Research article

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Production and Biochemical Characterization of Cellulase Enzyme by *Trichoderma* Strains from Harran Plain

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ABSTRACT

In this research, *Trichoderma* spp. fungus, a major producer of cellulase, that was isolated from Harran plain had been investigated. Morphological, microscopic and genetic identification of the six fungi isolates were carried out and their cellulase production ability were determined. The ITS region of four of the isolates were displayed over 90% similarity with the DNA sequences of *Trichoderma* spp. currently deposited into the databases. The molecular size of fungal cellulase was found as to be 32 kDa by SDS-PAGE analysis. Optimum working conditions of cellulase had also been studied. The optimal conditions for cellulase activity determined at 40 °C, pH 5.0, 60 min incubation time and 2% Carboxymethyl Cellulose (CMC). The fungal cellulase activity was compared with the activity of the commercial cellulase enzyme. The fermentation produced enzyme by using *Trichoderma* spp. isolated from Harran plain displayed remarkable cellulase activity.

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Introduction

Cellulose is the most common carbonhydrate polymer in the nature, and it is currently being used for the production of useful industrial materials such as biofuels and some chemicals. It is a biodegradable material and found in the structure of the plant cell wall and accounts for about 40% of the cell biomass [1]. Cellulose is a long chemical structure composed of glucose. Glucose molecules are linked to each other via β, 1-4 glycosidic bonds [2]. It can be directly converted into monomer by using chemicals, enzymes or by the combination of both. Chemical degradation is unuseful and uneconomical in comparison to the enzymatic degradation because it produces more byproducts at high temperatures and causes environmental problems. Cellulase enzymes which can hydrolyze cellulose can be divided

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into three types: endoglucanase (endo- 1,4-β-glucanase EC 3.2.1.4), exoglucunase (exo-1,4-β-glucanase EC 3.2.1.91), β glucosidase (1,4-β-glucosidase EC 3.2.1.21)[3]. Cellulases are important industrial enzymes used in various industrial applications such as in textile an detergent, food and feed, baking, pulp an paper and waste treatment. Cellulase mainly produced by microorganisms like bacteria and filamentous fungi. There are many microorganisms capable of producing extracellular cellulases such as Cellulomonas, Pseudomonads, Bacillus; Actinomycetes, Aspergillus, Humicola, Trichoderma and Penicillium [4]. Complexity of fungal cellulase is less than bacterial cellulase and former is produced extracellularly in large amount [3].

Trichoderma is known a microorganism found in nature especially in soil commonly, capability of utilizing various subsrates [5]. Trichoderma is a fast growing fungus and strains generally mature in 4 days. Colonies can be colorless, green or yellowish [6]. Some uses of Trichoderma are hyperparazism, antibiosis, competition, enzyme production [7]. The most important feature of Trichoderma is that it can produce cellulosic and hemicellulotic enzymes. These enzymes are extremely important as they are used in the recycling of waste. Because it is widely available in nature, high enzyme capability under favorable conditions and is prone to genetic manipulations, Trichoderma is preferred in researchs [8]. Studies on cellulase production show that Trichoderma viride, Trichoderma reseei, Trichoderma harzianum are the most productive strains [9].

Harran Plain is a major source of agricultural residues, such as lignocellulosic material, which could be used as a raw material for microbial production of enzymes. Microbial cellulase production is an important strategy for the development of sustainable processes of industrial based cellulase enzyme for some industries such as food, textile, animal feed, detergent etc. This study aims to isolate the Trichoderma which is capable of producing cellulase enzyme from Sanlıurfa Harran Plain soils. In this work we isolated some Trichoderma strains from soil and identificated by molecular technics. Moreover cellulase was produced via Trichoderma strains and characterized.

Materials and Methods

Soil material and microbial media

Soil samples were collected at the latitude of 37 ° 0 '0 "latitude, 38 ° 0' 0" longitude from Şanlıurfa Harran corn, cotton and wheat plain after harvest. Samples were taken from soil, rotting plant roots and stems.

Isolation of *Trichoderma* strains

The soil samples were diluted 10^{-1} - 10^{-6} with sterilized water, and then the dilutions were cultivated in DRBC(Dichloran Rose Bengal Chloramphenicol) medium at 28°C for 5 days. Colonies similar to *Trichoderma* were selected and cultivated on PDA (Potato Dextrose Agar) under the same conditions to obtain single colonies [10].

Microscobic and morphological identification of *Trichoderma* strains

Morphological definition is based on time dependent changes of colonies formed in PDA medium, structure of colony and hypha, color, of microorganism. Moreover a loopfull sample were taken from different parts of the colonies and spread over the coverslip in order to identify the strains microscopically. Samples were examined under the light microscope (Motic B series, Japan) and their definitions were made according to the spore structures [11].

Determination of cellulase activities of the *Trichoderma* strains

Cellulase activity ability was investigated on Carboxy Methyl Cellulose Agar (KH_2PO_4 1 g, $NaNO_3$ 3 g, KCl 0.5 g, $MgSO_4.7H_2O$ 0.5 g, $FeSO_4.7H_2O$ 0.01 g, CMCNa 15 g, agar 15 g per 1.0 litre distilled water) at 30 °C for 3 days and colonies forming zones were evaluated as cellulase positive and those without zones were evaluated as cellulase negative [10].

Molecular identification of *Trichoderma* strains

DNA extraction

The collected isolates of *Trichoderma* (5 isolates) were cultured in 100 ml Erlenmayer flasks containing 10 ml PD medium after 5 days incubation, mycelium was collected by filtration and ground to a fine powder in liquid N_2. 2 g culture was transferred to a 1.5 ml Eppendorf tube and diluted with 500 µl TE (Tris-Cl, EDTA) buffer. The TE buffer was decanted and 300 µl of extraction buffer (200 mM Tris HCl pH 8.5, 250 mM NaCl, and 25 mM EDTA,

0.5% SDS) was added to the pellet. The mycelium was crushed with a sterile toothpick. Subsequently, 150 µl of 3M Sodium acetate, pH 5.2 was added and tubes were placed at -20 °C for 30 minutes. Tubes were centrifuged 10000 rpm for 10 minutes. The supernatant was transferred to another sterile eppendorf tube. Equal volume of isopropanol was added and kept for 10 minutes at room temperature. DNA was precipitated by centrifugation at 12000 rpm for 10 minutes. After a wash with 70% ethanol, the pellet was dried for 5 minutes and re-suspended in 50 µl of TE to make DNA suspension [12].

ITS regions sequencing: ITS-PCR was done for each sample in a 25 µl reaction mixture. Each reaction mixture was contained with 2,5µl PCR buffer, 1.5µl $MgCl_2$, dNTP 1.25µl, 1µl ITS1 (forward primer), 1µl ITS4 (reverse primer), 0.25 Taq polymerase, 1µl DNA and 16.5µl of PCR water. The Thermal cycler was programmed for 30 cycles of initial denaturation at 94 °C for 2 minutes followed by denaturation at 94 °C for one minute. Primer annealing at 55 °C for 1 minute, elongation at 72 °C for 1 minutes, final extension at 72 °C for 5 minutes and final hold 4 °C (Shadid et al., 2013). PCR products were purified using illustraTM GFX ™ PCR DNA and Band Purification Kit (GE Healtcare, USA) and sequencing analysis was performed by Iontech compony, sequences were compared by NCBI GenBank, and then phylogenetic tree constructed by MEGA7.0.26 software.

Expression, purification and SDS-PAGE analysis of cellulases

For the production of cellulase in liquid state fermentation the fungus was grown in 250 mL erlenmayer flask containing 100 mL of fermentation medium. Concentrations of the nutrients were yeast extract 1 g/L, CMC.Na 5 g/L, NaCl 20 g/L, K_2HPO_4 0.5 g/L, $MgSO_4.7H_2O$ 0.5 g/L. pH of the medium was adjusted to 5.0. Microorganisms culture inoculated 10^{-6}. The inoculum preparations were completed by 28°C, 8 days in an shaker (150 rpm). Liquid state cultures were harvested by centrifugation at 4000 rpm, for 50 min, at 4°C. The resulting supernatant was called as crude enzyme preparation.

Polyacrilamide gel electrophoresis was used to determine molecular mass of crude enzymes. SDS-PAGE was performed in a 12% (w/v) polyacrilamide gel [13]. The proteins were stained using Coomassie Brillant Blue.

Enzyme activity assays

The activity of the cellulase was assayed with CMC.Na activity measurements. A crude enzyme sample of 0.5 mL was added into 0.5 mL of the reaction mixture (containing 0.05% (w/v) CMC.Na with pH citric acid buffer) and incubated at 40°C for 1 h. Then the reducing sugar released by the reaction was determined by DNS method [14]. One unit of the CMC enzyme activity was defined as the amount of enzyme that catalyzed to produce 1 μmol of reduced sugar per minute with the reduction of CMC.Na.

Characterization of crude cellulase enzymes

Effect of incubation period, temperature, pH, substrat concantration on enzyme activity

Incubation time, temperature, pH and substrat concantration was determined respectively 30 min., 60 min., 120 min. and 240 min.; 30°C, 40°C, 50°C, 60°C (1 hour); 3.0, 5.0, 7.0, 9.0, 11.0 (1 hour); 1% CMC, 1.5 CMC, 2% CMC, 2.5% CMC concantration (1 hour). Then cellulase activity measured at 560 nm by spectrophotometer.

Comparison of isolated enzyme activity with commercial enzyme activity

Crude enzyme activity from *Trichoderma* strains compared with commercial Vegazym HC (cellulolytic enzyme) activity by DNS method. Enzyme-substrate incubated at 40°C for 1 h and results were measured at 560 nm by spectrophotometer.

Results and Discussion

Identification of *Trichoderma*

Six *Trichoderma* samples isolated from Harran Plain soil. Morphological and microscobic identification of *Trichoderma* strains were carried out and then cellulase activity investigated on CMC agar medium by using congo red. After third days incubation microorganism colonies began to grow up on PDA and end of five days mycelia became tight and green color was observed locally.

Strain called as TH1 (corn), TH2 (corn), TH3 (corn), TH4 (wheat), TH5 (corn), TH6 (corn). Morphological and microscobic apperance of *Trichoderma* strains are shown in Fig.1 and Fig.2. T1, T2, T4, T5, T6 were observed similar to some *Trichoderma* strains but T3 sample was different *Trichoderma* colony shape and growth characteristics. Also color of T3 was different from *Trichoderma*.

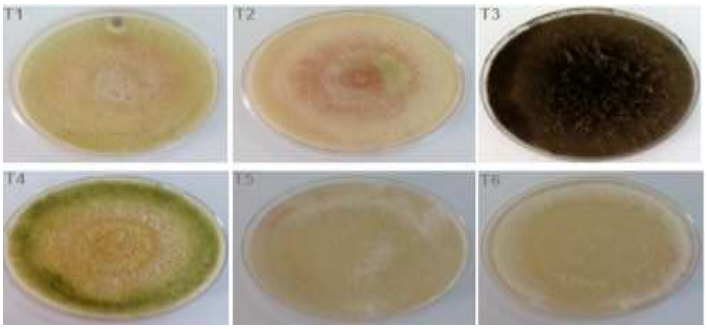

Fig. 1 Morphological apperance of strain TH1, TH2, TH3, TH4, TH5, TH6 on PDA

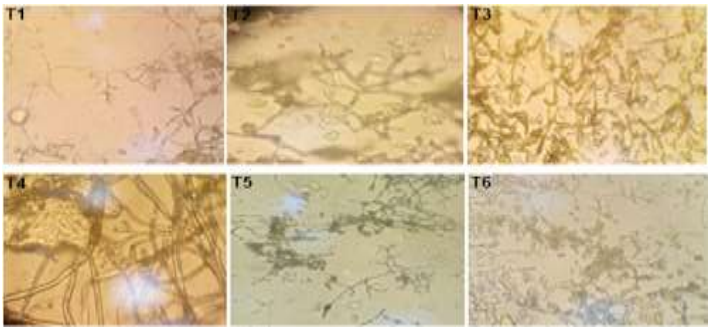

Fig. 2 Microscobic apperance of strain TH1, TH2, TH3, TH4, TH5, TH6

Looking at the microscopic view of the samples, main branches of conidophores formed lateral branches and spores were observed at the ends of the branches. The recorded image was observed to be the typical microscopic view of *Trichoderma*. It was observed that the sports structure of the T3 isolate is different from *Trichoderma* appearance.

Enzyme activity was calculated by dividing the hydrolysis zone diameter of the *Trichoderma* strain on the CMC agar medium by the diameter of the hydrolysis zone examined by using the congo red staining method. Cellulase activity of strains on CMC agar medium shown in Fig.3.

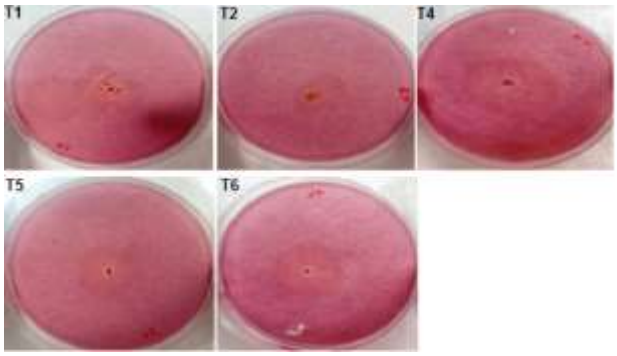

Fig. 3 Cellulase activity of *Trichoderma* strains on CMC kongo red medium

Table 1 Cellulase activity of *Trichoderma* isolates on CMC of strains

Sample	Colony diameter (cm)	Cellulase activity (EU)
T1	4.0±0.37	0.35±0.02
T2	2.7±0.25	0.29±0.01
T4	4.2±0.35	0.23±0.02
T5	2.6±0.20	0.15±0.01
T6	3.0±0.26	0.13±0.01

Similar results were also given by [10, 15, 16]. They measured the cellulose activity on CMC agar medium and found results similar to what we presented in here. Their obtained activity was slightly higher to the activity we regarded. While the slow growth of colonies in the PDA medium made it easier to observe colony development, the CMC solid fermentation medium developed within three days and completely covered the surface of the medium. The main problem of CMC method is that it is difficult to observe the formation of zones around the colony, since the hyphae are transparent in the outer wall.

Molecular identification of *Trichoderma* strains

Molecular identification of isolated *Trichoderma* strains was done by PCR method. ITS regions of the strains were amplified by ITS and ITS4 primers. The obtained PCR products were analyzed on Agarose gel and we were able to obtain bands at approximately 600 bp

size. The PCR products were purified and sent for the sequencing. The obtained sequences were compared to the DNA sequences deposited to NCBI database.

Results from ITS sequencing analysis indicated that the size of sequence was approximately 600 bp for all isolates, and similarities of T1 and T2 samples (95%) to *T. harzianum* and *T. piluliferum*, T4 (91%) to *T. citrinoviride*, T5 (91%) to *T. harzianum*. Although T6 sample showed similarity *T.harzianum*, sequence analyses resulted uncultured fungus. ITS sequencing analysis is shown in Fig 4 and phlyogenetic tree is shown in Fig 5.

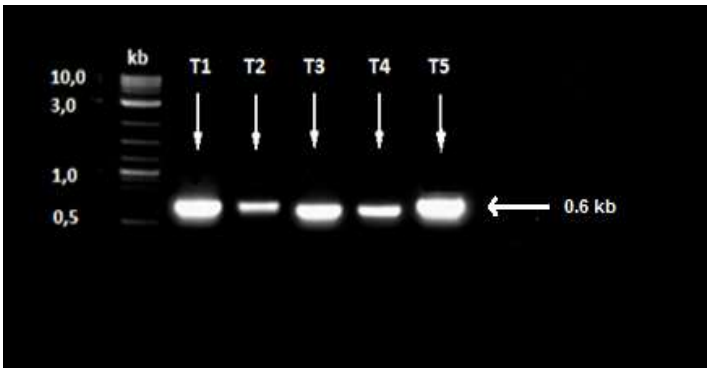

Fig. 4 1.2% agarose gel image obtained after amplification of DNA from T1, T2, T4, T5, T6 isolates using ITS1 and ITS4 primer pair. There are approximately 600 bp corresponding to the ITS1-5.8S-ITS2 gene region

Molecular identification reported by other researchers, DNA bands of about 660 bp were detected on the agarose gel as a result of studies performed using the ITS1 and ITS4 primer pair, and another study was resulted approximately 600 bp by using ITS1 and ITS2 primer [17]. Similar studies were carried out by other researchers. They performed PCR by using DNA from *Trichoderma* samples isolates. Shahid et al. (2013) studied a molecular characterization of the *Trichoderma longibrachiatum* 21PP strain isolated from soil. As a result of the studies conducted using the ITS1 and ITS4 primer pairs, DNA bands of approximately 660 bp size were detected on the agarose gel. Therefore, in order to reveal the molecular characterization of *Trichoderma* isolates obtained in this study, ITS1 and ITS4 primer pairs were used. As a result of PCR analysis by using ITS1 and ITS4 primers, approximately 600 bp DNA bands were detected on agarose gel, similar to the studies in the literature [18].

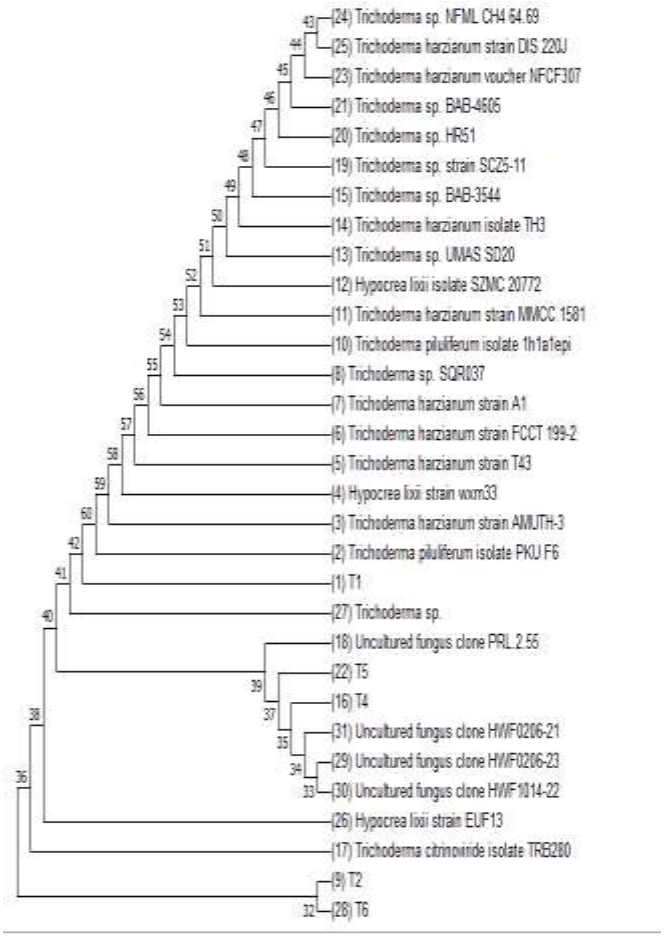

Fig. 5 Phylogenetic tree according to ITS1-5.8-ITS2 sequence results of isolates

Expression, purification and SDS-PAGE analysis of cellulases

Cellulase activity results measured by days are shown in Fig 6. We were able to detect enzyme activity at 5th day and also the highest activity was detected at 5th day.

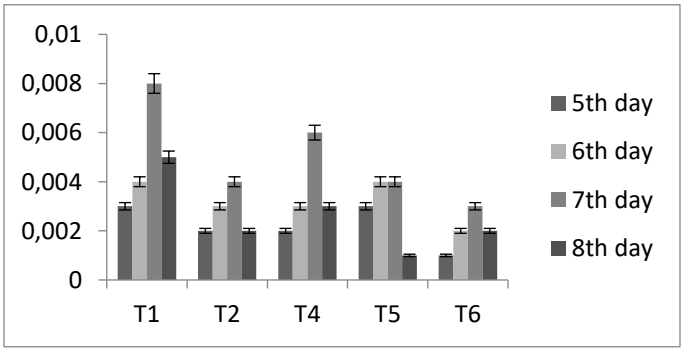

Fig. 6 Production of cellulase at CMC medium in shaker and activity by day (5, 6, 7, 8th day) by DNS method

It was observed that cellulase activity increased during fermentation. The activity started on the fifth day for all isolates. While activity increased on the sixth and seventh days of fermentation, there was a decrease on the eighth day. The day when it produced the highest amount of enzyme (the day the highest activity was measured) was observed as the seventh day. According to the activity data obtained, T1 isolate performed the highest activity with 0.008U / mL. Then the T4 isolate came with 0.006U / mL activity. T2 and T5 showed 0.004U / mL activity. The lowest activity was T6 with 0.003U / mL.

Although the time worked in the literature for fermentation is generally seen as 5 days, the activity varies depending on the microorganism and substrate difference. Mangalanayaki and Madhavan (2015) used *Trichoderma harzianum* and *Fusarium oxyporum* to produce cellulase by solid state fermentation in different substrates such as cotton seeds, corn cobs and rice bran. During fermentation, culture was taken from the medium and activity was determined. As a result, the highest activity in cotton seeds was observed in 144 hours, and the highest activity in corn cobs and rice bran was seen in 120 hours. Kim et al. (2002) produced cellulase from *Trichoderma harzianum* FJ1 strain. In the study, fermentation was followed up daily due to the increase in carbon source. The carbon source CMC was chosen for cellulase production. As a result, the highest activity was detected on the 5th day [19, 20].

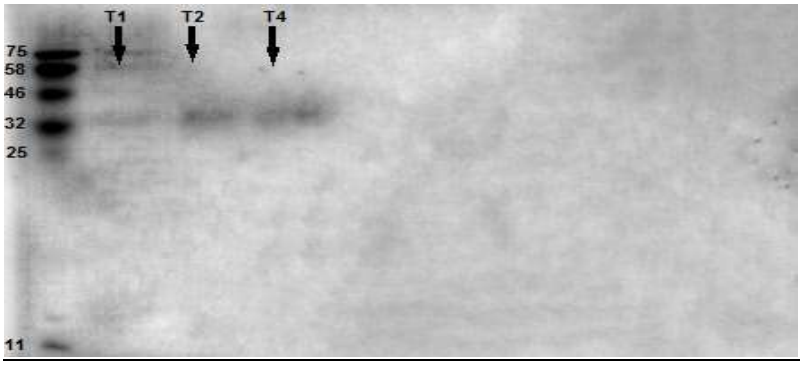

Fig. 7 SDS-PAGE analyses of *Trichoderma* isolates cellulase composition

Proteins isolated from *Trichoderma* strains are shown in Fig 7. Bands show protein size obtained from *Trichoderma* strains. Proteins were seperated on 12 % SDS-PAGE and their size were determined by using molacular weight markers. Bands of protein and enzyme

samples obtained from *Trichoderma* strains named T1, T2, T4 seems in the protein gel. According to the results obtained, 32 kDa protein bands are present in all three samples. In a study on cellulase production from *Trichoderma reseei*, crude cellulase showed 32kDa band in SDS-PAGE analysis. In another study with cellulase from *Bacillus*, it was seen that 32.5 kDa band. Noronha and Ulhoa (1999), produced β-1,3-glucanase from *Trichoderma harzianum*. In the study, SDS-PAGE analysis was performed to measure the size of cellulase. The enzyme purified by gel filtration and ion exchange chromatography showed a band of 29 kDa. Saravanakumar et al. (2016) worked with *Trichoderma* in biological control. In this study, they produced cellulase from *Trichoderma harzianum*. The produced raw cellulase showed two bands between 25-66 kDa in SDS-PAGE. Iqbal et al. (2011) detected 58 kDa band as a result of SDS-PAGE analysis in order to determine the molecular size of the cellulase enzyme produced from *Trichoderma viride* [21-25].

Enzyme activity assays

Effect of incubation period on enzyme activity

The enzyme and substrate were incubated for 30 minutes, 1 hour, 2 hours and 4 hours to determine the time at which the cellulase enzyme works best. Enzyme avtivity of *Trichoderma* samples are shown in Fig 8.

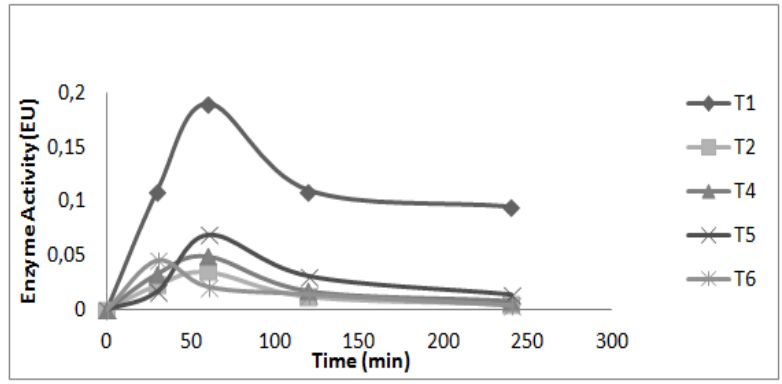

Fig. 8 Effect of incubation period on crude cellulase activity (U/mL)

When the data in the figure is examined, it is seen that cellulase activity starts at 30 minutes for all samples. While the activity in the T6 sample reached its maximum level in the 30th minute, the activity in the other samples continued to increase with advancing time.

Maximum activity levels for T1, T2, T4, T5 samples were measured at 60 minutes. In the determination of activity; T1 at 30 min; 0,11, T2; 0.023, T4; 0.033, T5; 0.017, T6; 0.045 U / mL, T1 at 60 min; 0.19, T2; 0.035, T4; 0.049, T5; 0.069, T6; 0.021 U / mL, T1 at 120 min; 0,11, T2; 0.012, T4; 0.017, T5; 0.031, T6; T1 at 0.014, 240 min; 0.095, T2; 0.005, T4; 0.008, T5; 0.014, T6; 0.004 U / mL was measured. According to the literature, 30-60 minutes was enough to break down the substrate of microbial cellulase[26-28].

Effect of temparature on enzyme activity

Temperature an important factor that influences the cellulase yield. In this study enzyme activity started by 30ºC for all samples. Optimum temperature is determined as 30-40ºC. Enzyme avtivity dependent temperature of *Trichoderma* samples are shown in Fig 9.

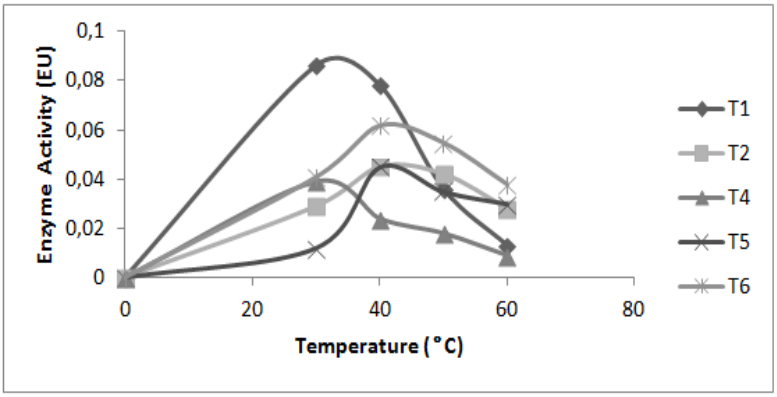

Fig. 9 Effect of temperature on crude cellulase activity (U/mL)

When the data were examined, the temperature-related activity started at 30 ºC for the enzymes obtained from all samples. The optimum temperature has been determined as 30-40 ºC in all. It was observed that enzyme activity decreased after 40 ºC, especially T1, T5, T6. Looking at the effect of temperature on the enzyme, enzyme activity T1 at 30ºC; 0.086, T2; 0.029, T4; 0.039, T5; 0.012, T6; 0.041 U / mL, T1 at 40 ºC; 0.078, T2; 0.045, T4; 0.024, T5; 0.045, T6; 0.062 U / mL, T1 at 50 ºC; 0.036, T2; 0.042, T4; 0.018, T5; 0.035, T6; 0.055 U / mL, T1 at 60 ºC; 0.013, T2; 0.028, T4; 0.009, T5; 0.030, T6; 0.038 U / mL was measured. The lowest cellulase activity was observed at 60 ºC for all enzyme samples.

Effect of pH on enzyme activity

Cellulase activity started by 3.0 pH and activity along with increased pH degree. Optimum pH was determined by 5.0. Enzyme avtivity dependent pH of *Trichoderma* samples are shown in Fig 10.

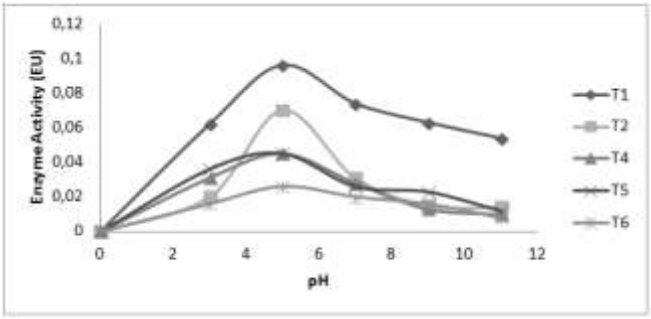

Fig. 10 Effect of pH on crude cellulase activity (U/mL)

Enzyme activity T1 at pH 3.0; 0.062, T2; 0.020, T4; 0.031, T5; 0.036, T6; 0.016 U / mL, T1 at 5.0; 0.096, T2; 0.070, T4; 0.045, T5; 0.045, T6; 0.026 U / mL, T1 at 7.0; 0.074, T2; 0.031, T4; 0.028, T5; 0.026, T6; 0.020 U / mL, T1 at 9.0; 0.063, T2; 0.015, T4; 0.013, T5; 0.023, T6; 0.016 U / mL, T1 at 11.0; 0.054, T2; 0.014, T4; 0.010, T5; 0.012, T6; It was calculated as 0.008 U / mL. The lowest activity was seen at pH 11.0 for all samples.

Cellulases are generally classified as acidic (pH 4.5-5.5), neutral (6,6-7,0), and alkaline (pH 9,0-10,0) cellulases, depending on their effective pH value. Since the enzyme obtained from *Trichoderma* has optimum activity at pH 5.0, it is accepted in the group of acidic cellulases[29].

In a study optimum conditions for cellulase activity were determined, the optimum temperature of the enzyme was 45 ºC and the optimum pH was 6,5, and in these conditions the enzyme activity was measured at 1,95 U / mL[27]. Also cellulase activity may vary depending on the microorganism in which it is produced. There are many studies on the effect of temperature and pH on cellulase activity. Researchers usually worked on cellulase from *Trichoderma harzianum* and *Trichoderma reseei* [1, 2, 4, 30-34].

Effect of substrat concentration on enzyme activity

Data presented in Fig 11 shown that cellulase activity was significantly influenced by the concentration of substrate.

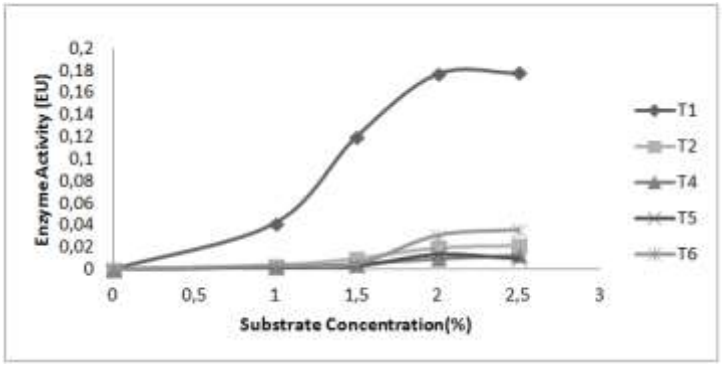

Fig. 11 Effect of substrate concentration on crude cellulase activity (U/mL)

The change of enzymes obtained from T1, T2, T4, T5, T6 Trichoderma isolates depending on the substrate concentrations is given. It was observed that enzyme activity increased as substrate concentration increased for all samples.

The increase in concentration varies greatly for the enzyme from T1 isolate, but no high increase has been observed for other samples. Enzyme activity increased with increasing substrate concentration showed the maximum activity value at 2% CMC concentration. The increase in the CMC concentration did not affect the enzyme activity after the 2% substrate, so the enzyme saturated with the substrate. Enzyme activity is T1 in 1% CMC substrate; 0.042, T2; 0.004, T4; 0.002, T5; 0.002, T6; 0.003 U / mL, T1 in 1.5% CMC substrate; 0.12, T2, 0.010, T4; 0.005, T5; 0.003, T6; 0.005 U / mL, T1 in a 2% CMC substrate; 0,177, T2; 0.020, T4; 0.010, T5; 0.014, T6; 0.031 U / mL, T1 in the 2.5% CMC substrate; 0,178, T2; 0.022, T4; 0.013, T5; 0,010, T6 was calculated as 0,036 U / mL.

Kim et al. (2002) tried 1.0%, CMC, 2.5% CMC and 5.0% CMC substrate concentrations to determine the substrate concentration in their enzyme characterization related to cellulase produced from *Trichoderma harzianum*. As a result of the study, the best substrate concentration was determined as 2.5% CMC[19].

Comparison of isolated enzyme activity with commercial enzyme activity

Max cellulase activity was determined for commercial Vegazyme enzyme by 1,8 U/mL activity.Then cellulase from T1 isolate followed by 0, 120 U/mL activity. And activity observed for cellulase from T2 0,045 U/mL, from T4 0,055 U/mL, from T5 0,067 U/mL and

from T6 0,025 U/mL. Min cellulase activity was registered for T6 sample's enzyme. Enzyme avtivity of *Trichoderma* samples are shown in Fig 12.

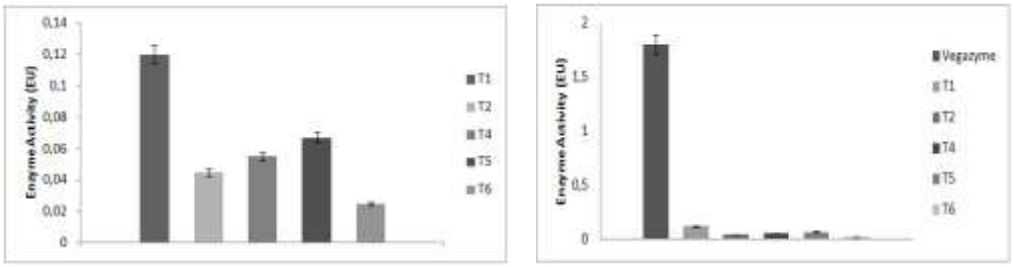

Fig. 12 Comparison of isolated enzyme activity with commercial enzyme activity (U/mL)

Conclusion

The successful use of cellulosic material as carbon source is dependent on the development of economically feasible process for cellulases production. The isolation *Trichoderma* strains and production of cellulases by *Trichoderma* strains investigated.

In the result of study T1 and T2 (95%) identified as *T. harzianum* and *T. piluliferum*, T4 (91%) identified as *T. citrinoviride*, T5 (91%) identified *T. harzianum*. Altough T6 sample showed similarity *T.harzianum*, sequence analyses resulted uncultured fungus. Enzyme wight 32 kDa are observed in all three samples. The optimum working temperature of the enzyme was determined to be 40 º C, optimum working pH of 5.0, optimum working time of 60 min and ideal substrate concentration of 2% CMC.

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