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Trichoderma Reesei Cellulase Produced by Submerged Versus Solid State Fermentations

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Abstract. The aim of this study was to produce and characterize a cellulase-rich fraction using submerged or solid state fermentation of *Trichoderma reesei (QM 1914)* strain. The carbon sources were the wheat bran or sawdust, the production yield of this enzyme production was higher in both fermentation types using sawdust substrate, and especially by solid state fermentation, after five days of fermentation. The optimum pH and temperature for the efficient crude enzyme production was established to be 5 and 60°C, respectively, but lost 50% of its activity after 30 minutes, when heated at 60°C. Comparatively with other fungi, the efficiency of *Trichoderma sp*. to synthesize cellulase rich extract was higher.

Keywords: enzymes, cellulase submerged fermentation, solid state fermentation, *Trichoderma reesei*.

INTRODUCTION

The filamentous *Trichoderma reesei* is an important fungus used to produce enzymes by fermentation. It secretes high amounts of cellulase and hemicellulase enzymes capable of degrading plant cell wall carbohydrate polymers (Vitikainen et al., 2010).

Nowadays, all industrial strains, used to prepare cellulases derive from Solomon Islands, the original isolate *T. reesei QM6a*. The initial strain improvements, to increase cellulase yields were based on classical mutagenesis techniques using UV light and mutagenic chemicals followed by growth on selective media (Seidl and Seiboth, 2010).

Solid-state fermentation has gained renewed interest from researchers in recent years and is often employed for the production of enzymes due to economical and practical advantages, such as simplicity, low capital costs for equipment and operating, high volumetric productivity, lower space requirements, and easier downstream processing (Assamoi et al., 2008).

Fungi are able to degrade cellulose, hemicellulose and lignin by a complex set of excreted hydrolytic and oxidative enzymes which degrade plant tissues (Abd El-Zaher, 2010). The differentiation of taxa of the cellulase producing strains in the *Trichoderma genus* is difficult and confusing. Fungi belong to one of the five kingdoms in ecosystem, which was defined by Robert Whittaker based on the way of taking nutrients into the cells (Whittaker, 1978).

Trichoderma strains have been used in cellulase production for two decades. The rich cellulase-producing strains were soon isolated, including the strain *Trichoderma viride QM6a* first selected from a soil sample at Bougainville Island. *T. viride QM6a* was subjected to genetic improvement for cellulase production. *T. reesei Rut C30* was a hyper-producing strain selected after UV light treatment (Montenecourt and Eveleigh, 1979).

Cellulases are distinguished from other glycoside hydrolases by their ability to hydrolyze specifically the β-1,4-glucosidic bonds. The enzymatic degradation of β-1,4-linkages in cellulose polymer result by acid hydrolysis. Hydrolysis products may result from a

process of inversion or retention of anomeric configuration of the C1 reducing end (Withers, 2001).

Natural cellulosic substrates (especially of plant cell walls) are polysaccharide chains composed of different, with different degrees of crystalinity and microfiber morphology (Bădărău and Neamtu, 1998). To degrade these materials, organisms produce a number of enzymes, generically called enzymatic systems (Warren, 1996).

Cellulase systems are not just a cluster of enzymes (endoglucanases, exoglucanases and β-glucosidase with or without carbohydrate-binding module), each one acting in a coordinated manner to a more efficient hydrolysis of cellulose (Teeri et al., 1997) from different substrates such as cotton fiber or textiles (Chi-Ming Lo, 2008).

The aim of our study was to prepare a cellulase crude enzyme from *Trichoderma reesei (QM9414)* using comparatively a submerged and solid state fermentation.

MATERIALS AND METHODS

Strain, media and fermentation conditions

The strain *Trichoderma reesei (QM 1914),* (Figure.1) was obtained from the Walloon Centre of Industrial Biology (Gembloux Agro-Bio Tech, Bioindustries Unit, Belgium).

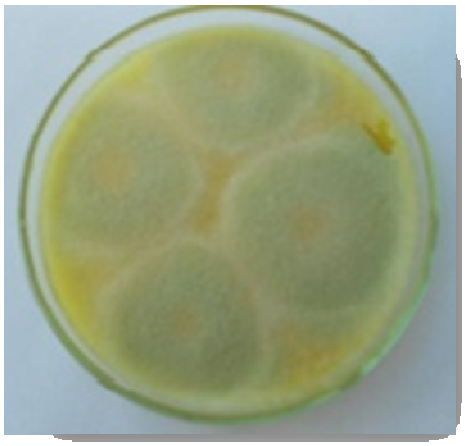

Fig. 1. *Trichoderma reesei (QM 1914)* strain.

The culture medium contained (g/l^{-1}): carbon source 50, yeast extract 10, glucose 10, $(NH_4)_2SO_4$ 1.4, KH_2PO_4 2, $CaCl_2 \cdot 2H_2O$ 0.4, $MgSO_4 \cdot 7H_2O$ 0.3, $FeSO_4 \cdot 7H_2O$ 0.005, $CoCl_2 \cdot 6H_2O$ 0.0037, $MnSO_4 \cdot H_2O$ 0.0016, $ZnSO_4 \cdot 7H_2O$ 0.0014 (Aftab and Vermette, 2008).

All chemicals used were of reagent grade (supplied by either Merck or Fluka) and used as supplied. For this study the carbon source was wheat bran and sawdust, particle size < 100 mm.

For the submerged fermentation (**SmF**), the medium was autoclaved for 20 min at 121°C and, after cooling, inoculated with a spore suspension (1.4 x 10^7 spores/g dry matters). Fermentation was carried in 250 ml Erlenmeyer flask by taking 50 ml medium at 30°C at 100 rpm.

The fermentation medium for solid state fermentation (**SSF**) had the same composition like the medium for submerged fermentation, with initial moisture values at 70%. The fermentation evolution was examined from 24 to 144 hrs.

The substrate selection to stimulate the enzyme biosynthesis in solid state fermentation took into account the following factors: a cheap agro-industrial product,

available at any time and without storage restrictions. The wheat bran was purchased from a specialized shop in selling agro - industrial products and sawdust was obtained from supermarket (bedding for animals).

Characterization of the optimum conditions for the cellulase crude enzyme activity

The total activity of cellulase was measured by hydrolyze of filter paper (Whatman nr.1,1x6 cm^2) using a standard assay method (Ghose T. K., 1987), based on the quantification of the final product, glucose. Shortly, the enzyme suspension was centrifuged and 0.5 ml supernatant was mixed with 1 ml of buffer solution, i.e., 0.05 M sodium citrate pH 4.8, in graduated test tube. A piece of Whatman filter paper (50 mg) was placed and submerged in the solution on the bottom of test tube. The test tube was incubated in a hot water bath for 60 min at 40°C. After the incubation, the total reducing sugar analysis was performed by the DNS method, for measuring the released glucose concentration. One unit of enzyme activity (U) was defined as the amount of enzyme which released one mmol of glucose per minute. Generally, the activity of cellulase was presented as FPU/ml, also known as U/ml.

The activity dynamics of cellulose obtaining during SSF was determinate using two substrates (sawdust and wheat bran).

To find the optimum conditions for the biosynthesis of cellulose obtained in solid state fermentation, we measured the activity on the pH range from 3 to 8 and the temperature range from 20 to 70°C.

Determination of optimal pH. In order to determine the optimum pH value for the crude enzyme obtained after fermentation, the activity of the enzyme was assayed between the pH values of 3.0 and 8.0.

Determination of optimal temperature. The crude enzyme was incubated with the substrates at different temperatures ranging from 20 to 70°C. The reaction mixtures were analyzed for cellulase activity.

Stability of the enzymatic activity at temperature (60°C). The crude enzyme was allowed to stand at 60°C for different durations (from 10 to 60 min), and the activity was assayed by the usual procedure.

RESULTS AND DISCUTIONS

Crude cellulase activity obtained on sawdust and wheat bran, by SmF and SSF

The activity of cellulase production in SmF, using sawdust and wheat bran as carbon sources are shown in Figure 2.

The influence of substrate on enzyme production is evident only after 96 hours of incubation. It can be seen that for wheat bran culture medium, maximum production was reached the 4^{th} day, while on sawdust medium the enzyme production was increasing until the last day of incubation (144 hrs).

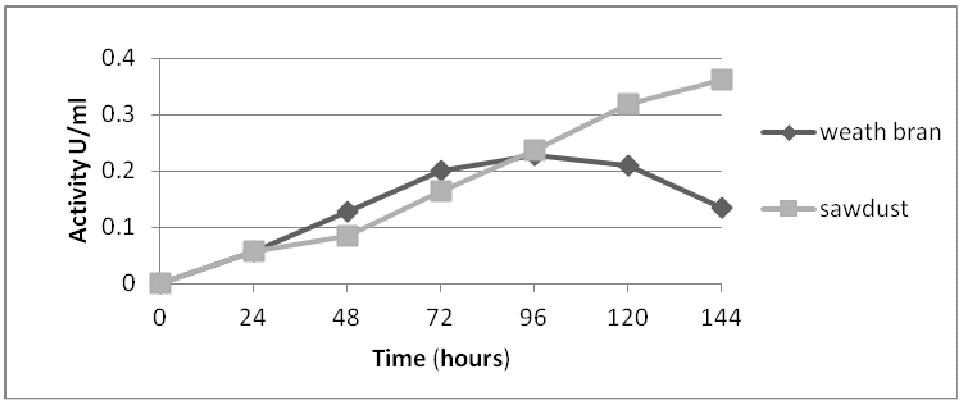

Fig. 2. Dynamics of enzymatic activity during submerged fermentation (144 hrs) on wheat bran and sawdust substrate.

Sawdust and wheat bran was also used as carbon source to obtain cellulase in solid state fermentation. To evidence the dynamics of enzymatic activity on solid fermentation (Figure 3) we used two types of substrates: CMC (carboxymethylcellulose) and FP (filter paper - Watman nr.1). Using CMC and FP we also evaluate the affinity of crude enzyme for these two substrates.

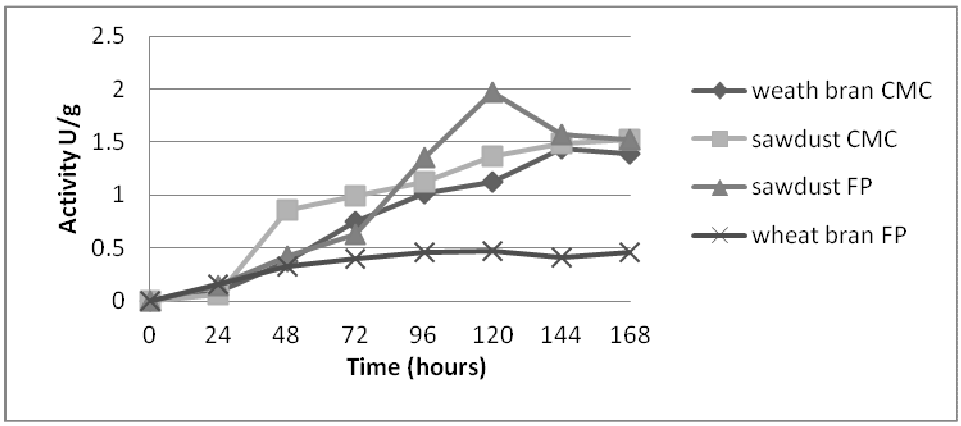

Fig. 3. Dynamics of enzymatic activity during solid state fermentation (168 hrs) on wheat bran and sawdust substrate. Carboxymethylcellulose (CMC) and filter paper (FP) were used as enzymatic substrate to evaluate the affinity of crude enzyme.

It can be seen that in both cases production begins from the first day of incubation. The crude enzyme obtained in medium with sawdust had the highest activity (1.971U/g) in 120 hours, then the activity decreased, gradually.

The solid state fermentation had a higher efficiency (1.971 U/g) compared to that of submerged fermentation, for cellulase production.

Also cellulase obtained on sawdust substrate has a higher selectivity for FP substrate than cellulase obtained on wheat bran substrate. The enzyme obtained on wheat bran substrate has a good affinity for CMC.

Latifian et al., (2007) obtained the maximum activity (1.1635 U/g) using rice bran like substrate and in same conditions for incubation. Yang et al. (2004) found similar results for a microbial consortium of *T. reesei* (AS3.3711), *Aspergillus niger* (3.316 U/g) and *Saccharomyces cerevisiaes* (AS2.399) on rice chaff in SSF. Similar results were also obtained using municipal solid waste residue and *Aspergillus niger*, with the maximum activity of exoglucanase (1.64U/g) and endoglucanase (1.84U/g) after 4 days (Gautam et al., 2011).

Characterization of the optimum conditions for the crude enzyme activity preparation.

Determination of the optimal pH (Figure 4.) was determined in the range from pH 3 to 8. pH 5 was found to be the optimum, at 45°C, with an enzymatic activity of 1.21 U/g.

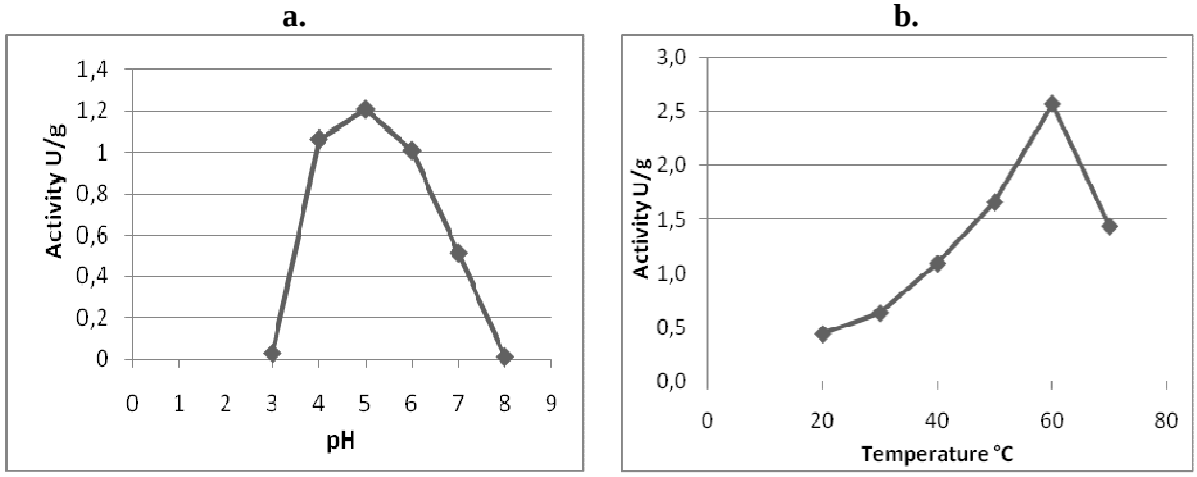

Fig. 4. Cellulase activity dependent on the pH (a.) and temperature (b.).

As presented in figure 4b. it can be seen that the temperature had a high influence on the enzymatic activity. For the cellulase obtained by sawdust fermentation, the optimal temperature was 60°C. At this temperature we obtained an enzymatic activity of 2.51 FPU/g, similarly to other previous reports. The endoglucanase maximum activity from *B. pumilus* EB3 was when incubation was done at 60°C (Ariffin et al., 2006; Ozaki, and Ito 1991; Onsori et al., 2005). The results reported for *Trichoderma* and *Aspergillus ssp*. has an optimum temperature of 40-55°C, were also close to our findings (Tangarone et al., 1989; Kotchoni et al., 2003).

Stability of the enzymatic activity at 60°C is shown in figure 5. In 30 minutes at 60°C the enzymatic activity decreased below 50%.

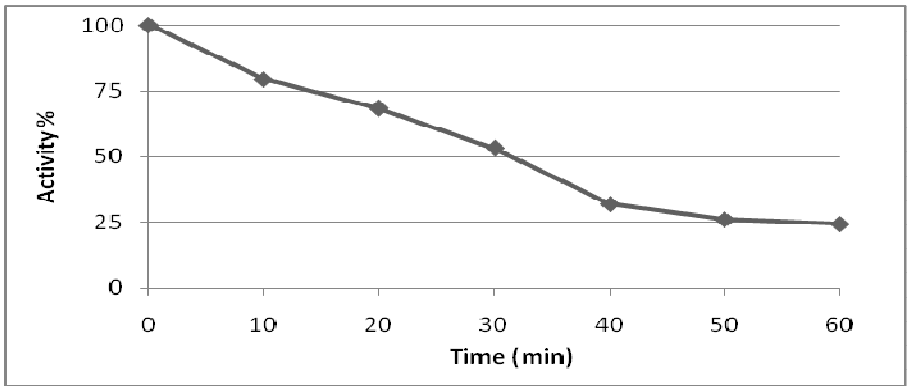

Fig. 5. Evolution of enzyme activity (FPU/g) at 60°C, depending on time (up to 60 min).

CONCLUSION

A rich cellulase crude enzyme preparation was obtained from *Trichoderma reesei (QM9414)* by a submerged or by solid state fermentation, using wheat bran or Sawdust waste as carbon sources.

The solid state fermentation had a higher efficiency compared to that of submerged fermentation, and the incubation time to obtain was around 144 hrs. Sawdust induced a higher cellulase production, especially by solid state fermentation, during five days. The optimum temperature was 60°C and pH at 5, to have the highest production yield and activity.Its activity decreased bellow 50% after 30 min of incubation at 60°C. This crude cellulase preparation obtained by the cheaper solid state fermentation procedure represents a good alternative for industrial applications and biorefinery using hydrolytic enzymes.

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