml) in the presence of 50 mg Whatman No. 1 filter paper (1 × 6 cm strip) and incubated at 50°C. Endoglucanases (carboxymethyl cellulase (CMCase) and endo-1,4-β-D-glucanase; EC 3.2.1.4) were assayed in the total reaction mixture of 1 ml containing 0.5 ml diluted enzyme and 0.5 ml of 1% (w/v) carboxymethyl-cellulose (CMC) solution in citrate buffer (0.1 M, pH 4.8). This reaction mixture was incubated at 50°C for 30 min. The amount of realized sugar was determined by the dinitrosalicylic acid method at 540 nm described by Miller (1959). A calibration curve was established with glucose (0 to 0.027 M). The activity was calculated as IU/ml. One unit of enzyme activity was defined as the amount of enzyme that forms 1 µmol glucose (reducing sugar as glucose) in 1 min/ml enzyme at 50°C, pH 4.8.

β-glucosidase activity was determined according to Norkrans (1957). A 0.1 ml of sample was mixed with 1 ml of 5 mM *p*-nitrophenyl-β-D-glucopyranoside in 0.1 M citrate buffer pH 4.8, and was kept at 50°C for 10 min. The reaction was stopped by addition of 2 ml of 1 M sodium carbonate solution followed by 10 ml of distilled water and the absorbance was measured at 400 nm. Standard curve was obtained using *p*-nitrophenol (0 to 2 mM). The activity in units (IU/ml) was calculated as µmol *p*-nitrophenol produced per min under the assay conditions. In all cellulase activities measurements, the blank was made in the same way excluding the substrate (filter paper for FPA, CMC for endoglucanase activity, and *p*-nitrophenyl-β-D-glucopyranoside for β-glucosidase). Every sample was analyzed in triplicate, mean values and standard deviations were calculated.

Determination of protein

The protein concentration was measured using the method of Lowry et al. (1951) with bovine serum albumin as a standard.

Characterization of endoglucanases and FPA activities

For cellulases characterization, the crude culture supernatant of 4 L fermenter was tested for the optimum temperature, the thermal stability, the optimum pH and the pH stability. The optimum temperature of the enzyme was measured by performing the enzyme activity assay at different temperatures ranging from 30 to 80°C in citrate buffer (0.1 M, pH 4.8). The heat stability of the cellulases in the crude supernatants was tested by preincubating enzyme samples at various temperatures (60, 70, 75, 80, and 90°C) for 5 h, and the remaining activity was quantified using the standard methods at different time intervals from 1 to 5 h with an increment of 1 h. For optimum pH activity, assays at different pH values were performed at the optimal temperature over a pH range of 3.0 to 9.0. The buffers used were 0.1 M citrate (pH 3.0 and 4.0), 0.1 M citrate phosphate (pH 4.0 to 7.0), 0.2 M phosphate (pH 7.0 and 8.0), and 0.2 M glycine-NaOH (pH 9.0), respectively. pH stability studies were performed by incubating the crude enzyme for 5 h at 50°C and at a pH ranging from 3.0 to 9.0. The residual activity of each sample was then measured using the standard methods.

Experimentation and analysis

All the values presented in graphs and tables are the means of three replications. Data were statistically analyzed by using the analysis of variance (ANOVA) and expressed as mean ± standard deviation (SD). Significance was considered established at $P < 0.05$.

RESULTS

Comparing *T. longibrachiatum* (GHL) with *T. reesei* Rut C-30 in cellulases production

In the aim of comparing the enzyme amounts produced by the isolated strain *T. longibrachiatum* (GHL) and the mutated *T. reesei* Rut C-30, fermentations were performed at 30 and 35°C in shake flasks, using an inoculum size of 10^7 spores/ml. as shown in Table 1 at 30°C, enzymes were produced slowly with the isolated strain, and they reached their maximum after 240, 168 and 192 h for FPA (1.72 IU/ml), endoglucanases (10.37 IU/ml) and β-glucosidase (0.49 IU/ml), respectively. However, the overall cellulase activity was secreted in larger amounts after 48 h with the mutant strain *T. reesei* Rut C-30, while to achieve the maximum enzyme activities, it takes time for cultures almost equivalent to *T. longibrachiatum*, 192 h with FPA (2.78 IU/ml) and β-glucosidase (0.62 IU/ml), and 240 h with endoglucanase (13.67 IU/ml), respectively. At 35°C, the isolated strain reached the maximum overall activity much faster than at 30°C (Table 1); the FPA maxima for the two *Trichoderma* were produced after 168 h of growth and for similar values (*T. longibrachiatum* (2.04 IU/ml) and *T. reesei*

Table 1. Comparing *T. longibrachiatum* (GHL) with *T. reesei* Rut C-30 in cellulase production.

Time (h)	*Trichoderma* (GHL) enzyme activities (IU/ml)			*Trichoderma reesei* Rut C-30 enzyme activities (IU/ml)		
	Endoglucanase	FPA	β-glucosidase	Endoglucanase	FPA	β-glucosidase
Cellulase production at 30°C						
48	6.15 ± 0.23	0.84 ± 0.06	0.20 ± 0.01	10.91 ± 0.27	2.38 ± 0.06	0.21 ± 0.01
72	8.62 ± 0.11	1.04 ± 0.03	0.22 ± 0.01	11.47 ± 0.13	2.42 ± 0.10	0.32 ± 0.06
96	9.57 ± 0.17	1.45 ± 0.03	0.26 ± 0.01	11.51 ± 0.21	2.36 ± 0.30	0.43 ± 0.02
120	10.07 ± 0.15	1.49 ± 0.06	0.30 ± 0.02	11.85 ± 0.24	2.75 ± 0.15	0.43 ± 0.02
144	9.79 ± 0.21	1.56 ± 0.03	0.32 ± 0.06	11.03 ± 0.23	2.72 ± 0.08	0.56 ± 0.04
168	10.37 ± 0.14	1.56 ± 0.04	0.44 ± 0.02	11.47 ± 0.21	2.67 ± 0.11	0.58 ± 0.05
192	9.84 ± 0.48	1.63 ± 0.04	0.49 ± 0.01	13.67 ± 0.20	2.78 ± 0.13	0.62 ± 0.02
216	9.83 ± 0.62	1.61 ± 0.08	0.48 ± 0.02	12.04 ± 0.42	2.53 ± 0.06	0.42 ± 0.01
240	9.38 ± 0.18	1.72 ± 0.08	0.37 ± 0.05	12.22 ± 0.14	2.53 ± 0.17	0.39 ± 0.01
Cellulase production at 35°C						
48	9.22 ± 0.15	1.09 ± 0.06	0.22 ± 0.01	9.72 ± 0.106	1.32 ± 0.04	0.21 ± 0.01
72	10.17 ± 0.27	1.39 ± 0.05	0.27 ± 0.01	10.73 ± 0.10	1.90 ± 0.07	0.42 ± 0.02
96	10.42 ± 0.18	1.71 ± 0.08	0.44 ± 0.06	10.39 ± 0.14	2.02 ± 0.07	0.59 ± 0.03
168	10.61 ± 0.13	2.04 ± 0.09	0.70 ± 0.03	10.11 ± 0.55	2.08 ± 0.09	0.65 ± 0.02
192	10.46 ± 0.25	2.02 ± 0.05	0.92 ± 0.03	9.68 ± 0.17	1.76 ± 0.11	0.60 ± 0.02
216	9.78 ± 0.20	1.78 ± 0.07	1.25 ± 0.03	9.38 ± 0.60	1.69 ± 0.10	0.64 ± 0.05
240	9.08 ± 0.15	1.61 ± 0.05	1.32 ± 0.06	8.68 ± 0.51	1.63 ± 0.09	0.72 ± 0.03

Data were expressed as mean values ± SD.

(2.08 IU/ml)). The maximum β-glucosidase activity of *T. longibrachiatum* (1.32 IU/ml) is obtained after 240 h. It is 2.69 times greater than at 30°C and 1.83 times greater in *T. reesei* at 35°C on the same culture time. In the other hand, *T. reesei* Rut C-30 secreted maximum enzyme amounts after 72 h for endoglucanase (10.73 IU/ml). The analysis of variance indicates that the production of the enzymes is influenced by the three factors tested (strain, temperature and time of fermentation). The strain has a significant effect on the production of endoglucanase activity (F = 271.67), FPA activity (F = 2180.01) (F: Fisher statistical test.), and β-glucosidase activity (F = 27.17). The temperature acts significantly on the production of endoglucanase activity (F = 74.13), FPA activity (F = 307.81), and β-glucosidase activity (F = 3196.10). Also, the time of incubation has a significant effect on the production of endoglucanase activity (F = 33.84), FPA activity (F = 152.89), and β-glucosidase activity (F = 1261.47).

Effect of inoculum size on cellulases production by *T. longibrachiatum* (GHL)

In order to check the effect of inoculum ratio on enzyme production by the isolated strain *Trichoderma* (GHL) in shake flasks, experiments were done with varying inoculum ratio (Table 2). The isolated strain *T. longibrachiatum* (GHL) secreted maximum endoglucanase activity (10.86 IU/ml) after 168 h of fermentation with 10^5 spores/ml, followed at the same time, by 10.78 and 10.61 IU/ml with 10^6 and 10^7 spores/ml, respectively. Using the rate at 10^8 spores/ml, endoglucanase activity reached 10.48 IU/ml value after 192 h of growth. However, the maximum FPA (2.26 IU/ml) was found with 10^6 spores/ml after 192 h of cultivation, followed at the same time, by 2.13 IU/ml with 10^5 spores/ml, but after

Table 2. Effect of inoculum size on enzyme production by the isolated strain *Trichoderma* (GHL): the value of β-glucosidase at 240H with the inoculum of 16^6sp/ml is: 1.52±0.04.

Time (h)	10^5 spores/ml enzyme activity (IU/ml)			10^6 spores/ml enzyme activity (IU/ml)		
	Endoglucanase	FPA	β-glucosidase	Endoglucanase	FPA	β-glucosidase
10^5 and 10^6 spores/ml						
48	6.56 ± 0.33	0.72 ± 0.04	0.20 ± 0.01	8.27 ± 0.70	0.96 ± 0.14	0.23 ± 0.04
72	9.71 ± 0.28	1.30 ± 0.13	0.22 ± 0.01	9.59 ± 0.18	1.31 ± 0.06	0.23 ± 0.01
96	10.46 ± 0.34	1.86 ± 0.03	0.28 ± 0.00	10.44 ± 0.14	1.88 ± 0.10	0.31 ± 0.02
168	10.86 ± 0.17	2.12 ± 0.12	1.03 ± 0.10	10.78 ± 0.09	2.25 ± 0.09	1.11 ± 0.09
192	10.39 ± 0.14	2.13 ± 0.19	1.11 ± 0.08	10.54 ± 0.34	2.26 ± 0.15	1.14 ± 0.08
216	9.45 ± 0.61	1.84 ± 0.05	1.50 ± 0.12	9.83 ± 0.30	1.91 ± 0.09	1.53 ± 0.07
240	8.74 ± 0.52	1.54 ± 0.05	1.49 ± 0.06	8.88 ± 0.20	1.70 ± 0.07	1.52 ± 0.04
10^7 and 10^8 spores/ml						
48	9.22 ± 0.15	1.09 ± 0.06	0.22 ± 0.01	9.24 ± 0.10	1.13 ± 0.07	0.20 ± 0.01
72	10.17 ± 0.27	1.39 ± 0.05	0.27 ± 0.01	10.05 ± 0.13	1.37 ± 0.09	0.29 ± 0.01
96	10.42 ± 0.18	1.71 ± 0.08	0.44 ± 0.06	10.24 ± 0.21	1.52 ± 0.05	0.48 ± 0.02
168	10.61 ± 0.13	2.04 ± 0.09	0.70 ± 0.03	10.22 ± 0.11	1.75 ± 0.08	0.74 ± 0.06
192	10.46 ± 0.25	2.02 ± 0.05	0.92 ± 0.03	10.48 ± 0.17	1.75 ± 0.22	0.80 ± 0.07
216	9.78 ± 0.20	1.78 ± 0.07	1.25 ± 0.03	10.12 ± 0.15	1.58 ± 0.04	1.08 ± 0.08
240	9.08 ± 0.15	1.61 ± 0.05	1.32 ± 0.06	9.06 ± 0.09	1.38 ± 0.04	1.17 ± 0.06

The data were expressed as mean values ± SD.

168 h, with 2.04 and 1.75 IU/ml values using the inoculums sizes of 10^7 and 10^8spores/ml, respectively. For β-glucosidase, maximum activity was measured at 1.53 and 1.50 IU/ml with the rates at 10^6 and 10^5 spores/ml, after 216 h of fermentation. Both 10^7 and 10^8 spores/ml rates showed maximum activity at the end (240 h) of fermentation (1.32 and 1.17 IU/ml, respectively). Statistically, the inoculum size has a significant effect on the production of endoglucanase activity (F = 15.57), FPA activity (F = 51.71), and β-glucosidase activity (F = 36.30). Also, the incubation time acts significantly on endoglucanase activity (F = 122.49), FPA activity (F = 377.83), and β-glucosidase activity (F = 745.57).

Cellulase production in 4 L fermenter

The process of cellulase production by the isolated strain *T. longibrachiatum* (GHL) was performed in a 4 L fermenter. The time course of enzymes production using 10 g/L cellulose Avicel as the substrate are as shown in Figure 1. Overall, maximum endoglucanases, FPA and β-glucosidase were obtained after 144, 128 and 120 h, respectively. The recorded activities were 11.22, 1.88 and 0.64 IU/ml. The volumetric enzyme productivity of endoglucanase and FPA was 166.66 IU/L/h and 22.03 IU/25 L/h, respectively. The specific endoglucanase and FPA were measured at 5.81 and 1.17 IU/mg, respectively.

Characterization of the enzyme synthesized by the isolated strain *T. longibrachiatum* (GHL)

Different properties of the crude enzymes contained in the supernatants from culture of the isolated strain were analyzed. Enzymatic assays

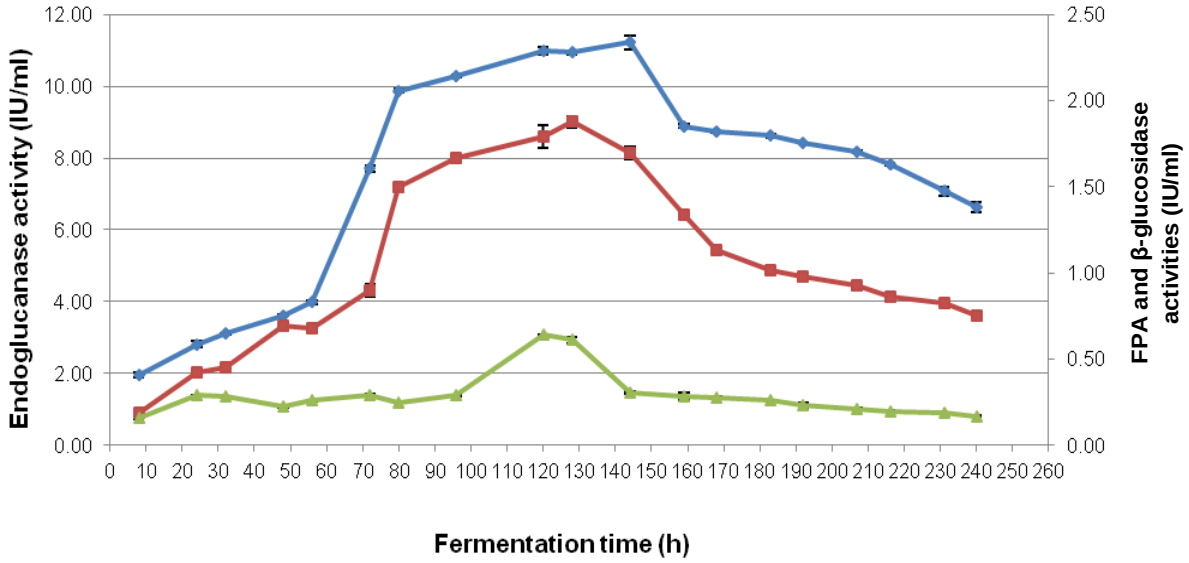

Figure 1. Fermentation profile for *T. longibrachiatum* (GHL) grown on Mandels medium with 10 g/L cellulose Avicel in stirred fermenter at 35°C. ♦, Endoglucanase; ■, FPA; ▲, β-glucosidase activities.

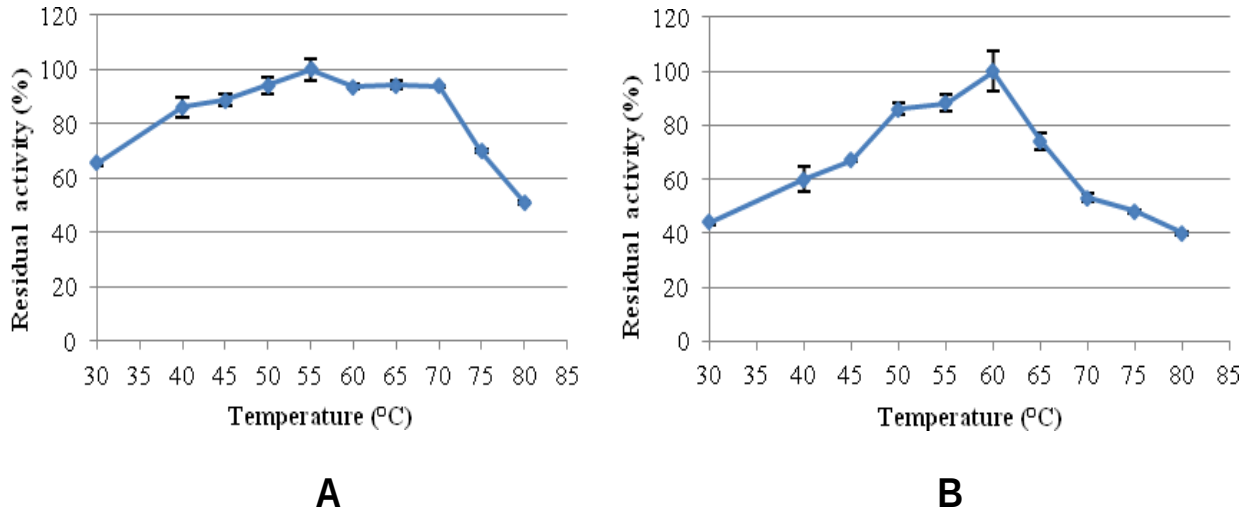

Figure 2. Effect of temperature on the activity of endoglucanase (A) and FPA (B). The values are means ± SD, n = 3. Absence of bars indicates that errors were smaller than symbols.

at different temperatures revealed that endoglucanase and FPA had an optimal activity at 55 and 60°C, respectively (Figure 2). Endoglucanase activity (80 to 100%) was preserved from 40 to 70°C. At 80°C, only 51% of the endoglucanase activity was maintained, and 60% of the FPA was lost. The effect of temperature on the enzymes was significant ($F = 127.67$ for endoglucanase activity, $F = 67.42$ for FPA activity). Thermal stability was tested by preincubating the crude enzyme for 5 h at various temperatures, and the remaining activity was measured by standard cellulase assays conditions. The endoglucanase was stable at 60 and 70°C, and the total activity retained was 93 and 80% respectively, after 5 h incubation at these temperatures (Figure 3A). At 80 and 90°C, about 20% of the activity was still retained, after 5 h of incubation. However, FPA was more affected by the incubation temperature. At 60°C, after 5 h of incubation, only 48% of the original activity was retained, with a half life after 3 h of incubation at the same temperature (Figure 3B). At above 70°C, about 72% of the FPA was lost after 5 h incubation at 75°C.

Using a temperature of 55°C for endoglucanase and 60°C for FPA, the optimum pH of the enzymes was

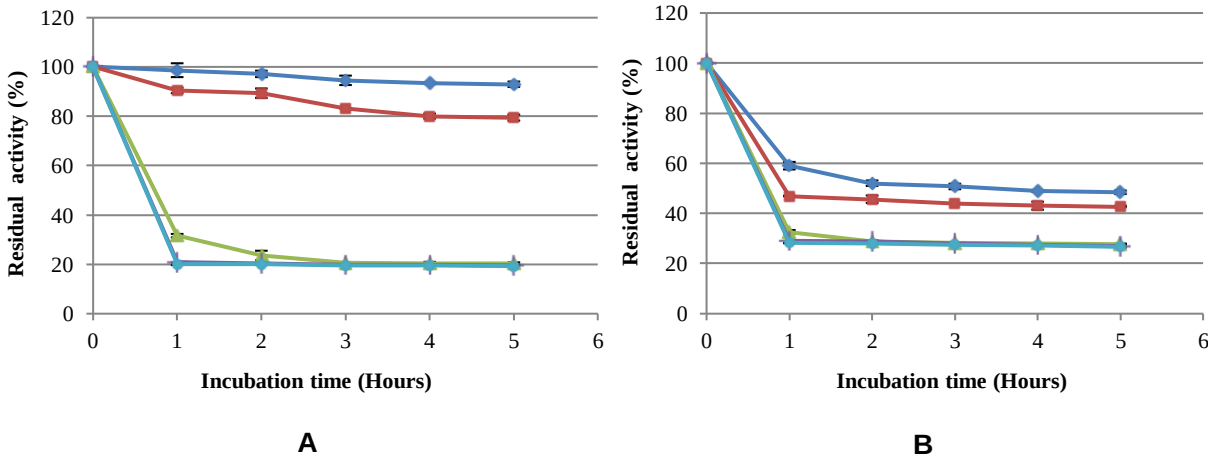

Figure 3. The thermostability of endoglucanase (A) and FPA(B) activities produced by the isolated strain at various temperatures: 60°C(♦), 70°C(■), 75°C(▲), 80°C(+) and 90°C(•). Data are presented as means±SD, n=3. Absence of bars indicates that errors were smaller than symbols.

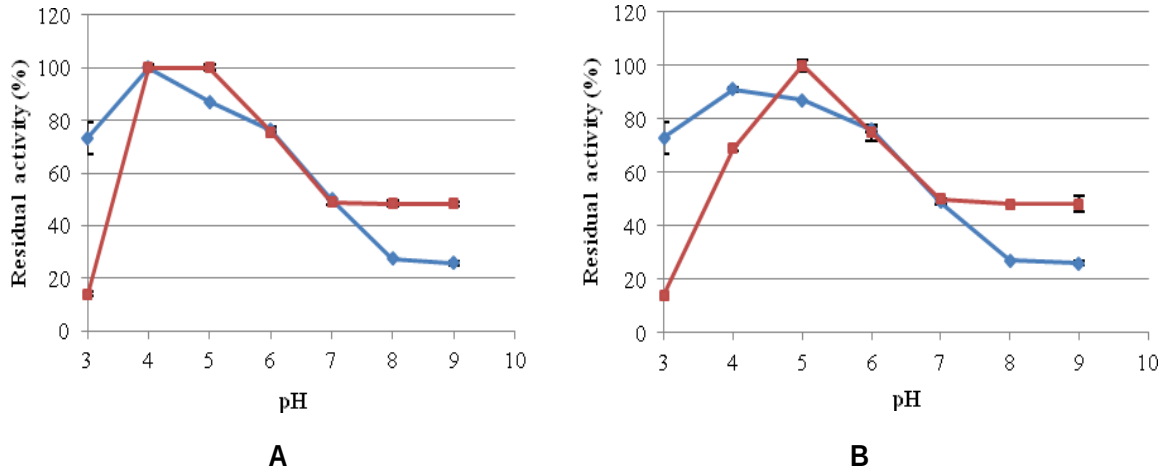

Figure 4. Effect of pH on the activity of endoglucanase (♦) and FPA (■). A: citrate buffer pH 3.0 and 4.0, citrate phosphate buffer pH 5.0, 6.0, and 7.0, and phosphate buffer pH 8.0 and 9.0. B: citrate buffer pH 3.0, citrate phosphate buffer pH 4.0, 5.0, and 6.0, and phosphate buffer pH 7.0, 8.0, and 9.0. Data are presented as means ± SD, n = 3. Absence of bars indicates that errors were smaller than symbols.

determined (Figure 4A). Endoglucanase showed an optimal activity at pH 4.0 (citrate buffer) and the FPA showed an optimal activity at both pH 4.0 (citrate buffer) and pH 5.0 (citrate phosphate buffer). Besides, the endoglucanase activity was active on a broad range of pH from 3.0 to 6.0. The analysis of these results by ANOVA revealed that the pH affect considerably the activities of endoglucanase (F = 330.19) and FPA (517.17). In addition, we noted the effect of the buffer nature (Figure 4B). With citrate buffer pH 4.0, the optimal endoglucanase activity was reached, but about 91% of the maximum activity was obtained with citrate phosphate buffer pH 4.0. Despite, only about 69% of the maximum FPA was obtained with the citrate phosphate buffer pH 4.0, in contrast with a maximum activity with citrate buffer pH 4.0. However, at pH 7.0, even with citrate phosphate buffer or phosphate buffer, there was no difference in enzyme activities of both endoglucanase and FPA.

The pH stability of the crude enzyme was also examined at various pH ranging from pH 3.0 to 9.0, as shown in Figure 5. More than 80% of the original endoglucanase activity was maintained at broad pH ranging from pH 3.0 to 6.0 after 5 h incubation at 50°C. The endoglucanase retained 100 and 95% of its activity after 3 and 5 h incubation in the citrate buffer pH 4.0, respectively. At pHs of 7.0, 8.0, and 9.0, only about 20% of the original activity was preserved after 5 h incubation (Figure 5A). On the other hand, FPA was not stable at pH

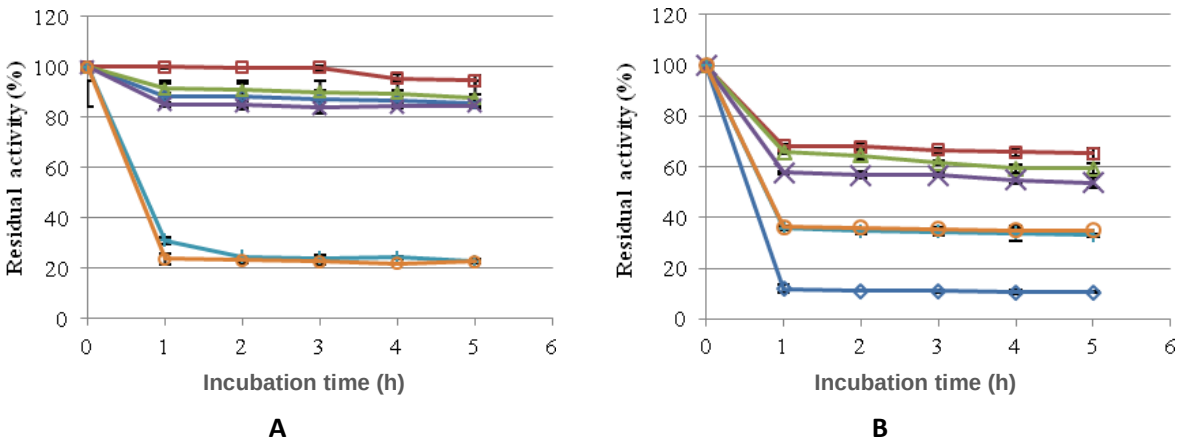

Figure 5. The pH stability of the endoglucanase (A) and FPA (B) activities produced by the isolated strain at pH 3.0 (◊), 4.0 (□), 5.0 (Δ), 6.0 (×), 7.0 (+), and 8.0 (o). Data are presented as means ± SD, n = 3. Absence of bars indicates that errors were smaller than symbols.

3.0, and more than 50% of the original activity was maintained over the pH ranging from 4.0 to 6.0, after 5 h incubation at 50°C. Above pH 6.0, about 35% of the FPA was retained after 5 h incubation (Figure 5B).

DISCUSSION

In this work, we adopted a locally isolated stain of *Trichoderma* (GHL), from sample soils collected near the hydrothermal vent of Guelma situated in the North-east of Algeria. We successfully isolated a strain which showed approximately equivalent amounts in activities of endoglucanase and FPA, in shake flask culture, in comparison with the hyper producing cellulolytic fungus, mutant *T. reesei* Rut C-30. The culture of the isolated strain in the standard Mandels medium with 1% of cellulose Avicel, at 35°C indicated a rapid growth with a total clearance of the fermentation broth over the end of the fermentation; this means that the fungus has hydrolyzed the cellulose that troubled the medium. This may be explained by the result of the synergism of different types of enzymes produced by the evaluated fungus. In this work, we studied the influence of varying spores concentration on enzyme synthesis by the isolated strain *T. longibrachiatum*, with 10^5 spores/ml rate, the enzyme production was slow as compared to the other ratios, and it reached the maximum values of endoglucanase, FPA, and β-glucosidase after 168, 168 and 216 h, respectively. In general, from these data, there was no significant effect of different ratios used on cellulase activities. Our results are in good agreement with those indicated by Roussos and Raimbault (1982) for the production of cellulase *by Trichoderma harzianum*, in which there was no significant difference between enzyme activities obtained at elevated spore concentration (10^{10} spores/ml) with those measured at small inoculums size (10^7 spores/ml). In contrast, Domingus et al. (2000) reported that higher protein and enzyme activity were produced using more concentrated inoculums of *T. reesei* Rut C-30 cultivated on a modified Mandels medium.

Fermentation temperature and pH are two important factors affecting the biosynthesis of enzymes. In this study, the production of cellulases by the isolated strain was favored by a temperature of 35°C, corresponding to its optimum growth temperature, whereas, with the mutant strain *T. reesei* Rut C-30, the enzyme production was favored at 30°C. At 30°C, *T. reesei* Rut C-30 produced more enzyme activities (Endoglucanase, FPA, and β-glucosidase) than the isolated strain with respective differences of 3.3, 1.06, and 0.13 IU/ml. Unlike at 35°C, there was no significant difference in endoglucanase and FPA liberated by the two strains, but the isolated strain produced a β-glucosidase of 1.32 IU/ml, corresponding to two fold more than that produced by *T. reesei* Rut C-30. The production of these cellulases by the isolated strain *T. longibrachiatum* in 4 L fermenter was comparable to the work of Wen et al. (2005), that obtained endoglucanase and FPA of 12.22 and 1.74 IU/ml, respectively by *T. reesei* grown on agricultural wastes. However, the endoglucanase activity measured in our investigation was higher than the value of 4.2 IU/ml obtained by Ahamed and Vermette (2008), but FPA was higher (5.02 IU/ml) than that obtained in our study. Also, Domingus et al. (2001) reported a value of 2.8 U/m FPA, which was obtained with *T. reesei* Rut C-30 grown on a mixture of 15 g/L lactose and 30 g/L glucose. In another study, the FPA was 1.9 IU/ml when *T. reesei* was grown on 45 g/L cellulose Avicel PH 101 (Weber and Agblevor, 2005). However, the maximum FPA produced by *T. reesei* was 5.48 IU/ml after four days cultivation on corn

cob residue of 40 g/L in a 30 m^3 stirred fermenter (Liming and Xueliang, 2004). However, β-glucosidase activity was very low (0.64 IU/ml) after 120 h of fermentation, a value inferior to that obtained in shake flasks. Jun et al. (2009) obtained 0.39 and 0.42 IU/ml of β-glucosidase within six days when lactose (1%) and wheat bran (1%), respectively, were used as substrates from a mutant NU-6 of *T. reesei* Rut C-30. Sohail et al. (2009) obtained 0.25 IU/ml of β-glucosidase after 225 h from *Aspergillus niger* MS 82 grown on Mandels medium with 1% CMC. The lower activity of β-glucosidase as compared to other enzyme components has been reported as a common feature of *Trichoderma* strains (Sternberg et al., 1977; Ryu and Mandels, 1980). Due to the variety of cellulosic media used to cultivate fungi, and to the different reported expression of cellulase activities, it is difficult to compare the efficacy of the enzymes analyzed in this work with others already described. Although, the Mandels medium is widely used for cellulase production, but the concentration of carbon and nitrogen sources in this medium are insufficient for high yield of enzyme synthesis; the cellulase production was relatively low. It has been reported that high concentration of nutrient is necessary for high cellulase productivity (Sternberg and Dorval, 1979; Tangnu and Blanch, 1981).

Given the aforementioned, this study reveals important enzyme activities in the Mandels medium with 1% cellulose Avicel, but they could be enhanced using other media or cellulosic biomass. In this study, the produced enzyme was fully characterized. As indicated in Figure 4, one advantage of the endoglucanase activity produced by the isolated strain is the broad range of temperatures (from 40 to 70°C) at which it achieves elevated activities (more than 80% of the original activity was retained), with a peak at 55°C, and retained 51% of its activity at 80°C, whereas the FPA activity showed a peak at 60°C. Cellulase activities from *Trichoderma* spp. and other mesophilic fungi are at their optimum when assayed at about 50°C (Mandels et al., 1974; Kawamori et al., 1987). In another study, the optimum temperature of CMCase activity from *T. reesei* was 60°C (Busto et al., 1996). Jun et al. (2009) reported 50°C as optimum temperature for both FPA and endoglucanase produced by the mutant strain NU-6 of *T. reesei* Rut C-30. The present findings coincide with the notion that enzymes from thermophilic fungus present higher optimal temperature which could be related to longer “shelf-lives” for industrial applications (Maheshwari et al., 2000). Concerning the thermal stability, endoglucanase activity was shown as thermostable, and it retained at least 93 and 80% of its total activity after 5 h incubation at 60 and 70°C, respectively. On the other hand, FPA was moderately thermostable and inactivated rapidly above 70°C. Jun et al. (2009) reported that both FPA and endoglucanase were stable at 50°C, and the total activity retained more than 80% after 30 min incubation at this temperature, and inactivated rapidly above 60°C. Quiroz-Castaneda et al. (2009) indicated that the endoglucanase activity from the white rot fungus, *Pycnoporus sanguineus*, resisted up to 60°C showing approximately 80% of the original activity after 1 h of incubation, above 70°C, the endoglucanase activity was lost in 1 h of incubation. It is obvious that the stability of an enzyme varies considerably, depending on the strain used for its production and the nature of the habitat requiring the microorganism to adapt to extreme conditions (Busto et al., 1996). Also, enzyme which acquires its structural stability is an excessive glycosylation (Vieille and Zeikus, 2001a), either to the presence of hydrophobic bonds, hydrogen bonds, disulfide bridges (Kumar et al., 2000b; Sedlak et al., 2001; Vieille et al., 2001b; Mallick et al., 2002; Roovers et al., 2004), salt bridges, and cofactors (Vieille et al., 2001a). These elements were involved certainly in the maintenance of the spatial structure of the enzyme, despite thermal treatment. Besides, thermostable cellulolytic enzymes also have great potential to be used in industrial processes such as food processing, textiles, and bioconversion (Bhat and Bhat, 1997; Murray et al., 2004). High thermostability is an important property of cellulases, since the hydrolysis of cellulose will proceed faster at higher temperatures (Tong et al., 1980). So, cellulolytic enzymes that are stable at high temperatures can be used in cellulose saccharification processes at elevated temperatures to protect both substrate and products of the enzymic reaction from microbial contamination and deterioration (Hagerdal et al., 1980). Towards pH, endoglucanase was active over a broad range of pH from 3.0 to 6.0 (retained more than 70% of its original activity); while FPA was active over a broad range of pH 4.0 to 9.0 (retained more than 50% of the original activity). Jun et al. (2009) described that FPA and endoglucanase produced by the mutant strain NU-6 of *T. reesei* Rut C-30, have an optimal activity at pH 5.0 and 6.0, respectively, and below pH 3.0 or above pH 9.0, only 20 to 30% of the maximum activity was reached. In another study conducted by Busto et al. (1996), the endoglucanase activity of *T. reesei* showed a narrow range of pH (4.0 to 6.0) with a maximum activity at pH 5.0.

However, Jun et al. (2009) revealed that both FPase and CMCase produced by the mutant strain NU-6 of *T. reesei* Rut C-30, have an optimal activity at 50°C, pH 5.0 and 6.0, respectively, and both FPase and CMCase were stable at 50°C, and the total activity retained more than 80% after 30 min incubation at this temperature.

Conclusion

The results presented in this report show that cellulase activities produced by the isolated strain *T. longibrachiatum* (GHL), display no significant differences using different spores concentration as inoculum. Comparing the isolated strain with the hyper producing

cellulolytic fungus, at 35°C, the two strains liberated approximately equivalent amounts of endoglucanase and FPA, but the β-glucosidase produced by the isolated strain was twofold more than that secreted by *T. reesei* Rut C-30. Lower activity of β-glucosidase as compared to other enzyme components has been reported as a common feature of *Trichoderma* strains, for this reason, it was necessary to complete their enzyme extracts with β-glucosidase from other fungi, especially *Aspergillus.* The cellulase produced by the isolated strain was also characterized. The endoglucanase was optimally most active at 55°C, and more than 80% of the original endoglucanase activity was maintained at broad temperatures ranging from 40 to 70°C, while FPA showed an optimum at 60°C. The endoglucanase was thermostable and preserved 80% of its total activity after 5 h incubation at 70°C, while FPA showed a half-life of 3 h at 60°C. The optimal pH for the endoglucanase activity was 4.0, and more than 80% of the original endoglucanase activity was maintained at broad pHs ranging from pH 3.0 to 6.0 after 5 h incubation at 50°C. FPA activity was optimal at both pH 4.0 and 5.0, and more than 50% of its activity was maintained over the pH ranging from 4.0 to 6.0.

Coupled with its enzyme yields, occurrence of a relatively greater amount of the β-glucosidase component and the characteristics with the thermostable property of the endoglucanase enzyme (80% of its activity after 5 h incubation at 70°C), we are convinced that this strain should be a good candidate for industrial enzyme production. Therefore, this study needs to be extended further to develop an economical process for cellulase production using cellulosic wastes as inexpensive carbon sources.

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