Current Research in Microbiology and Biotechnology
Vol. 6, No. 2 (2018): 1518-1524
Research Article
Open Access

ISSN: 2320-2246

Cellulase enzyme production from solid state fermentation and submerged fermentation using *Trichoderma* species isolated from Iraqi soils

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Received: 19 January 2018 Accepted: 12 February 2018 Online: 01 March 2018

ABSTRACT

In this study, forty-eight soil samples were collected from fields cultivated with wheat and barley from different governates of Iraq to isolate fungi producing cellulolytic enzyme. Fungal isolates were screened for enzymes production on selective media containing carboxymethyl cellulose (CMC) as a sole source for carbon and energy. Results of semi-quantitative screening showed that forty-eight isolates were able to produce cellulase with variable degrees depending on the formation of halo zones around colonies of these isolates. These isolates in producing cellulase were selected and subjected to quantitative screening by determining cellulase specific activities in the culture filtrates. Results showed that the isolate symbol N48 was the most efficient among the other isolates in cellulase production since it produced the highest yield of cellulase enzyme. The specific activity of cellulase of this isolate reached 50.1U/mg protein.

Keywords: Cellulase, *Trichoderma spp*, Submerged Fermentation, Solid-state fermentation.

1. INTRODUCTION

Cellulases are glycoside hydrolases (GHs) that decompose cellulose a hydrophilic, water-insoluble polymer composed of repeated units of D-glucose interlinked by β-1,4- glycosidic bonds into shorter chain polysaccharides such as cellodextrin, cellobiose, and glucose [1]. Cellulases are industrially important enzymes with a market share of 500 million dollars that is expected to rise to 1.5 billion dollars by 2018. Cellulases play a crucial role in generating sugar feedstock for lignocellulosic based biorefinery platform. In addition, their demand in textile, paper, feed and food industries is rising steadily. However, these industrial applications require thermostable, catalytically highly efficient cellulases for making the processes commercially viable [2]. Cellulase has been produced from different organism, mainly fungi, bacteria, and protozoans, but the fungus *Trichoderma spp* was recorded as one of the most important commercial cellulase producers and has been widely used in a variety of industries. *Trichoderma* sp. are widely distributed all over the world and occur in nearly all soils and other natural habitats, especially in those containing organic and inorganic matter [3]. They are ubiquitous colonizers of cellulosic materials and can thus often be found wherever decaying plant material is available as well as in the rhizosphere of plants, where they can induce systemic resistance against pathogens [4].

Submerged Fermentation is a type of fermentation which is used for most of the industrial enzyme production because of its ease of process and separation and good control of environmental factors such as temperature, aeration, agitation and pH, but this technique is not only expensive but also of energy intensive[5]. It involves the production of enzymes by microorganism in a liquid nutrient media which utilizes the liquid substance such as molasses and broth [6].

Solid-state fermentation (SSF) is a process whereby an insoluble substrate is fermented with sufficient moisture but in the absence of free-flowing water. However, the substrate must possess enough moisture to support growth and metabolism of the microorganism [7]. Solid state fermentations have been reported to have high enzyme productivities as compared to submerged fermentations [8]. The exact reasons for higher titres in SSF as compared to submerged fermentations are currently not wellknown. There are however suggestions that higher biomass and lower protein breakdown contribute to better production in SSF. SSF holds a tremendous potential for the production of enzymes especially where the crude fermented products may be used directly as an enzyme source. SSF processes have lower energy requirements and produce less wastewater [9]. SSF offers numerous advantages lower cost and reduced need for asceptic techniques compared to submerged fermentations (SmF) [10].

This study aimed to investigate the efficient fungal isolate from all among fungal isolates in cellualse production through solid state fermentation and submerged fermentation.

2. MATERIALS AND METHODS

2.1 Isolation, maintenance, Identification of *Trichoderma* spp.

Soil samples were collected from fields cultivated with wheat and barley crops from different locations in some Iraqi governorates. About 100 g portions of each soil sample was taken from a depth of about 15 cm from the soil surface and placed in a sterile polyethylene bag and transported to the laboratory of the college of biotechnology Al-Nahrain University. Serial dilutions of each soil samples were prepared by mixing 1g with 9 ml of sterile distilled water, and shaked vigorously, then dilutions of 10^{-4} and 10^{-5} were carried out [11]. Aliquots of 0.1ml of each dilution was spread on TSM agar medium as a selective medium for isolation *Trichoderma* spp. which was constituted as g/L(K_2HPO_4, 0.9 , $MgSO_4.H_2O$, 0.2 , KCl, 0.15, Glucose, 3, NH_4NO_3,1, Chloramphenicol, 0.25, Metalaxyl, 0.01, PCNB, 0.2, Rose Bengal, 0.15, Agar, 20) [12], plates were then incubated at 28°C for 7 days. After incubation, pure fungal isolates were subcultured on PDA agar plates as an inrichment medium for maintenance and identification of fungal isolates. Then plate were incubated at 28°C for 7 days, and kept at 4°C for further analysis. These isolates were identified of fungal isolates according to the genus [13].

2.2 Inoculation of cellulase production medium

Inoculation of cellulase production medium was achieved by transferring 1ml from each fungal fresh cultures of *Trichoderma* spp. which were cultured these isolates on PDB medium and incubated 28°C for 4 days. After that, the spores number were calculated from all isolates using haemocytometer.

2.3 Screening for Cellulase production

2.3.1 Qualitative Screening

Cellulolytic activity of *Trichoderma* spp on CMC agar medium (K_2HPO_4 1, $MgSO_4$ 0.5, KCl 0. 5, CMC Sodium salt 5,Peptone 2, Na NO_3 1, Agar 15) was determined qualitatively by iodine plate assay method which was described by [14]. Fresh culture (6days old) of *Trichoderma* spp. from PDA slant were cultured on CMC agar medium and incubated for 5 days at 30°C, then plates were flooded with Gram‘s iodine solution (It was prepared by dissolving 1 g iodine crystals and 2 g of potassium iodide in 100 ml D.W and stored in a dark bottle) for 3 to 5 min. [15] and incubated for 15 min at 28 °C, then excess stain was drained off in order to observe and measure zone of clearance around each fungal colony which indicates the hydrolysis of CMC. The highest cellulase activity assumed by the largest clear zone of hydrolysis .The Zone of cellulose hydrolysis was appeared as a clear area around the colony [16].

2.3.2 Semi –Quantitative Screening

Semi–quantitative screening for cellulase production by fungal isolates was achieved by using agar diffusion method. Aliquot of 1ml of fresh culture of each fungal isolates were used to inoculate 50 ml of mandel medium containing 1% CMC in conical flasks then incubated at 30°C for four days in shaker incubator (100 rpm). After incubation cultures were centrifuged at 8000 rpm for five minutes, then 0.5ml of culture filtrates were added into wells of CMC – agar plates(0.5% CMC, 1.7% agar) and incubated at 30 °C for 24 hours, then wells were rinsed out using tap water. After that plates were flooded with congo red solution (0.25%)(It was prepared by dissolving 1g of Congo red in 400 ml distilled water, and kept at 4°C until use) and left to stand for 15 minutes, then rinsed with 1M NaCl. Wells containing cellulase activity gave a clear zone measured in mm showing a promotion idea of cellulase activity[17]

2.3.3 Quantitative Screening

Quantitative Screening for cellulase production by fungal isolates was achieved by determining cellulase activity (U/ml) in culture filtrates according to [18] as follows:

1. Conical flasks containing 50 ml of CMC broth medium were inoculated with 1ml fresh culture of each fungal isolates, and incubated in shaker incubator (100 rpm) at 30 °C for four days.
2. After incubation each culture was centrifuged at 8000 rpm for five minutes.
3. Aliquot of 100µl of supernatant (crude enzyme) was transferred to test tubes containing 0.9 ml of 0.5% CMC in 50 mM sodium acetate buffer solution (pH5), and vortexed for few seconds.
4. Test tubes were incubated in water bath at 50 °C for 30 minutes, then reaction was stopped by adding 3ml of 3,5-dinitrosalycilic acid.
5. Test tubes containing reaction mixtures were placed in boiling water bath for five minutes, then

left to cool at room temperature and the absorbance at 540nm was read.

6. Cellulase activity was calculated according to glucose stranded curve[19].

Cellulase activity (U\ml) =

$$\frac{ABS\ (540\ nm)}{Slope} \ x \ \frac{1000}{Glucose\ MW} \ x\ 0.1\ x\ 30min.$$

Specific activity (U\mg protein) =

$$\frac{cellulas\ activity\ (U\backslash ml)}{Protein\ concentration\ (mg\backslash ml)}$$ [20]

Enzyme activity was defined as the amount of enzyme that release 1µmol of glucose per minute under experiment conditions.

2.4 Estimation of protein concentration in culture filtrates

Protein concentration was determined by adding 0.1 ml of crude filtrate to 0.4 ml of Tris-HCl , then 2.5 ml of Coomassie brilliant blue was added. After shaking for 2min., absorbance at 595 nm was measured, and the protein concentration(mg/ml) was calculated from the standard curve for BSA[21].

2.5 Cellulase production by *Trichoderma* spp.

Cellulase production by *Trichoderma* spp was achieved in liquid medium and on solid medium to examine the higher Cellulase production in both culture medium.

2.5.1 Submerged Fermentation

Conical flasks containing 50ml of mineral salt medium (Mandel medium) which was constituted as g/L(K_2HPO_4 2, $MgSO_4 7.H_2O$ 0.3, CMC-Na 7.5, Urea 0.3, NH_4SO_4 1, $ZnSO_4.7H_2O$ 0.0014, $CoCl_2.6\ H_2O$ 0.002, pepton1, Tween80 2 ml, $CaCl_2.2\ H_2O$ 0.3, $FeSO_4.7H_2O$ 0.005, $MnSO_4.H_2O$, 0.0016, Triton X-100, 1 ml) pH 6.5 were inoculated with 1 ml of fresh culture of the each fungal isolates and incubated at 30°C in shaker incubator (100 rpm) for seven days. After incubation cultures were centrifuged at 8000 rpm for five minutes, then cellulase activity and protein concentration were determined in crude filtrates as shown in (2.3.3) and (2..4) respectively.

2.5.2 Solid State Fermentation

To examine the ability of the fungal isolates of *Trichoderma* spp in cellulase production by using solid state fermentation and compare it with the enzyme productivity in submerged fermentation medium, the fungal isolates were grown on wheat bran medium [22].

Ten grams portion of wheat bran in conical flasks were wetted with 20 ml of mineral salt solution which was constituted as g/L(K_2HPO_4 ,5, NH_4NO_3 , 5, $MgSO_4 7H_2O$, 21, Urea, 2, $ZnSO_4.7H_2O$, 0.001, $CoCl_2$. ,0.0002, Pepton, 5, Tween80, 0.5, NaCl ,5, $CaCl_2$, 1, $FeSO_4.7H_2O$, 0.005,$MnSO_4.7H_2O$, 0.001) [23], pH was adjusted to 5, then flasks were sterilized by autoclaving , after cooling to room temperatures flasks were inoculated with 1ml of fresh culture of each fungal isolate and incubated at 30 °C for five days, then 50 ml of D. W. was added to each flask, and shaked vigrously for 1-2 minutes, then centrifuged at 8000 rpm for 5 min., cellulase activity and protein concentration was determined in supernatants as shown in (2.3.3) and (2. 4) respectively . Because enzyme production (specific activity) on solid state fermentation is higher than enzyme production in submerged fermentation, the former was used for cellulase production under the optimum conditions.

3. RESULTS AND DISCUSSION

3.1 Identification of fungal isolates

The results of the identification fungal isolates observed that all isolates are *Trichoderma* spp. according to the taxonomic key [13].

3.2 Ability of local isolates in producing cellulase enzymes

3.2.1 Screening of isolates for cellulase production

To select the most efficient isolates for cellulase production, the local isolates were screened using solid and liquid medium supplemented with CMC as sole source of carbon.

3.2.1.1 Qualitative screening

Cellulases are known to break down cellulose into monomeric structures, i.e. glucose subunits. To test the activity of the enzyme isolated from the fungus, pure carboxymethyl cellulose (CMC, 1%) was used in plate test. After 5days of incubation of cultures of all isolates of *Trichoderma* species in the medium supplemented with CMC, a zone of clearance was produced around the fungal colony because of the enzyme exuded after staining the dye iodine. The width of the zone of clearance indicated the ability of enzyme to hydrolyze cellulose. In the present study, Isolate *Trichoderma* N48 proved to be the best cellulose degrader among all the fungi tested, with maximum clearance zone as shown in table 1 which reported higher zone hydrolysis at 42 mm than all another isolates of *Trichoderma* sp. This result suggests that the enzyme degrades pure CMC and is known to show a degradation zone. The enzyme breaks down the polysaccharide because of which the area surrounded by the enzyme gets reduced to smaller monosaccharides. The dye cannot bind effectively to the monosaccharide and which results in the formation of a clear zone. Similar results have been reported by Pachauri [24] and Zeng [25].

Table 1: Qualitative activity of cellulase enzyme produced by fungal isolates after incubation for 5 days at 30ºC.

Isolate No.	Zone of Hydrolysis (mm)	Isolate No.	Zone of Hydrolysis (mm)	Isolate No.	Zone of Hydrolysis (mm)
N1	18	N17	19	N33	40
N2	17	N18	19	N34	33
N3	19	N19	27	N35	13
N4	18	N20	25	N36	40
N5	12	N21	38	N37	34
N6	15	N22	39	N38	40
N7	15	N23	40	N39	33
N8	41	N24	38	N40	40
N9	40	N25	39	N41	32
N10	10	N26	28	N42	39
N11	23	N27	38	N43	40
N12	33	N28	35	N44	39
N13	20	N29	36	N45	22
N14	29	N30	27	N46	38
N15	28	N31	29	N47	14
N16	40	N32	41	N48	42

3.2.1.2 Semi-quantitative screening

The semi-quantitative method was used to determine the capacity of the studied fungi in the production of cellulase enzyme. Appearance of pale zone around the fungal colony is an evidence of the degradation of carboxymethyl cellulose (CMC-Agar) by the enzyme cellulase produced by these fungi as a result of the disintegration of cellulose into simple sugars. All fungi gave positive results with differed variable levels in their ability to produce cellulase enzyme as reported in table 2. Isolate N 48 produce the highest efficiency in the degradation of carboxymethyl cellulose, it was observed that the clear zone diameter of decomposition around the fungal colony began from the second day of incubation that reached (18) mm in diameter. Similar results have been reported by Rathore [26].

Table 2: Semi quantitative activity of cellulase enzyme produced by fungal isolates after incubation for 24hours at 30ºC.

Isolate No.	Zone of Hydrolysis (mm)	Isolate No.	Zone of Hydrolysis (mm)	Isolate No.	Zone of Hydrolysis (mm)
N1	9.3	N17	10	N33	16.3
N2	9	N18	12	N34	12
N3	10	N19	11.5	N35	8.8
N4	9.5	N20	11.5	N36	14
N5	7.5	N21	14.9	N37	13
N6	8.9	N22	13	N38	12.7
N7	8.5	N23	15	N39	15.8
N8	15	N24	14.7	N40	13.3
N9	14.1	N25	13.5	N41	12.1
N10	8	N26	11.7	N42	16.2
N11	10.5	N27	15	N43	14.9
N12	13	N28	14	N44	15.9
N13	9.1	N29	16	N45	9.7
N14	12	N30	8	N46	15.1
N15	11.8	N31	12.4	N47	8.3
N16	12.9	N32	15.5	N48	18

3.2.1.3 Quantitative screening for cellulase production

3.2.1.3.1 Submerged State Fermentation

All fungi showed variable ability for enzyme production in Smf medium supplement with CMC, but among all the fungi isolated, the strain N48 was selected because they showed the highest level of cellulase production which is amounted 50.1 U/mg as shown in table 3.The weakness of some isolates in the production of cellulase enzyme can be explained as either the incubation period is not enough to stimulate fungi for enzyme production, or due to the difference in the ability of fungi to exploit the media of breeding, and

inappropriate pH of the media of this fungi[27]. However the efficiency of the cellulase enzyme production by the fungi varies by different species [28].

Similar results have been reported by Miklaszewska [29] that found the highest CMCase activity at 0.197 IU/ml was observed for *Trichoderma* isolate 1.12 grown on CMC when compared with other strains of *Trichoderma*. Additionally, Patel [30] who reported that specific activity of cellulase in Smf was higher in *Penicillium* sp. than *Aspergillus* and *Trichoderma* sp.

Table 3: Specific Activity of Cellulase produced by different fungal isolates by Submerged State Fermentation.

Isolate No.	Activity (U/ml)	Specific activity (U/mg)	Isolate No.	Activity (U/ml)	Specific activity (U/mg)
N1	35.35	33.0	N25	1.47	0.6
N2	26.49	20.2	N26	3.69	1.5
N3	37.97	26.0	N27	1.39	0.6
N4	28.05	22.2	N28	4.26	2.5
N5	47.08	35.6	N29	11.48	6.1
N6	29.03	22.3	N30	4.92	3.7
N7	41.42	27.4	N31	19.1	9.1
N8	54.87	35.8	N32	1.8	0.6
N9	28.54	20.8	N33	10.33	6.6
N10	20.99	13.8	N34	4.42	1.4
N11	20.67	15.7	N35	5.08	3.0
N12	30.51	22.4	N36	10.82	5.2
N13	56.10	43.1	N37	5.49	2.6
N14	45.60	32.1	N38	11.48	7.6
N15	75.05	4.3	N39	2.70	0.6
N16	7.38	8.5	N40	4.34	1.6
N17	9.43	5.6	N41	0.57	0.1
N18	8.85	5.0	N42	1.39	0.6
N19	8.77	11.6	N43	2.78	2.05
N20	3.3	2.5	N44	6.64	36.8
N21	3.52	3.3	N45	2.95	1.85
N22	3.93	2.6	N46	23.62	4.2
N23	12.79	6.6	N47	22.31	47.7
N24	3.69	2.1	N48	17.55	50.1

Table 4: Specific Activity of Cellulase produced by different fungal isolates by Solid State Fermentation.

Isolate No.	Specific activity (U/mg)	Isolate No.	Specific activity (U/mg)	Isolate No.	Specific activity (U/mg)
N1	32.6	N17	4.2	N33	1.1
N2	49.8	N18	1.7	N34	4.55
N3	25.0	N19	3.0	N35	0.4
N4	30.5	N20	4.0	N36	8.7
N5	48.0	N21	1.9	N37	23.0
N6	11.6	N22	0.3	N38	29.4
N7	15.6	N23	0.9	N39	24.8
N8	2.4	N24	0.6	N40	20.6
N9	51.6	N25	4.3	N41	31.0
N10	33.2	N26	2.9	N42	11.8
N11	6.0	N27	2.5	N43	26.4
N12	38.3	N28	4.2	N44	18.52
N13	53.7	N29	0.3	N45	0.6
N14	33.5	N30	0.7	N46	28.3
N15	1.6	N31	4.1	N47	38.1
N16	5.0	N32	2.6	N48	56.4

3.2.1.3.2 Solid State Fermentation

In total, forty-eight fungi were isolated and tested for cellulase production under solid state fermentation. Higher yield of cellulase in SSF cultures compared to Smf was reportd by vantila [31], Longwei [32] which considered the SSF process are strongly recommended as systems for producing higher concentration cellulases at lower price than submerged cultures along with reducing the step in downstream processing, in turn reducing the cost of operation. The CMCase activities obtained by SSF, through the action of all isolates, indicting a potentially variable degrees in productivity with the substrate wheat bran, but isolate N48 gave highest efficiency of cellulase enzyme with that substrate after 5 days of the incubation, reaching to 56.4 U/mg as shown in table 4. It should be mentioned that wheat bran has been employed in numerous reports for enzyme production in SSF, because it is a good source of nitrogen and carbon [33]. This result also agrees with the previous reported results by El-shishtawy [34] indicated that the *Trichoderma virens* reported higher CMCase activity with wheat bran compared with other *Trichoderma* species. On the basis of these results, the wheat bran was selected as the indicated substrate for further experiments.

4. CONCLUSION

The selected fungal isolate *Trichoderma* spp. N48 showed and reported maximum cellulase activity among all fungal isolates using several screening and fermentation procedures mentioned.

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