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Some Production Comparisons of Two Celluloytic Fungi

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Abstract

Eighteen fungal strains, with cellulolytic capabilities, were isolated from the microflora of Iran. All of the isolated fungal strains produced, under the preliminary conditions of growth and enzyme production, higher extracellular cellulolytic enzymes relative to *Trichoderma reesei*. For one of the isolated fungal strains, which turned out to belong to the Botrytis family and *Trichoderma reesei* optimized growth conditions were separately established. Maximum enzyme production for *T.reesei*, 400 mFPU/ml was achieved during 13 days of growth in a citrate/phosphate buffered media (pH=5) at 29°C. However, the isolated *Botrytis sp*. produced maximally 670 mFPU/ml by day 15th of growth at 29°C and in an unbuffered citrate system (pH=7).

Key Words*: Botrytis sp. cellulose system, screening, 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(Spreinat *et al*.,1990; Saxena *et al*.,1991; Xia *et al*.,1999; Paice *et al*.,1987; Bailey *et al*.,1987; Haltrich *et al*.,1996; Christov *et al*.,1999). Chemical methods produce

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more byproducts and they are performed at high temperatures compared to the enzymatic hydrolyses, which are technologically important approaches. In addition, chemical degradations of the cellulose materials, due to the environmental problems, are unfavorable and uneconomical approaches. Therefore, extensive effort has been made to make cellulose economically hydrolyzable 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 been 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 present, the promising microorganisms include aerobic fungi e.g. *Trichoderma sp*.(Okada, 1976) anaerobic fungi e.g. *Neocallimastix sp*.(Bar *et al.*,1989;Bauchop *et al.*,1981) aerobic bacteria e.g. *Thermomonospora sp*.(Moreita *et al*.,1981), and anaerobic bacteria [e.g. *Clostridium sp*.(Coughlan *et al*.,1985).

The objectives of this study were to screen for cellulolytic fungi in microflora of Iran and to establish the optimized conditions of growth and enzyme productions for one of the isolated fungi and *Trichoderma reesei* (CBS 383.73).

Materials and Methods

Chemicals

All chemical reagents the best grade available, were purchased from Merck(E.Merck, D-6100 Darmstadt, F.R.Germany), Sigma(St. Louis Mo, USA), or Aldrich (Gillingham, Dorset, England) and were used without further purifications. Filter paper (Whatman No.1) was used for assaying enzyme activities. Walseth cellulose was prepared from Avicel PH101 in our laboratory according to the method of wood (Wood *et al.*1978) and used immediately for media preparation without any storage. The fungus *Trichoderma reesei* CBS 383.78, was

parchased from Iranian research organization for science and technology.

Isolation method

The aerobic fungal strains were isolated by enrichment cultures. The enrichment cultures were initiated by inoculating 1 gr of the source materials (soil Collected from agricultural fields from Ardabil province of Iran) into screw-capped test tubes (1.8x18cm) each containing 10 ml of the modified Petterson medium(Almin *et al*.,1975) with the following composition (per liter, medium No.1): $(NH_4)_3PO_3$,0.89g; KH_2PO_4, 0.6 g; K_2HPO_4, 0.4 g; $MgSO_4.7H_2O$, 0.5 g; $FeSO_4.5H_2O$, 10mg; $ZnSO_4.7H_2O$, 4.4 mg; $MnSO_4$, 2.5 mg; $CaCl_2.2H_2O$, 0.55 mg; Yeast extract, 1g ; $CoCl_2.6H_2O$, 1mg; Thiamine.HCl, 100µg; walseth cellulose, 2g. The PH of the medium was adjusted to 6 prior to sterilization. The tubes were incubated at 25 °C for 5-10 days. At different time intervals 2 ml of each culture medium was transferred to an agar plate containing the medium No.1 which contains 1.5-% agar. The inoculated plates were kept at 25°C and frequently subcultured on the same agar plates up to complete purification. After 18 days of incubation at 25°C, the colonies with clear zones of cellulose digestion were selected and maintained on sabouraud dextrose agar slants and transferred monthly. Eighteen cellulolytic fungi were isolated in this investigation.

Inoculum preparation

An agar plate of medium No.1 was inoculated with an isolated cellulolytic fungus and incubated at 25°C for 7 days, at which time a good spore crop was evident. Two milliliters of sterile saline solution (0.9% NaCl) was added to the plate, swirled about gently, and then withdrawn. This spore suspension was used to inoculate preculture flasks (1 ml suspension /20 ml) containing medium No.2 with the following composition (per liter): $(NH_4)_2SO_4$, 1.4g ; KH_2PO_4, 2g; $MgSO_4.7H_2O$, 0.3g ; $CaCl_2.2H_2O$, 0.4g ; Urea, 0.3g ; Peptone, 1gr ; Tween 80, 0.2g; $FeSO_4.7H_2O$, 5mg; $MnSO_4.H_2O$, 1.6mg; $CoCl_2.6H_2O$,

0.2g; $ZnSO_4.7H_2O$, 1.4mg; Xylose, 10g. The pH was adjusted to 5.0 prior to sterilization. The flasks were incubated in an incubator/shaker at 25°C and 130 rpm. for 3 days. Aliquots of these inocula were used for enzyme production.

Analyses

pH

Samples of the 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.

Cell Mass

An aliquot of the inoculated medium, at different time, was withdrawn and kept at 4°C for 2 hours. The lighter mycelial buttons was separated from the heavier cellulose deposite by decantation. A sample of the decanted filtrate was centrifuged at 20,000 rpm. for 30 min. The supernatant was discarded and the pellet washed twice with distilled water before drying at 100°C for 24 hours.

Enzyme assay

Crude enzyme broth of each microorganism was used in all hydrolysis experiments and the cellulose activity was measured by the filter paper assay and the activity was expressed as milifilter paper unit per ml of the broth (mFPU/ml) according to Mandel's method (Mandels,1975) An aliquot of the centrifuged culture media was incubated with a 1x6cm (50mg) strip of whatman No.1 filter paper for 2 hours at 50°C. The reducing sugars liberated were measured by the dinitrosalisylic 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.

Cellulase Production

Two-milliliter aliquot of the inoculated preculture medium was transferred to 100 ml of medium No.3. The chemical composition of medium No.3 is exactly like that of medium No.2, except that walseth cellulose (0.5% w/v) was substituted for xylose. The inoculated flasks were incubated in the incubator/skaker at 25°C and 130 rpm. and assayed for their Celluloytic activities at different time intervals.

Results and Discussion

Figure 1 shows the kinetic behavior of Celluloytic enzyme production from acid treated Avicel. As it is avident from Figures 1a and 1b and under the preliminary growth conditions, almost 73% of the isolated microorganisms showed more than 50 mFPU activity per millilitter after eight days at 25°C in comparison to the strain of *Trichoderma reesei* which shows an activity of almost 100 mFPU/ml. On the other hand, only 11% (2 out of 18) of the microorganisms show higher cellulolytic activities after 14 day. Figures 1c and 1d demonstrates that the pH of the production media for almost 50% of the microorganisms decreased bellow 5 after eight days of growth and it rised slightly above 5 for the other 50% of the isolated fungi. The exception is observed for fungus no.15 which is faced with a rapid rise in the pH of the growth medium. It has been reported that pH has a pronounced impact on cellulose production and it varies depending upon the carbon source in the growth media (Cochet,1991;Kume&Fujio,1991).

Using pure cellulose, it has been shown that for *Trichoderma reesei* strains the pH usually drops from 5 to as low as 2.5 after 2 days and then keeps stable at that level for few days and rises slightly at the end (Allen &Roche, 1984). As it is evident from Figure1c, the pH of *Trichoderma reesei* strain varied from 5 to about 4 after 4 days and then rised gradually to about 4.8 and remained fairly stable up to the end.

From the comparison of the data presented in Table 1 and the graphs of Figure 1a and 1b it can be concluded that for some of the isolated microorganisms, the enzyme production profiles do not parallel the rate of cell mass growth. For example, the fungus with code no. 4, shows (per milligram of cell mass) a celluloytic activity of 1.89 mFPU and a cell mass of 17.50 mg/ml after 14 days of growth; or the other isolated fungus (code no. 16) after 14 days of growth shows a celluloytic activity of 3.90 mFPU/mg and a cell mass of 10.00 mg per milliliter.

Figure 1-Patterns of cellulolytic enzyme production from walseth cellulose by the isolated fungi and *Trichoderma reesei* CBS 383.73(a,b), and their corresponding pH profiles (c,d)?y The numbers next to each graph represent the fungi code numbers. Microorganisms presented: *T.reesei* (?—?);1,13(?----?); 2,14 (? ----?); 3,15 (?`—?`); 5,12(?`—?); 6,11(?`----?); 7,10(?`?`?`); 8,16(?`----?`); 9,18(?«--?).

Table 1 - Cell masses and the corresponding ratio of activity/cell mass (mFPU/mg) of the cultivated fungi after 14 days of growth at 25 ?C and 130 rpm. For experimental details refer to the material and method section.

Fungi code no.	**Cell mass after 14days?b**mg/ml	**Activity/cell mass after 14 days** (mFPU/mg)
1	3.00	12.00
2	2.10	6.19
3	3.75	13.33
4	17.50	1.89
5	7.00	2.85
6	7.00	12.14
7	2.50	37.60
8	4.25	16.24
9	2.00	34.50
10	2.55	49.02
11	2.50	40.80
12	3.50	23.14
13	3.50	24.29
14	3.45	7.54
15	1.50	47.33
16	10.00	3.90
17	2.25	51.50
18	4.00	3.25
T.reesei	2.00	50.00

The comparison of the ratios of activity/cell mass (mFPU/mg) of the isolated fungi with the corresponding ratio obtained for *Trichoderma reesei* strain (Table 1) demonstrates that despite the relatively good growth rates, the cellulolytic potentials of the majorities of the isolated fungi are less than that of *Trichoderma reesei* strain. In contrast to these observations, most of the isolated fungi showed higher cellulolytic activities on solid agar plates (data not presented). From these observations it may be concluded that the optimized enzyme production systems should be established for each individual microorganisms.

Better cultivation and production media were investigated for *Trichoderma reesei* CBS 383.73 and one of the isolated fungi, code no. 17, Which was characterized to belong to Botrytis family. A significant enhancement in the extent of enzyme production, almost by a factor of two, was observed for both fungi by changing the incubation temperature from 25°C to

29°C. In addition, Figure 2a indicates that enzyme production by *T. reesei* will get depressed after 10 days of growth under high concentration of walseth cellulose (more than 1% w/v) and a cellulose concentration of 1% seems to be suitable for optimal enzyme production for both microorganisms. Figure 2c and 2d shows the patterns of pH changes, for both microorganism, during 18 days of growth under different concentrations of walseth cellulose. On the other hand, another enzyme production enhancement was observed for *Trichoderma reesei* strain (about 50%) and the *Botrytis sp.* (about 33%) by adjusting the initial media pHs to 5 and 7, respectively (Figs. 3a and 4a). The patterns of pH changes during 18 days of growth at 29°C and under different initial pHs are shown in Figures 3b and 4b.

Figure 2 - Effects of pure cellulose concentration on the cellulolytic activities of the culture filtrates of *Treesei* (a) and the Botrytis strain (b); and the pattern of pH changes with respect to growth time for *T.reesei* (c),and the Botrytis strain (d). Carbon concentration: 0.5% (?Ï?Ï ?); 1% (?Ï?Ï ?Ï); 1.5% (?&?&?&); 2% (?&?&?&).

Figure 3 - The effects of inital pHs on the extent of cellulolytic enzyme productions by *Trichoderma reesei* strain (a) and the patterns of pH changes (b) during 18 days of growth at 29?C and a walseth cellulose concentration of 1%. Initial pH of the production media: 4 (?Ç?Ç?Ç), 5 (?Ç?Ç?Ç), 6(?Ç?Ç?Ç), and 7(?Ç?Ç?Ç).

Figure 4 - The effects of initial pHs on the extent of cellulolytic enzyme productions by the *Botrytis sp*. (a) and the patterns of pH changes (b) during 18 days of growth at 29 ?‡C and a walseth cellulose concentration of 1%?Initial pHs of the production media: 4 (?¯?¯?¯), 5 (?¯?¯?¯), 6(??¯?¯), and 7(?¯?¯?¯ or ??¯?¯).

The effect of buffering the production media was also investigated on the extent of enzyme production using citrate or citrate/phosphate systems. As it is evident from Figure 5a, the enzyme production by *Trichoderma reesei* strain is enhanced compared to Figure 3a by almost 66% in citrate/phosphate buffer (citrate, 24mM ; phosphate, 50mM) with initial pH of 5. On the other hand, the enzyme production was quenched significantly for the *Botrytis sp*. in both buffer systems with initial pH of 7 (Figure 5b); meaning that these buffer systems are not flexible media for enzyme production by the Botrytis strain. However, our investigation indicated that the extent of enzyme production in *Botrytis sp*. enhanced about 62% after 15 days of growth by using 0.8% NaH_2PO_4 relative to the data presented in Figure 4a (Figure 6). The enzyme production by *Trichoderma reesei* strain was slightly quenched under different concentration of NaH_2PO_4 (data not presented).

Figure 5 - The effect of citrate (50 mM) and citrate-phosphate (24mM-50mM) buffers on the extent of cellulolytic enzyme production by *Trichoderma reesei* strain at pH 5 (a) and by the *Botrytis sp*. at pH 7 (b) at different time intervals. Buffer system: Citrate (?b?b?b), Citrate-Phosphate (?b?b?b).

Figure 6 - The effect of different concentrations of NaH_2pO_4 on the extent of cellulolytic enzyme production by the *Botrytis sp.* at the initial pH of 7 at different time intervals. Phosphate concentration: 0.4% (?ŠŠ?Š), 0.6% (?ŠŠ?Š), 0.8% (?ŠŠ?Š), 1%(?ŠŠ?Š)

Conclusions

Eighteen aerobic fungi with different cellulolytic capabilities were isolated from the microflora of Iran. One of the high producing fungi was identified as a strain of Botrytis family. The cellulolytic enzyme production of *Trichoderma reesei* CBS 383.73 and the isolated *Botrytis sp*. were investigated to improve the enzyme production yield using 1% (w/v) walseth cellulose as the only carbon source in the production media. Our results demonstrate that *Trichoderma reesei* strain will produce more cellulolytic enzymes in citrate-phosphate buffer

system, pH=5, at 29°C and the *Botrytis sp*. will show higher enzyme production capability in unbuffered phosphate system (0.8% w/v) at 29°C with the initial pH of 7. From the collected data it may be concluded that the cellulolytic systems of these two fungi are totally different. Further investigation is in progress to compare these enzymatic systems in more details.

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