

Cellulase Production by *Trichoderma reesei* (CBS383.73) and an Isolated *Botrytis* Strain Using Several Agricultural Wastes

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Abstract

Variation in the composition of the production media were investigated to optimize the excretion of the cellulolytic enzymes by *Trichoderma reesei* (CBS383.73) and a *Botrytis sp.* isolated from the microflora of Iran. The culture filtrate of *Trichoderma reesei* showed a cellulolytic activity of 300 mFPU/ml after 13 days of growth in a medium containing walseth cellulose (0.5%, w/v) at pH 5. Under similar production conditions, except for the initial pH of 7, the culture filtrate of *Botrytis sp.* showed an activity of 360 mFPU/ml. Replacement of walseth cellulose with H₂O₂ – treated bagasse, Barely husks, wheat husks, or sawdust (0.5% w/v) in the presence of the inducer (0.5% w/v, walseth cellulose) enhanced the extent of enzyme production by *Trichoderma reesei* to 780 mFPU/mL. The *Botrytis sp.* did not excrete cellulolytic enzyme using (as the main sources of carbon), the H₂O₂-treated bagasse or sawdust plus 0.5% walseth cellulose. However, the culture filtrate of the *Botrytis sp.* showed a cellulolytic activity of 679 or 463 mFPU/ml using the H₂O₂-treated wheat or barely husks (1% w/v) plus 0.5% walseth cellulose, respectively. In addition, the Calcium hydroxide treatment of the agricultural wastes depressed totally the enzyme secretion by the *Botrytis sp.* except for the treated sawdust which enhanced the enzyme production almost by a factor of seven in the absence of the inducer.

Keywords: Agricultural wastes, *Botrytis sp.*, Cellulase, *Trichoderma reesei*.

Introduction

Cellulose is the world's most abundant natural biopolymer and a potentially important source for the production of industrially useful materials such as fuels and chemicals. Degradation of the cellulosic materials is achieved either chemically, enzymatically, or by the combination of both chemical and enzymatic methods (Bailey *et al.*, 1987; Christov *et al.*, 1999; Haltrich *et al.*, 1996; Paice *et al.*, 1987; Saxena *et al.*, 1991; Spreinat *et al.*, 1990 and Xia *et al.*, 1999). Chemical methods produce more by products and they are performed at high temperatures compared to the enzymatic hydrolyses which are mostly performed at low temperature and pH. In addition, chemical degradations of the cellulosic materials, due to the environmental problems, are unfavorable and uneconomical approaches. Therefore, extensive effort has been made to make cellulose hydrolysable under mild conditions. In spite of huge extent of research for finding more active enzyme preparations from a large variety of microorganisms, the enzymatic saccharification of cellulose, so far, has not reached to the level of conversion of starch to glucose by the microbial enzymes. Thus, much work and research is needed to produce enzymes capable of saccharifying plant materials. At the present, the promising microorganisms include aerobic fungi, e.g. *Trichoderma sp.* (Okada, 1976), anaerobic fungi e.g. *Neocallimastix sp.* (Bar *et al.*, 1989), aerobic bacteria, e.g. *Thermomonospora sp.* (Moreita *et al.*, 1981) and anaerobic bacteria, e.g. *Clostridium sp.* (Coughlan *et al.*, 1985).

Due to presence of huge amount of annual agricultural wastes with no effective and systematic industrial application, it is economically ideal to apply these endless source for cellulolytic enzyme productions and other industrially important chemicals. Therefore, the objective of this investigation is to look for better conditions of growth and enzyme productions for one of the high producing fungi isolated from the microflora of Iran (Zolnorian *et al.*, 2000) and *Trichoderma reesei* using four different types of agricultural wastes, with and without chemical modifications.

Materials and methods

Chemicals: All chemical reagents, of the best grade available, were purchased from Merck (Darmstadt), and Aldrich (England) and were used without further purification. Filter paper (Whatman No.1) was used for assaying the cellulolytic activities of the culture filtrates. Walseth cellulose (H₃PO₄-swollen cellulose) was prepared from Avicel PH101 according to the method of Wood (1978) and used immediately for media preparations without any storage. The fungus *Trichoderma reesei*, CBS383.78, was obtained from "Iranian Research Organization for Science and Technology", and the *Botrytis* sp. was isolated from microflora of Iran as described previously (Zolnorian *et al.*, 2000). Wheat and barely husks, bagasse and sawdust were purchased from local shops.

Inoculum preparation: Basal agar plates of the following composition (Almin *et al.*, 1975) were inoculated with the microorganisms (g/l): (NH₄)₃PO₃, 0.85; KH₂PO₄, 0.6; K₂HPO₄, 0.4; MgSO₄. 7H₂O, 0.5; FeSO₄. 5H₂O, 0.01; ZnSO₄. 7H₂O, 4.4; MnSO₄, 0.0025; CaCl₂.2H₂O, 0.0055; Yeast extract 1.0; CoCl₂, 0.001; Thiamine. HCl, 0.1; walseth cellulose, 20, agar, 15. The plates were incubated at 25°C for 7 days, at which time a good spore crop was evident. Two milliliters of sterile water was added to the plate, swirled about gently, and then withdrawn. The spore suspension (1 ml) was used to inoculate the preculture flasks containing 20 ml of the medium with the following composition (g/l): (NH₄)₂SO₄, 1.4; KH₂PO₄, 2.0; MgSO₄. 7H₂O, 0.3; CaCl₂.2H₂O, 0.4; Urea, 0.3; peptone, 1.0; Tween 80, 0.2; FeSO₄. 7H₂O, 0.005; MnSO₄. H₂O, 0.0016; CoCl₂.6H₂O, 0.002; ZnSO₄. 7H₂O, 0.0014; Xylose, 10. The pH was adjusted to 5.0 prior to sterilization. The flasks were incubated in an incubator/shaker at 29°C and 130 rpm for 3 days. Aliquots of these inocula were transferred to flasks containing the production media as it follows (Almin *et al.*, 1975).

Cellulase Production: Two milliliter aliquots of the above inoculated preculture medium, was transferred to 100 ml of the production medium. The chemical composition of the production medium is exactly like that of preculture media except that walseth cellulose (10%, w/v) or treated agricultural wastes were substituted for

xylose. The inoculated flasks were incubated in the incubator shaker at 29°C and 130 rpm and at different time intervals, aliquots were withdrawn for assaying the corresponding cellulolytic activities.

Treatment of the agricultural wastes

H₂O₂- treatment: One gram of the partially milled agricultural material was mixed in 50 ml of 1% (V/V) solution of H₂O₂ and the pH was adjusted to 11.5 using NaOH(0.1 N). The mixture was kept at room temperature while mixing for 18 hours. The mixture was then filtered and washed thoroughly with distilled water to neutrality (Ramos, et al., 1993).

Ca(OH)₂-treatment: Five grams of the partially milled agricultural waste was mixed with 100 ml Ca(OH)₂ solution (2% W/V). The mixture was mixed at room temperature for 18 hours and then it was filtered and washed thoroughly with distilled water to neutrality (Ramos, et al., 1993).

Analyses

pH: Samples from each inoculated medium were withdrawn at different time intervals. Centrifuged at 20,000 rpm for 15 min. An aliquot was then taken for pH determination and the remaining filtrate was used for the enzyme assays.

Enzyme assay: Crude enzyme broth of each microorganism was used in all hydrolysis experiments and the cellulolytic activity was measured by the filter paper assay and the activity was expressed as millifilter paper unit per milliliter of the broth (mFPU/ml) according to Mandel's method (Mandels, 1975). An aliquot of the centrifuged culture media was incubated with a 1x6 cm (50 mg) strip of Whatman no. 1 filter paper for two hours at 50°C. The reducing sugars liberated were measured by dinitrosalicylic acid method (Miller, 1959) or by the Symogyi-Nelson method (Somogyi, 1952; Nelson, 1952). Results were expressed as millifilter paper units (mFPU) defined as (μmol reducing sugar (glucose equivalent) produced per minute.

Results and Discussion

The effects of different walseth cellulose concentrations on the patterns of cellulolytic enzyme productions by *Trichoderma reesei* and the isolated *Botrytis sp.*, at 29°C were reported previously (Zolnorian *et al.*, 2000). According to this report, It appeared that a cellulose concentration of 1% is suitable for enzyme productions by both fungi.

The effects of substituting various agricultural wastes for the walseth cellulose, were investigated for *Trichoderma reesei* (at initial pH of 5) and the *Botrytis sp.* (at initial pH of 7). These pH values have been established to be the best pHs for the optimal production of the cellulolytic enzyme by these two microorganism (Zolnorian *et al.*, 2000). The agricultural wastes, including barely, wheat husks, bagasse and sawdust were treated before use with hydrogen peroxide (1%, v/v) or calcium hydroxide (2% , w/v). Enzyme secretion was not observed in the production media made of untreated agricultural wastes as their sole carbon source (data not shown). The enzyme productions by both microorganisms were not satisfactory in the absence of an inducer such as walseth cellulose (Figures 1 and 2, a-d). However, as it is evident from (Fig.1, a-d), the production media containing any one of the H₂O₂-treated agricultural wastes along with the inducer (0.5% w/v) enhanced the enzyme production by *T. reesei* almost by a factor of five. The corresponding pH profiles (Fig. 1, e-h) indicate that, in the first few days of growth, there is generally a slight decrease in pH followed by a gradual increase to a maximum of 7.0. The only exception is observed for the medium made of bagasse in which the pH of the medium increased slightly in the first week of growth. In contrast to the enhancement observed in the extent of enzyme secretion by *Trichoderma reesei* on H₂O₂- treated agricultural wastes, calcium hydroxide treatment of wheat, Barely, and bagasse did not significantly, increased the enzyme production by *Trichoderma reesei* (Figure 1, a-c), but the microorganism was still capable of secreting cellulolytic enzyme using Ca(OH)₂ – treated sawdust in the presence of 0.5% of the inducer (Fig.1, d).

The effects of chemically treated – agricultural wastes on the extent of cellulolytic enzyme production by the *Botrytis sp.* was also

Figure 1 - The effects of agricultural wastes on the extent of cellulolytic activities of the culture filtrates (a-d), and the corresponding pH profiles (e-h) of the *Trichoderma reesei* at different time intervals. (a, e), wheat husk; (b, f), barely husk; (c, g), bagasse; (d, h), sawdust; H₂O₂-treated (?); Ca(OH)₂-treated (?); H₂O₂-treated plus 0.5% walesth cellulose (?); Ca(OH)₂-treated plus 0.5% walesth cellulose (?).

investigated at 29°C at the initial pH of 7. Similar to *T. reesei*, the *Botrytis sp.*, did not produce cellulolytic enzymes using untreated agricultural wastes as the only source of carbon. In addition, the extent of enzyme production in the media of the treated agricultural wastes was no significant in the absence of an inducer (Fig. 2, a-d). Addition of walsyth cellulase (0.5%, w/v) to the media increased the cellulolytic activity in the production media made of H₂O₂ – treated wheat and barely husks by almost a factor of four. There was no cellulolytic activity in the culture filtrate of the medium made of H₂O₂ – treated bagasse (Fig. 2, c) and the enzyme secretion in the medium of H₂O₂–treated sawdust was very low (with and without the inducer) and Ca(OH)₂–treated sawdust in the presence of the inducer. However, in the absence of the inducer the production rate increased by almost a factor of seven (Figure 2d). Therefore, it seems that wheat and barely husk treated with H₂O₂ solution (1%) are good carbon sources for the enzyme production by the *Botrytis sp.*, compared to expensive walsyth cellulose. In addition, sawdust treated with Ca(OH)₂ without need to an inducer, can increase the production of the enzyme by the *Botrytis sp.*, and it seems that for *T. reesei* each of the four agricultural wastes, treated with 1% H₂O₂ solution, are fairly good substitutes for the walsyth cellulose.

Conclusion

The effects of four different agricultural wastes on the extent of cellulolytic enzyme production by *Trichoderma reesei* and an isolated *Botrytis sp.*, were evaluated. The agricultural wastes were subjected to H₂O₂ (1%) or Ca(OH)₂ (2%) treatments before use. Replacement of walsyth cellulose from the production media with the H₂O₂ – treated husks (wheat and barley) bagasse, or sawdust, along with 0.5% walsyth cellulose enhanced the extent of enzyme secretion by *Trichoderma reesei* almost by a factor of two. However, enzyme production was totally quenched in the absence of the inducer. Calcium hydroxide-treated sawdust was also a good source for enzyme production by *Trichoderma reesei*. Similarly, H₂O₂ - treated

Figure 2 - The effects of agricultural wastes on the extent of cellulolytic activities of the culture filtrates (a-d), and the corresponding pH profiles (e-d) of the *Botrytis* sp. at different time intervals. (a, e), wheat, (b, f), barely husk, (c, g), bagasse, (d, h), sawdust, H₂O₂-treated (?); Ca(OH)₂-treated (?);

H₂O₂-treated plus 0.5% walseth cellulose (?); Ca(OH)₂-treated plus 0.5% walseth cellulose (?).

wheat husk and barely along with 0.5% walseth cellulose enhanced enzyme production by the *Botrytis sp.* almost by a factor of three, but the enzyme production by Ca(OH)₂ -treated bagasse, with or without the addition of 0.5% walseth cellulose, was totally quenched. However, Ca(OH)₂- treated sawdust increased the level of the enzyme production by almost a factor of seven without the need for the inducer. Further research is in progress to improve the cellulolytic enzyme production based on the agricultural wastes as the main carbon source.

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