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Increased Production and Activity of Cellulase Enzyme of *Trichoderma reesei* by Using Gibberellin Hormone

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Abstract

Cellulolytic complex are enzymes capable of hydrolyzing cellulose. Due to rapid growth in population and industrialization, most countries are required to produce more fuel. Production of bioethanol from lignocellulosic biomass is very challenging due to environmental pollution by fossil fuels. Cellulases play a significant role in biotechnological processes. The cost of production of cellulase is very high. Approximately 50% of the cost of producing bioethanol is used to produce cellulose. Gibberellin regulates the growth of fungi and plants. Cellulase production was significantly improved by gibberellins hormone. The cellulase activity was 4.29 FPU/ml (when we used 50 μmol/ml of gibberellins in culture medium). This was 12- fold higher (0.34 FPU/ml in control solution) compare with untreated hormone and was comparable to that achieved with concentrated by n- butanol. In this experiment, the enzyme activity was increased 223- fold higher compare with control solution.

Keywords: *Trichoderma reesei*; Gibberellins; Cellulose; Butanol.

Introduction

Cellulase is one of the most useful enzymes in industry. Cellulase can be produced by fungi, bacteria or actinomycetes, but the most common producer is fungi [1]. Agricultural and industrial wastes are among the main causes of environmental pollution. Their conversion into useful products may reduce the intensity of the problems caused by them. The enzyme is used in various industries including food, animal feed, agriculture, biomass refining, pulp and paper, textile, and laundry. Increased air pollution due to the use of fossil fuels will cause the countries to produce energy from other sources, particularly bioethanol fuels. Bioethanol fuels can be produce from cellulose waste, but the most difficult part of the process is the conversion of cellulose to glucose. Cellulase convert cellulose into glucose, but this enzyme is complex and composed of several different enzymes that act synergistically. Production of cellulose from *Trichoderma reesei* reported by using substrates like cellulose, xylose and lactose [2]. Cellulases have been studied for most of the 20th century. The enzyme activity was increased by about 30 – 80 percent. Cost of cellulose production may be brought down by multifaceted approaches which include the use of cheap lignocellulosic substrates and the use cost efficient fermaentation strategies like solid state fermentation. Cellulolytic enzymes comprise three main activities: chain end- cleaving exoglucanases or exo- 1,4- β- D-cellobiohydrolases (CBH) (EC. 3.2.1.91), endoglucanase or endo- 1,4- β- D-glucanase (EG) (EC.

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3.2.1.4) and β- glucosidase or cellobiase (BGLs) (EC. 3.2.1.21) which hydrolyze soluble short- chain gluco-oligosaccharides to glucose [3 – 6]. Investigation into enzymatic degradation of cellulose has lasted about 30 years, researchers are investigating information about the structure and function of cellulose recently obtained [7].

Cellulase activity is very low and much research has been done to increase the activity of the cellulase enzyme system. Synergism between the each component of cellulolytic enzyme acting upon insoluble cellulose adds further complexity of the investigation into mechanisms and action of the enzymes. The action of each enzyme is different from the action of these enzymes in complex. The use of bioethanol fuel as an energy source is highly regarded [8]. Consequently, how to obtain strain of high cellulase activity is the backbone to improve ethanol production.

Gibberellin increased cell division and caused the activation of cell cycle from G1 to S phase. This hormone induces expression of genes which involve cyclin- dependent- protein- kinase [9]. RGA (for repressor of ga1- 3) is a likely repressor of gibberellin signaling and gibberellin signal appears to depress the gibberellin signaling pathway by degrading the repressor protein RGA. Silverstone and colleagues showed that RGA protein presented in the nucleus, when there was not gibberellin in the cells [10]. Both proteins GA1 and RGA contain a protected area at the N- terminal domain which is called DELLA (aspartic acid, glutamic acid, leucine, and alanine). This motif is involved in gibberellin responses. In this case the RGA protein is continuously synthesized, but in the presence of gibberellins, this protein is degraded. Therefore, this motif is essential for the response [11]. Gibberellin has two receptors: (1) membrane receptor which is located on the cytoplasmic membrane of cells and (2) soluble receptors in the nucleus [12]. Varner and Johner (1968) proposed that gibberellin has an effect on the transcription of RNA synthesis. Gibberellin increases synthesis of hydrolase enzymes via rough endoplasmic reticulum and regulation of the endoplasmic reticulum is the first effective site of gibberellin. Gibberellin increases the activities of phosphorylcholine cytidyl transferase, phosphorylcholine glyceride transferase, and membrane enzymes [13]. The most important research on gibberellin was its role in the regulation of protein biosynthesis. In this case, physiological or biochemical processes may be affected.

In this report we tried to increase the activity of cellulase enzyme which purified from *T.reesei* by the hormone gibberellins. Much research has been done on this hormone and it turned out that the hormone causes the growth of plants, but its function of fungi still questionable. In this experiment, different amounts of the hormone gibberellins were added to the medium containing *T. reesei* resulting in the increased protein content of the medium. Add gibberellin to the medium containing *T. reesi* increase the activity of cellulase. The results are reported on the first time for this fungus. For purification of cellulase enzyme, we used "Vroman effect". This method was previously experienced [14].

Materials and Methods

Material: *Trichoderma reesei* strain CBS 383.78 was obtained from the Iranian Research Organization for Science and Technology (Tehran, Iran). DNS (3, 5-dinitrosalicylic acid), Walseth cellulose (H_3PO_4-swollen cellulose) was prepared from microcrystalline cellulose (Merck), all other chemical reagents were purchased from Merck.

Growth of the *Trichoderma reesei*: Stock cultures of the *T.reesei* were maintained on potato dextrose agar (PDA) plates at 28 °C under constant illumination. Conidia was harvested from mycelia grown for 7 - 9 days on PDA plates and were used to inoculate liquid suspension cultures. Liquid suspension cultures were grown in 100 – ml glass bottles for 2 days. This preculture medium was Mandels culture and the carbon source was glucose [15]. The growth medium (1 ml) was added to 100 ml liquid Mandels cultures in 250- ml glass bottles for 16 days at 28 °C an orbital shaker (120 rpm) the carbon source was Walseth cellulose. Six flasks were prepared that each containing 100 ml of the culture medium. To each of the above culture medium was added 10, 50, 200, 400, 600 and 800 μmol/ml gibberellin hormones.

Enzyme assay

After various incubation times, the content of each flask was centrifuged at 1000 ×g for 3 min by a Sigma 3K20 centrifuge. The cellulase activity was measured by the filter paper assay and the activity was expressed as a filter paper unit per ml of the broth (FPU/ml). An aliquot of the centrifuged broth was incubated with 1× 6 cm (50 mg) strips of filter paper (Whatman no.1) for 2 h at 50 °C. The reducing sugars liberated were measured by dinitrosalicylic acid method [16]. The purified enzyme can be stored on a shelf in diethyl ether at room temperature for a period of one year. Cellulase enzyme remains fully active in diethyl ether solvent and will not be attacked by microbes. Cellulase enzyme is a multi-enzyme. Accumulation of enzymes can be done better in the solvent diethyl ether.

Protein determination

Protein determination was achieved according to the Lowry method with BSA as standards [17].

Cellulolytic enzymes adsorption and desorption

After 16 days, the culture medium containing cellulase enzymes and other compounds. When we add to this medium cellulose, just cellulase enzyme binds to cellulose. In this stage we have cellulase enzyme which bind to cellulose (adsorption), Now we need a method to separate the enzymes from cellulose (desorption). The following methods were used to carry out the above steps.

The broth containing cellulolytic enzymes after 16 days was centrifuged at 1000 ×g and 4 °C. Ten grams of microcrystalline cellulose were added to 100 ml of the supernatant and was stirred for 60 min (at 4 °C). Then the mixture was centrifuged and the pellet containing the cellulolytic enzymes was collected. 100 ml of diethyl ether was added to pellets containing the enzymes (at 4 °C) and after 60 min, 100 ml citrate buffer 0.05 M was added. All of the cellulolytic enzymes were transferred to citrate buffers.

Enzyme concentration

To 100 ml of solution containing cellulase, 100 ml n-butanol was added. The solution was shaken, the two phases were formed. Then aqueous phase was separated which the volume was 80 ml. Again, 80 ml new n-butanol was added and shaken. In this step, the aqueous phase became 64 ml. The above procedure was repeated 6 times, and final solution volume was 6 ml. In each step, the protein concentration and enzyme activity was measured. All these experiments were carried out with the control (without GA_3) and sample containing 50 μmol/ml GA_3.

Using different amounts of the hormone gibberellins

To 100 ml of culture medium containing *T.reesei* 10, 50, 200, 400, 600, and 800 μ moles/ml gibberellins were added. The amount of protein and cellulose activity was determined in each of them.

Results

T.reesei growth was performed without the use of hormone (gibberellins, GA_3) for 16 days (control). At intervals, 2 ml supernatant was removed, centrifuged for 2 min and then assayed for the protein content and unabsorbed cellulase activity. The next experiment was carried out with different amounts of gibberellins and the results are shown in Table 1. All assays have been done in duplicates. In 100 ml culture medium containing *T.ressei* was added varying amounts of GA_3. Culture medium which labeled control did not contain gibberellin hormone. In this culture, the enzyme activity was 0.34 FPU/ml and protein concentration was 5.05 mg/ml. In this experiment in 100 ml of culture medium containing *T.ressei* varying amounts of gibberellin hormone was added. Enzyme activity and protein concentration in each sample was measured.

One hundred milliliter solution containing cellulase was concentrated by n- butanol in 6 steps. Enzyme activity was compared between hormone- free medium (control) and medium containing 50μmol/ml of GA_3. This method was previously used in this laboratory. In

Table 1. Determination of cellulase activity and protein concentration in varying amounts of GA_3.

GA_3 (μmol/ml)	Enzyme Activity (FPU/ml)	Protein conc. (mg/ml)
Control	0. 34	5.05
10	3.58	13.69
50	4.29	34.27
200	1.98	11.30
400	3.30	11.30
600	2.97	20.80
800	3.30	26.70

Table 2. The comparison of enzyme activity between medium containing 50 μmol/ml of GA_3 and hormone- free medium (control)

Volume (ml)	Control Activity (FPU/ml)	50μmol/ml of GA_3 Activity (FPU/ml)	Increased Activity (%)
100	0.34	0.58	70.60
80	5.02	6.65	32.50
45	8.24	9.76	18.50
22	12.21	20.15	65.10
16	14.80	57.00	285.20
6.0	18.00	76.30	323.90

each step, we added the same volume of n- butanol to solution containing cellulase. Two phases were formed and the aqueous phase was separated. For each step we had control. The control sample just did not have gibberellin hormone (Table 2).

Discussion

Our results from GA_3 in this study suggest that this hormone can stimulate *T.reesei* and causes to produce more proteins. One of these proteins is cellulolytic enzymes. This experiment showed that cellulase activity is increased by adding gibberellin hormone. Our results suggest that this hormone is used directly as a growth supporting substrate because the color of media changed and production of protein increased.

Regulating the activity of certain enzymes such as β-1,3- glucanase by GA_3 action in plants has been demonstrated [18]. Several genes induce by *Xanthomonas axonopodis* pv. *Citri* in leaf of Citrus seems to be regulated by the hormones auxin and gibberellin [19]. The number of fungi that produce gibberellin was investigated and to date, eight fungi were detected. Five species of *Sphaceloma, Neurospora crassa, Gibberella fujikuroi,* and *Phaeosphaeria* sp. [20]. Both *G.fujikuroi* and *Sphaceloma* sp. are phytopathogenic fungi that cause superelingation diseases, while *N.crassa* has no phytopathogenicity [21].

The results showed that after the addition of gibberellins in the medium containing *T.reesei*, the amount of proteins were increased compared to control. The total amount of proteins in media lacking GA_3 was 5.05 mg/ml of culture, but after adding 50 μmol/ml of GA_3 to the medium, the protein concentration was increased to 34.27 mg/ml (Table.1), It means that the protein concentration was increased 578%. Cellulase activity of a hormone- free medium was 0.34 FPU/ml, but after adding 50 μmol/ml hormone to the medium, the activity was increased to 4.29 FPU/ml. Thus, the enzyme activity of cellulase by adding 5 μmol/ml of gibberllin was increased 1161% (compared to control). By increasing the amount of GA_3 in the medium (for quantities more than 50 μmol/ml, for example 200, 400, 600 and 800 μmol/ml), it was observed that the protein content decreased. According to the results obtained, may be increased GA_3 to the medium, protease increased and caused to reduced the amount of proteins. Then the enzyme was concentrated by n- butanol. This step was repeated several times. The volume of the first solution contains cellulase enzyme was 100 ml, then by adding butanol at each step, volume decrease to 80, 45, 22, 16 and 6 ml. this experiment was carried out with the control solution and solution containing GA_3. By using the above method, the activity of cellulase enzyme was increased from 0.34 FPU/ml to 0.58 FPU/ml (in the first stage of concentration, compared with the control) (Table 2). In the first stage, the cellulose activity increased to 70%, but the solution was concentrated to a volume of 80 ml. This process was repeated 6 times. Finally, the solution volume was 6 ml and enzyme activity was increased to 76 FPU/ml. This level of increases is incredible and for such an enzyme is an important event.

By adding gibberellin to the medium containing *T.reesei*, fungal growth increased. In this article, we could raise the activity of cellulase from 0.34 FPU/ml (in control without gibberellins) to 34.27 FPU/ml (in 50 μmol/ml of gibberellins). To check the accuracy of the test, the activity was carried out on a blank sample containing hormone + growth medium. In this experiment, we found that absorption was zero. It means that gibberellins does not interfere in DNS. When diethyl ether added to the solution, the enzyme complex separated from cellulose (desorption). In this condition, the purification of the enzyme can be done easily. This purification procedure was carried out previously in this laboratory. Please refer to Article [14]. Disadvantage of the earlier method was that it was difficult to separate the enzymes from cellulose. But by adding diethyl ether we were able to apply the simplest method for this separation. Ultra filtration is used for concentrating the enzyme, but in this article we were adding n- butanol to the solution containing the enzyme and butanol absorbed some water from the solution that caused the enzymes were concentrated [21].

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