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Improvement of Fungal Cellulase Production by Solid State Fermentation

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Abstract: Cellulase production studies have been carried out using the fungal strains *Trichoderma longibrachiatum* and *Aspergillus terreus* by using two different lignocellulosic materials for solid state fermentation (SSF). The effect of basic fermentation parameters (substrate, moisture content and the fermentation time) on enzyme production was studied. Maximum cellulase production (FPA and endoglucanase) of *Trichoderma longibrachiatum* were 33.83 U/gds and 167.4 U/gds, respectively, which represented a 1.75 fold improved activities than that of *Aspergillus terreus* (21.5 U/gds and 95.82 U/gds, respectively) using wheat bran as substrate. The optimal conditions for cellulase production with wheat bran were found to be: initial moisture content 70% and the optimal incubation time for production was three days. The endoglucanase of *Trichoderma longibrachiatum* was thermostable and showed a half-life of 1 hour at 70°C, while the filter paper activity (APF) lost its total activity after 1 hour at 70°C. Results indicate the scope for further optimization of the production conditions to obtain higher cellulase titres using the *Trichoderma longibrachiatum* strain under SSF.

Keywords: Cellulase, *Trichoderma longibrachiatum*, *Aspergillus terreus*, Solid state fermentation, Lignocellulosic material, Wheat bran.

Introduction

The need for utilizing renewable resources to meet the future demand for fuel has increased the attention on cellulose, the most abundant and renewable resource in the world. Over the last three decades, lignocellulosic biomass conversion has received a great interest, mainly due to the potential use of carbohydrates and lignin as sustainable sources of bioethanol or bioproducts precursors in a biorefinery concept (Kamm and Kamm, 2004).

Among the different feed stocks, wheat represents one of the most important crops produced worldwide with 653.6 million tons produced in 2010 and the most wide spread on earth with 217.2 million hectares harvested in 2010 (FAOSTAT, 2012). Wheat milling industry generates a large amount of bran, a co-product corresponding to the outer part of the grain, which generally accounts for 14-19% of the total mass. The main components of this fibrous material include (glucurono) arabinoxylans, mixed linkage β-glucans, cellulose, proteins, lignin and variable proportions of starch dependent on the milling conditions (Maes and Delcour, 2001). This agro-industrial residue remains an important source of lignocellulose with low value-added.

Cellulose is degraded by cellulases to reducing sugars and is fermented by yeast or bacteria to ethanol (Duff and Murray, 1996), which is an attractive alternative fuel to petroleum.

Cellulases are produced by various fungi and bacteria. *Trichoderma reesei* is a popular source of commercial cellulase, as it displays high cellulase activity, owing to the high protein secretion capacity of mutant strains obtained by random mutagenesis (Durand et al., 1988). *Aspergillus* speices also have

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been widely exploited for production of cellulases (Kang et al., 2004 ; Sohail et al., 2009). The biodegradation of complex polymeric structure of cellulose is primarily attributed to multi-component enzyme system that works in a synergistic manner. These enzymes are comprises of endoglucanase (EC 3.2.1.74), which attack cellulose in amorphous zone and release oligomers, cellobiohydrolases (CBHI & CBHII) (EC 3.2.1.91), that liberate cellobiose from reducing and non-reducing ends, and β- glucosidase (EC 3.2.1.21), which hydrolyze cellobiose to glucose (Lynd et al., 2002). Due to intensive research on the structural, catalytic and functional roles, both bacterial and fungal cellulases have led their use in various fields (Horn et al., 2012; Narra et al., 2014). In addition to their potential use in the biofuel industry, for degradation of agro-residual waste into simple sugars which can be further fermented, cellulases are widely employed in increasing the yield of fruit juices, beer filtration, paper and pulp industry as well as improving animal feed stock (Soni et al., 2008; Kaur et al., 2014).

Two technologies are currently utilized to achieve the production of enzymatic complexes: traditional submerged fermentation (SmF), which occurs in the presence of excess water submerging nutrients and microorganisms, and solid-state fermentation (SSF), which happens on insoluble solids in the absence or near absence of free water. Most enzymes companies apply classical SmF technology to manufacture their biocatalysts because this process is regarded to be more easily controlled. However, SSF technology could be an interesting alternative pathway, which is nowadays underexploited, to generate enzymatic complexes. One of the main SSF advantages is the direct use of raw materials (i.e., lignocellulose), which are employed to induce the production of a broad range of enzymes. This technology has also been recognized to have lower consumption of water and energy, high side activities, and it has been generally claimed that product yields are higher in SSF than in SmF (Singhania et al., 2010; Pandey, 2003).
To date, research concerning strain improvement and optimization of culture conditions has been studied. Thus, to maximize cellulase production, the exploration of microorganisms from pristine environments as valuable source of this commercial enzyme and optimization of culture conditions should be performed consequently. With this knowledge, the present study report two wild type strains of *Trichoderma longibrachiatum* and *Aspergillus terreus* from soil near the hot spring of Guelma (North-east of Algeria) and optimize culture conditions to enhance cellulase production.

Materials And Methods

Microorganisms and inoculum preparation

Trichoderma sp and *Aspergillus sp* were isolated at 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. The *Trichoderma* strain was identified as *Trichoderma longibrachiatum* Rifai by German Collection of Microorganisms and Cell Cultures (DSMZ, GmbH laboratory). *Aspergillus* sp was identified by macroscopic and microscopic tests, in our laboratory, as *Aspergillus terreus*. The strains were maintained on Sabouraud agar. Sabouraud Petri plates were incubated at 30°C 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 distilled water. The spore concentration was determined in a counting chamber after appropriate dilution. Stock cultures were stored at -20°C in distilled water with 20% (v/v) glycerol.

Solid substrates

Two different agro-industrial residues wheat bran (WB) and wheat straw (WS) were tried as substrates for cellulase production without pre-treatment. Wheat straw was precouped on strips (between 0.2 and 2cm length).

Solid state fermentation for cellulase production

The solid substrate (5 g) was weighed in 250 ml Erlenmeyer flasks and wetted with a moistening agent containing (g/L): glucose 10; sulfate d’ammonium 0.46; tartrate double Na, K 0.46 and distilled water. The contents were sterilized at 121°C for 20 min by autoclaving and were inoculated with $2x10^7$ spores per g of substrate. The flasks were incubated for 7 days at 30°C. Samples were withdrawn every day as whole flasks and enzyme extraction was performed using simple contact method. Citrate buffer (0.1 M, pH 4.8) was added to the fermented substrate to a total volume of 200 ml, mixed and centrifuged (10000 rpm for 10 min at 4°C) for further clarification and the supernatant was used as the crude enzyme preparation for assay of enzyme activity. Every experiment was performed in triplicate.

The parameters studied were initial moisture level (40, 50, 60, 70 and 80%) and time course for maximum enzyme production.

Analytical methods

Determination of enzyme activities

Cellulase activity was analyzed by the: filter paper assay and the carboxymethyl cellulase (CMCase) assay. 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. Endoglucanase (carboxymethyl cellulase (CMCase) was 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 0.0167 M/L). One unit of enzyme activity was defined as the amount of enzyme that forms 1 µmol glucose (reducing sugar as glucose) per minute under the standard assay conditions and was expressed as units per gram dry substrate (U/gds). Every sample was analyzed in triplicate, mean values and standard deviations were calculated.

Determination of thermal stability

The heat stability of *Trichoderma longibrachiatum* cellulases in the crude supernatants was tested by preincubating enzyme samples at various temperatures (60°C and 70°C) for 1 hour, and the remaining activity was quantified using the standard methods at different time intervals from 15 minutes to 60 minutes with an increment of 15 minutes.

Results and Discussion

Optimal conditions for SSF

Substrate

Selection of a suitable substrate is a crucial parameter in optimizing SSF. Presently, among the tested substrates, wheat ban resulted in a favorably high production of cellulase as indicated by the highest enzyme yield, for the two fungal studied, while the other solid media wheat straw did not (Tab 1). Therefore, solid wheat bran was chosen for further experiments. The cellulase activities (FPA and endoglucanase) of *Trichoderma longibrachiatum* were 33.83 U/gds and 167.4 U/gds, respectively, which represented a 1.75-fold improved activities than that of *Aspergillus terreus* (21.5 U/gds and 95.82 U/gds, respectively). Wheat ban resulted in a favorably high production of endoglucanase (19.2 U/g) by the mutant *Aspergillus sp.* SU 14 (Vu et al., 2011). However, Nara et al., (2012) obtained 9.73 U/gds of filter paper activity from *Aspergillus terreus* cultivated on rice straw under solid state fermentation.

Table1 Cellulase production on several solid media by *Trichoderma longibrachiatum* and *Aspergillus terreus*.

	Trichoderma longibrachiatum **Enzyme activities (U/gds)**		***Aspergillus terreus*** **Enzyme activities (U/gds)**	
Substrate	**FPA**	**Endoglucanase**	**FPA**	**Endoglucanase**
Wheat bran	33.83	167.4	21.5	95.82
Wheat straw	8.11	37.03	8.2	28.3

The use of wheat bran for cellulase production have been previously reported (Kang et al., 2004 ; Ikram-ul-Haq and Khan, 2006; Vu et al., 2011 ; Maurya et al., 2012). Soluble enzyme inducers are reported to be generally weaker in their inductive power when compared to insoluble substrates (Margaritis and Merchant, 1986).

Effect of initial moisture level of the medium on cellulase production

Moisture content is generally considered to be a crucial factor that affects oxygen transfer and nutrient accessibility for cell growth and enzyme production under SSF, which determines the outcome of the process. As shown in figure 1, the optimum initial moisture level was 70% for cellulase (APF and endoglucanase) production (51.15 U/gds and 262.47 U/gds, respectively, with *Trichoderma longibrachiatum*, again 23.48 U/gds and 113.56 U/gds, respectively, with *Aspergillus terreus*), that was much higher than obtained at other moisture levels.

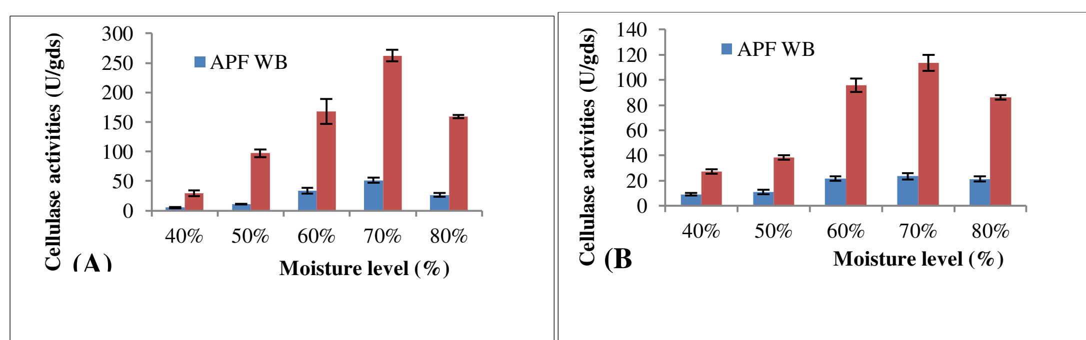

Figure 1 Effect of moisture level on cellulase production on wheat bran (WB) by *Trichoderma longibrachiatum* **(A)** and *Aspergillus terreus* **(B)**, after 7 days at 30°C. The values are means ± SD, n = 3.

High moisture encourages fungal growth, nutrient transportation and enzyme activities, but limits oxygen transfer and facilitates contamination (Mekala et al., 2008 ; Mrudula and Murugammal, 2011). Optimum moisture content may also be influenced by properties of the substrate such as porosity and particle size (Mekala et al., 2008 ; Maurya et al., 2012).

Moisture contents below 40% or above 70% were not suitable for high cellulase production. In SSF, moisture level plays an important role in biosynthesis and secretion of many kinds of enzymes, especially cellulases. Very high moisture content in solid medium results in decreased substrate porosity as well as reducing oxygen penetration among the substrate particles (Vu et al., 2010), but excessively low moisture levels in solid medium leads to poor microbial growth, poor development and poor accessibility to nutrients (Vu et al., 2010). Our results are in good agreement with those indicated by Sun et al., (2010) for the production of cellulase by *Trichoderma sp on apple pomace*, in which the optimum initial moisture level was 70%. Similarly, the maximum yield of endoglucanase 2.29 U/ ml was obtained at 70% moisture level of wheat bran with *Trichoderma reesei* NCIM 992 (Maurya et al., 2012). In contrast, Vu et al., (2011) reported that a 50% moisture content in wheat bran resulted in higher cellulase production (76.6 U/g) by the mutant *Aspergillus sp.* SU 14. Unlike, Singhania et al., (2006) reported a maximum yield of filter paper activity produced by *Trichoderma reesei* NRRL 11460 at 66.4% initial moisture content of pre-treated sugar cane bagasse.

Time course of cellulase production

As indicated in figure 2, the enzyme activities (APF and endoglucanase) with the two fungal studied, increased progressively with the incubation time and reached their maximum at 72 hours. After 72 hours, the enzyme activities began to decrease. Enzyme activities of *Trichoderma longibrachiatum* were maximum with respective activities of APF and endoglucanase measured at 180 U/gds and 1087,3 U/gds, while that of *Aspergillus terreus* were maximum with respective values (APF and endoglucanase) of 48,6 U/gds and 221,3 U/gds.

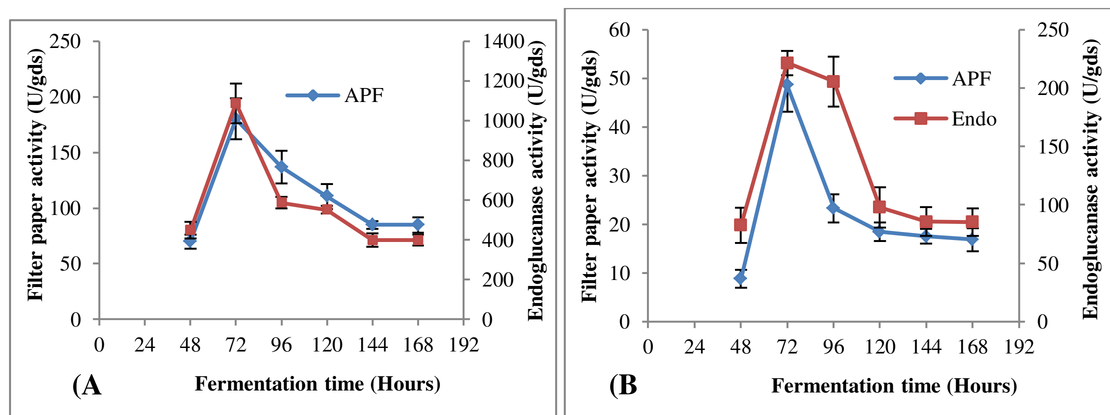

Figure 2 Effect of fermentation time on cellulase (APF and endoglucanase) production with *Trichoderma longibrachiatum* (A) and *Aspergillus terreus* (B). The values are means ± SD, n = 3.

Longer fermentation times were not explored as they were felt to be commercially insignificant. The length of incubation period is a prime concern for the development of a commercial cellulase production process and 7 days may not be viable (Abdullah et al., 2016).

The organisms have different period for optimal cellulase yield. The time was shorter when incubated on pure cellulose compared to the cellulosic waste materials. Time course required to reach maximum level of cellulase activity may be affected by several factors, including the presence of different ratios of amorphous to crystalline cellulose (Ogel *et al*., 2001). Various studies showed that maximum cellulase production from *Trichoderma reesei* could be achieved within 72-96 hours, the optimal was at 72 hours using cassava bagasse, wheat bran or rice straw (Singhania et al., 2006). Similarly The highest production of endoglucanase (28.31 U/g) was observed after 3 days of fermentation with the mutant *Aspergillus sp.* SU 14 (Vu et al., 2011). Likewise, Cellulase were produced from *Aspergillus niger* KK2 at 120 h incubation (Kang *et al*., 2004). Also, cellulolytic enzymes were produced by *Aspergillus phoenix* at 120 h incubation (Dedavid *et al*., 2008). The maximum endoglucanase activity of *Trichoderma reesei* NCIM 992, was obtained by SSF on wheat bran yielding 2.63 U ml-1 during incubation time of 6 days (Maurya et al., 2012). A

requirement of 3 days of cultivation for the production of cellulase under SSF of corn-stover and wheat-bran by *Aspergillus niger*, *Trichoderma reesei* and *Penicillium oxalicum* was reported by Gong et al., (2015) indicating that many fungal species take longer periods to produce cellulases under SSF of crude substrates. However, Gao et al. (2008) mentioned 96 h as the optimum duration for the cellulase production from a thermoacidophilic strain of *Aspergillus terreus* M11.

Thermal stability

Thermostability was tested by preincubating the crude enzyme of *Trichoderma longibrachiatum* for 60 minutes at various temperatures (60°C and 70°C), and the remaining activity was measured by standard cellulase assays conditions. The filter paper activity (APF) was more affected by the incubation temperature. At 60°C, after 1 hour of incubation, only 9% of the original activity was retained, at 70°C, about 99% of the APF was lost after 1 hour incubation (figure 3A). However, the endoglucanase was stable at 60 and 70°C, and the total activity retained was 57% and 52.5% respectively, after 1 hour incubation at these temperatures (Figure 3B).

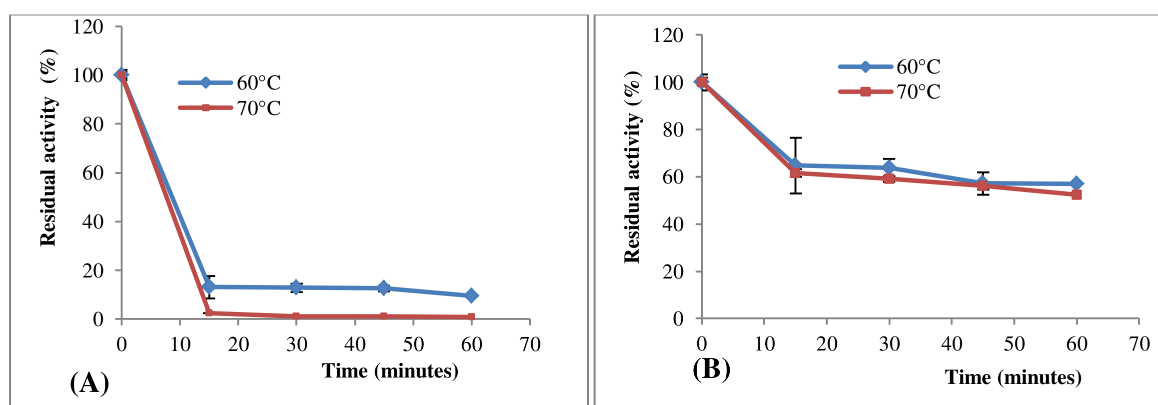

Figure 3 The thermostability of filter paper APF (A) and endoglucanase (B) activities produced by *Trichoderma longibrachiatum* at 60°C and 70°C. Data are presented as means±SD, n=3.

From these results, it seems that the endoglucanase activity of *Trichoderma longibrachiatum* was shown as thermostable. 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). Besides, thermostable cellulolytic enzymes have great potential to be used in industrial processes such as food processing, textiles, bioconversion and cellulose saccharification processes (Bhat and Bhat, 1997; Murray et al., 2004 ; Hagerdal et al., 1980).

Conclusion

The wild strains of *Trichoderma longibrachiatum*, *Aspergillus terreus* are capable of producing cellulases from wheat bran and wheat straw. Of the two cellulosic materials, wheat bran recorded the highest yield of the enzyme for the two organisms. Wheat bran is therefore the most suitable low-cost substrate for cellulase production. The waste cellulosic material is a potentially useful for commercial cellulase production. Its use will undoubtedly result in production of cheaper cellulase for the transformation of the huge waste cellulosic materials available in our environment. Initial moisture level of the medium and incubation time influenced the cellulase production greatly. The optimum initial moisture level and incubation time were 70% and 72 hours of incubation, respectively. The endoglucanase of *Trichoderma longibrachiatum* was thermostable and showed a half-life of 1 hour at 70°C, while the filter paper activity (APF) lost its total activity after 1 hour at 70°C. From this findings, the strategy to produce cellulase from wheat bran under solid state fermentation, was successful as it resulted in a considerably good amount in cellulase enzymes produced by newly strain *Trichoderma longibrachiatum* under laboratory conditions.

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