JJBS

Volume 11, Number 1,March 2018
ISSN 1995-6673
Pages 31 - 36

Jordan Journal of Biological Sciences

Optimization of Factors Influencing Cellulase Production by Some Indigenous Isolated Fungal Species

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Received June 16, 2017; Revised September 7, 2017; Accepted September 17, 2017

Abstract

The aim of the present study is to isolate and characterize efficient cellulose degrading fungi from their common natural habitats and optimize the factors influencing the production of cellulase enzyme. Fungi were isolated from soil, tomatoes and oranges samples which were collected from different sites in Khartoum and Khartoum North. Cellulase production was studied after cultivation of fungi in cellulose containing media. The conditions were optimized by using production media containing Carboxymethyl Cellulose (CMC) and inoculated by fungi with different biotechnological parameters, such as temperatures (28 ºC, 37 ºC, and 50 ºC), pH (3.0, 5.0 and 9.0) and substrate specificity of carbon (lactose, maltose and sucrose) & nitrogen sources (urea, yeast extract and sodium nitrate). Among eight initial isolates, three isolates (A, B, and C) were finally screened as the most efficient cellulase producer fungal isolates. These isolates were confirmed as (A) *Aspergillus niger* (B) *Fusarium solani* and (C) *Trichoderma viride*. Out of these isolates, the maximum zone of hydrolysis (54.33±1 mm) was obtained for 'isolate A, whereas the minimum zone of clearance (19.67±1mm) was recorded for *Trichoderma viride.* Cellulase activity and amount of cellulase produced by the three test microorganisms were determined and compared. The results obtained from the fermentations showed that *Aspergillus niger* produced the highest amount of cellulase among the test microorganisms (2.9 IU/ml) at pH 5 and temperature of 50 ºC on Day 5 of fermentation.

Key words*:* Cellulase, Cellulose, *Trichoderma, Fusarium, Aspergillus.*

1. Introduction

Cellulose is the most abundant component of plant biomass, exclusively in plant cell walls (Lee *et al*., 2002). Cellulose is totally insoluble in water (Lederberg, 1992). It is a linear, unbranched homopolysaccharide consisting of glucose subunit joined together via 1-4 glycosidic linkages. Individual cellulose molecules (polymer) vary widely in length and are usually arranged in bundles or fibrils (Walsh, 2015). Within the bundles, cellulose molecules can occur in crystalline or paracrystalline (amorphous) structures (Walter, 1998).

The hydrolysis of cellulose can be done by using enzymes known as cellulases to produce glucose, which can be used for the production of ethanol, organic acids and other chemicals (Koomnok, 2005). Cellulase refers to a class of enzyme that catalyze the hydrolysis of 1, 4 β-D glycosidic linkages in cellulose. These enzymes are mainly produced by fungi, bacteria and protozoans (Jagdish and Pawandeep, 2012).

Cellulases can convert world's most abundant biopolymer, 'cellulose' into reducing sugars and used in many biotechnological applications (Bhat, 2000). A hefty portion of these applications are accounted for instance cotton handling, paper reusing and as creature encourage added substances. (Yano *et al*., 2012). It is used for bioremediation, wastewater treatment and also for single cell protein (Alam *et al*., 2005). Cellulases are likewise utilized for deinking of fiber surfaces in paper ventures and to improve mash seepage in material businesses. (Penttila *et al*., 2004).

This compound is significant in nourishment sciences, like sustenance handling in espresso, drying of beans by for effective cleansing of juices when utilized blended with pectinases. It is also useful in plant protoplast isolation, plant viruses' investigations, metabolic and genetic modification studies (Chandara *et al*., 2005; Shah, 2007). This enzyme has also pharmaceutical importance, treatment of phytobezons - a type of bezoar cellulose existing in human's stomach - (Ali and Saad, 2008).

Fungi are the main natural agents of cellulose degradation; they are widely distributed in nature and used for commercial production of cellulases. Most of the fungi elaborate one or more cellulolytic enzymes including endoglucanase, exoglucanase and β- glucosidase (Bhat and Hazlewood, 2003). Amongst fungi, species of *Trichoderma* and *Aspergillus* are well known for cellulolytic potential (Lee *et al*., 2002).

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Generation of cellulases by fungal isolates requires ideal conditions for their development which prompts the obtaining of extracellular chemical molecules. The development conditions and additionally extracellular protein generation conditions are probably going to shift among test microorganisms. The real parts of generation medium, like carbon and nitrogen sources and physical parameters like temperature, pH and brooding time, were observed to be basically influencing the cellulase creation consequently should be enhanced for each isolate (Polyanna *et al*., 2011).

Therefore, the present study aims to investigate high level production of extracellular Cellulases by *Trichoderma*, *Aspergillus* and *Fusarium* and optimizing cultural parameters to enhance cellulase enzyme production.

2. Materials and Methods

2.1. Area of Study

The present study was conducted at the Department of Microbiology and Molecular Biology, Faculty of Science and Technology, Al-Neelain University, Khartoum – Sudan. All experiments were done aseptically in the laboratory of microbiology.

2.2. Collection of Sample

Five random soil, tomatoes and oranges samples were collected from different areas in Khartoum and Khartoum north. The samples were then brought to the laboratory of Microbiology.

2.3. Isolation of Test Microorganisms

Potato Dextrose Agar (PDA) (Hi-media, India) was prepared according to the manufacturers' recommendations with pH 5.6, sterilized at 121 ºC and 15 Ibs for 15 minutes and poured into the pre-sterilized Petri dishes, the plates were let to solidify at room temperature.

One gram of soil was transferred to aliquots of 10 mL sterile distilled water in test tube. It was shaken vigorously at constant speed for 10 min. The soil suspension was then subjected to serial tenfold dilutions. An amount of 0.1 ml soil suspension from the appropriate dilution (10^{-2}) was transferred to Petri dish containing PDA medium. While an infected spot in tomato and orange samples were swabbed and placed on the middle of the plate of PDA media. The plates were incubated for 5 days at 28ºC. The well-growing spread single colonies were picked up and further sub-cultured on potato dextrose agar (Aneja, 2005).

To confirm their purity and their viability, the isolates were examined macroscopically by determination their culture characteristics. Also they examined microscopically using Lactophenol cotton blue stain technique and compared with those listed in standard reference books (Domsch *et al*., 1980).

2.4. Screening for Cellulase Enzyme Production

Associated fungi were tested for their ability to produce cellulase enzyme by the plate assay method using 1% carboxymethyl cellulose in a modified basal salt media. According to Hankin and Anagnostakis, (1977) at the incubation period, 0.1% Congo red solution was added and counterstained with 1 M NaCl for 15 minutes. The zone of cellulose hydrolysis appears as a clear area around the colony.

2.5. Production of Cellulase Enzyme

Cellulase production was carried out by using cellulose as the sole carbon source in 250ml an Erlenmeyer flask containing broth media. The composition of the medium was in (g/l in distilled water peptone (0.1%), urea (0.03%), $MnSO_4.7H_2O$ (0.0016%), $ZnSO_4.7H_2O$ (0.0014%), $(NH_4)_2SO_4$ (0.14%), $MgSO_4.7H_2O$ (0.03%), $FeSO_4.7H_2O$ (0.05%), $CaCl_2$ (0.01%), $CON_2O.6H_2O$ (0.0029%), KH_2PO_4 (0.2%) and cellulose (1%), the pH value of the media was 5. For the cellulolytic fungi test, 1% CMC, 1.5% agar and 1 ml of Triton-X-100 were also added to the media.

An Erlenmeyer flask (250ml) containing this media was autoclaved at 121ºC and 15 Ibs for 15 minutes. The cellulose medium was inoculated with two plugs (5mm diameter) of fungi isolates from 5 days old culture and incubated on a shaker (Orbital shaker, Gerhardt, Bonn) at 121rpm. After 5 days of cultivation the culture filtrates were filtered off (Whatman filter paper No.1) and transferred into falcon tube to centrifuge (China), at 10,000 rpm for 15 minutes to remove cell debris. The supernatants were used to determine the cellulolytic activity by the standard assay method (Jadhav *et al*., 2013).

2.6. Cellulase Assay for Enzyme Production

Filter paper activity (FPase) for total cellulase activity in the culture filtrate was determined according to the standard method (Eveleigh *et al*., 2009). Aliquots of appropriately diluted cultured filtrate as enzyme source were added to Whatman no. 1 filter paper strip (1 × 6 cm; 50 mg) immersed in one milliliter of 0.05 M sodium citrate buffer of pH 5.0. After incubation at 50ºC for 1 hour, the reducing sugar released was estimated by dinitrosalicylic acid (DNS) method (Ghose, 1987). One unit of filter paper (FPU) activity was defined as the amount of enzyme required to release 1 μmole reducing sugars from filter paper per ml per min under standard assay condition (Gilna and Khaleel, 2011).

2.7. Optimization of Culture Conditions for Cellulose Enzyme Production under Submerged State Fermentation (SmF)

Certain factors were examined and optimized to obtain the highest enzymatic yields, those include:

2.7.1. Effect of pH on Cellulase Enzyme Production:

To determine optimal pH, fungus cultures were cultivated in a 250 mL flask containing 50 mL optimized medium with different pH values 3.0, 5.0 and 9.0. The pH of the medium was adjusted by using 1 N HCl and 1 N NaOH. The flasks were kept in stationary stage at 28 ºC for 5 days of cultivation. After 5 days of incubation the culture broths were filtered off (Whatman No.1 filter paper) and transferred into falcon tube to centrifuge at 10,000 rpm for 15 minutes to remove cell debris. The supernatants were used to assay cellulase enzyme activity by using DNS method (Ghose, 1987). The absorbance was measured using UV-Spectrophotometer at 540nm.

2.7.2. Effect of Temperature on Cellulase Enzyme Production:

In order to determine the optimum temperature for cellulase production by the fungal species, fermentation was carried out at 28 ºC, 37 ºC, and 50 ºC. The flasks were incubated for 5 days of cultivation. After 5 days of incubation the culture broths were filtered off (Whatman No.1 filter paper) and transferred into falcon tube to centrifuge at 10,000 rpm for 15 minutes to remove cell debris. The supernatants were used to assay cellulase enzyme activity by using DNS method (Ghose, 1987). The absorbance was measured using UV-Spectrophotometer at 540nm.

2.7.3. Effect of Carbon Sources on Cellulase Enzyme Production:

Various carbon compounds namely, sucrose, lactose and maltose were used. The broth was distributed into different 250 ml flasks and 1% of each carbon sources were then added before inoculation of the strain and after culture inoculation, the flasks were incubated for 5 days at 28 ºC. After 5 days of incubation the culture broths were filtered off (Whatman No.1 filter paper) and transferred into falcon tube to centrifuge at 10,000 rpm for 15 minutes to remove cell debris. The supernatants were used to assay cellulase enzyme activity by using DNS method (Ghose, 1987). The absorbance was measured using UV-Spectrophotometer at 540 nm.

2.7.4. Effect of Nitrogen Sources on Cellulase Enzyme Production:

The fermentation medium was supplemented with organic and inorganic compounds (Sodium nitrate, urea and yeast extract) replacing the prescribed nitrogen source of the fermentation medium. After 5 days of incubation the culture broths were filtered off (Whatman No.1 filter paper) and transferred into falcon tube to centrifuge at 10,000 rpm for 15 minutes to remove cell debris. The supernatants were used to assay cellulase enzyme activity by using DNS method (Ghose, 1987). The absorbance was measured using UV-Spectrophotometer at 540 nm.

3. Results and Discussion

3.1. Test Microorganisms

Eight initial fungal isolates including molds (*Aspergillus*, *Trichoderma*, *Fusarium*, *Penicillium*, *Alternaria*, and *Curvularia*) and yeasts (Two *Candida* species) were isolated from soil samples, tomatoes and oranges. Three isolates were selected as active cellulase producers, namely *Aspergillus niger*, *Trichoderma viride* and *Fusarium solani*. Other excluded either as saprophytes or non-cellulase producers.

Figure 1. Morphological and cultural characteristics of *Apergillus niger* in PDA using 10 X objective lens

Figure 2. Morphological and cultural characteristics of *Trichoderma viride* in PDA using 10 X objective lens

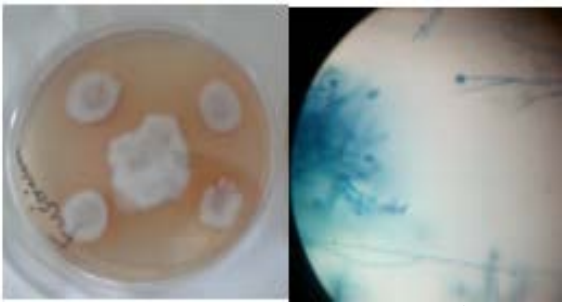

Figure 3. Morphological and cultural characteristics of *Fusarium solani* in PDA using 10 X objective lens

3.2. Screening of Fungi for Cellulase Enzyme Activity

Screening of fungi for their cellulase activity was carried out by the hydrolysis of substrate incorporated in the basal salt medium. After an incubation for 15 minutes, enzyme activities were detected by the appearance of zones either by substrate clearances or coloration and discoloration around the fungal colonies. Three fungal isolates, *Apergillus niger*, *Trichoderma viride* and *Fusarium solani*, showed the highest zone around the colony (Figure 4 (a-d)), used for further study. All the fungal isolates exhibited cellulase activity.

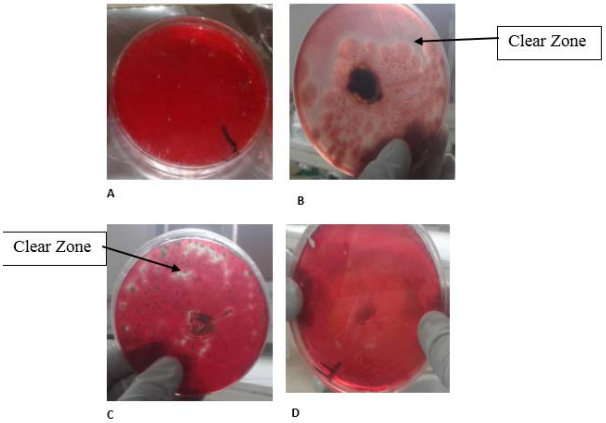

Figure 4. Screening for cellulase enzyme production in BSM at 28 ºC for 15 minute using 1% Congo red (a) Negative control (un-inoculated Petri plate) (b) *A. niger* (substrate clearance) (c) *T. viride* (discoloration around the fungal colonies) (d) *F. solani* (change of indicator colour entire the Petri plate from deep red to pink which indicates the cellulase production)

3.3. Cellulase Enzyme Assay

The cellulase enzyme was detected as yellow coloration after the addition of 3 ml DNS reagent to mixture of sodium citrate and metabolite filtrate of pre-inoculated basal medium (Figure 5).

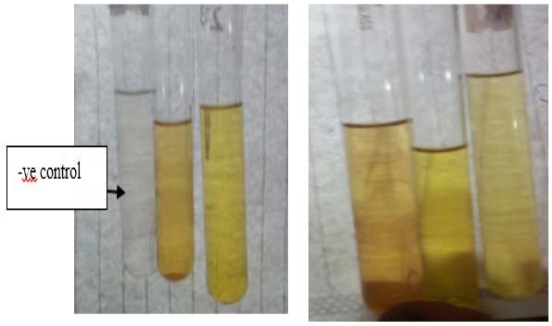

Figure 5. Production of cellulase indicated by the yellow color (Right), the clear solution indicates the negative control which was mixture of sodium citrate and metabolite filtrate of pre-inoculated basal medium without the addition of DNS reagent (Left)

3.4. Optimization of Culture Conditions for Cellulase Enzyme Production

3.4.1. Effect of pH on Cellulase Enzyme Production

Among physical parameters, pH of the growth medium plays an important role by inducing morphological changes in microbes and in enzyme secretion. The pH change observed during the growth of microbes and affects product stability in the medium. The optimal pH varies with different microorganisms and enzymes. All the three isolates were allowed to grow in media of different pH values 3, 5, and 9. The maximum enzyme activity was observed in medium of pH 5.0 in case of *Aspergillus niger* (1.3U/ml) showed (Figure 6, Table 1). The findings of the present study are in agreement with many scientists, such as Beldman *et al*. (1985) who reported that *Aspergillus* species grow and metabolize well in acidic pH medium between pH 3.0 –5.0. Their study investigated that maximum cellulase production from *A. oryzae* was reported when the pH of the medium was adjusted to 5. Ali *et al*. (1991) also stated similar results and he noted that there are certain fungal genera have an ability to produce cellulases include *Aspergilli*. Also the present findings were in line with Pothiraj *et al*. (2006) who determined that the *Aspergillus niger* and *Aspergillus terreus*, *Rhizopus stolonifer* and *Trichoderma* species have an ability to produce cellulases. Shafique and Bajwa, (2009) revealed that pH was the key factor that affects the production of cellulases enzymes from *T. harzianum*. The findings of the present study are more than the findings of Lee *et al*. (2002) who noted that CMCase, Avicelase and FPase activities exhibit a pH optimum of approximately 4.

Table 1. Yield of cellulase enzyme U/ml under different pH value

pH value	Test microorganisms		
	A. niger	*F. solani*	*T. viride*
3	0.91	1.14	0.35
5	1.3	1.25	0.46
9	0.53	0.16	0.18

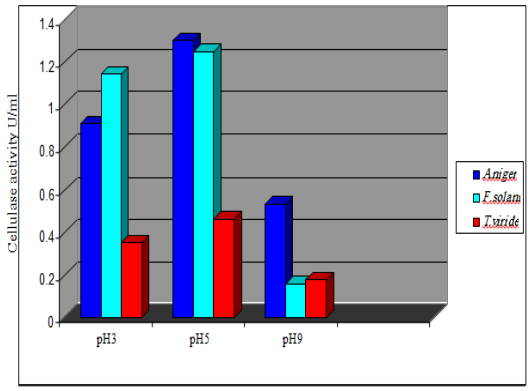

Figure 6. Effect of pH on enzyme production, the most effective pH value was 5. *A. niger* and *F. solani* produce pronounced cellulase at acidic pH and less at alkaline pH

3.4.2. Effect of Temperature on Cellulase Enzyme Production

The results of the test made at different temperatures value (28 ºC, 37 ºC, and 50 ºC) showed that the optimal temperature for cellulase activity (2.9 U/mL) produced by *A. niger* at 50 °C (Table 2, Figure 7). Many researchers have reported different temperatures for maximum cellulase production either in flask or in fermenter studies using *Aspergillus* sp. and *Trichoderma* sp. suggesting that the optimal temperature for cellulase production also depends on the strain variation of the microorganism (Lu *et al*., 2003). The present results disagreed with Immaneul *et al*. (2007) who estimated the optimum temperature for cellulase enzyme production by *A. niger* and *A. fumigatus* at 40 ºC.

Table 2. Yield of cellulase enzyme U/ml under different temperature

Temperature (ºC)	Test microorganisms		
	A. niger	*F. solani*	*T. viride*
28	2.3	2.6	0.96
37	2.19	1.35	1.81
50	2.9	2.44	0.35

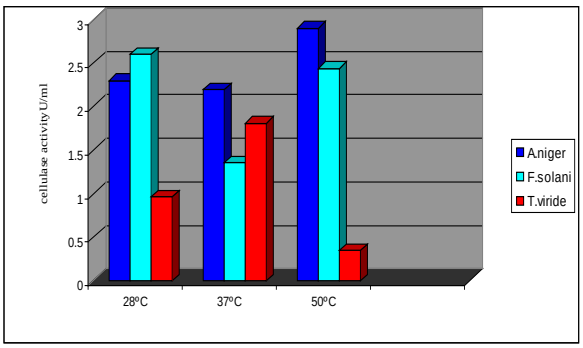

Figure 7. Effect of temperature on enzyme production, *A. niger* and *F. solani* have a wide range of temperature to produce cellulases while *T. viride* produces cellulases with very good amount at ambient temperature 37 ºC. The production of cellulase was reduced when *T. viride* grown at high temperature (50 ºC)

3.4.3. Effect of Carbon Sources on Cellulase Enzyme Production

Various sources of carbon, such as lactose, maltose and sucrose, were used to replace glucose which was the original carbon source in growth media. Results obtained showed that *Fusarium solani* in presence of sucrose brought about the maximum cellulase production compared to other carbon sources (Table 3, Figure 8). This result is in line with Asma *et al*. (2012) who reported that sucrose was the most effective as a sole carbon source for the cellulolytic enzymatic activity. Also, the findings of the present study are in line with Kilikian *et al*. (2014) who estimated that the production of cellulases by certain species of *Trichoderma* was enhanced by the presence of sucrose as a carbon source. Findings of the present study are contrary to those of Vinod *et al*. (2014) who observed that glucose was a sole carbon source.

Table 3. Yield of cellulase enzyme U/ml under different carbon sources

Carbon source	Test microorganisms		
	A. niger	*F. solani*	*T. viride*
Sucrose	1.44	2.14	2.12
Lactose	2.09	1.9	0.53
Maltose	1.18	1.49	0.15

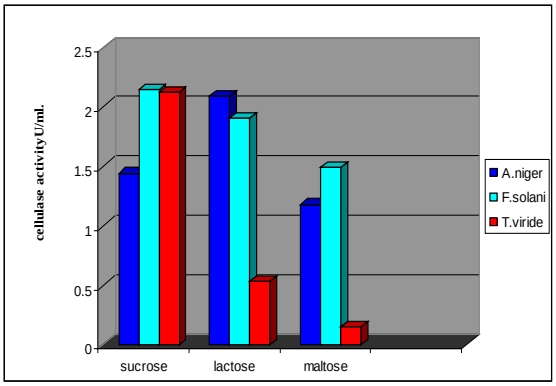

Figure 8. Effect of Carbon sources on enzyme production, the best Carbon source for cellulase production by all test fungi was sucrose; *A. niger* and *F. solani* have an ability to utilize other Carbon sources (lactose and maltose) and produce cellulases but *T. viride* affected negatively when subjected to medium containing lactose and maltose

3.4.4. Effect of Nitrogen Sources on Cellulase Enzyme Production

Results indicate that the sources of nitrogen greatly affected the production of cellulase enzyme. Sodium nitrate was the best nitrogen source for *Aspergillus niger* (Table 4, Figure 9). The current results are in accordance with Swati *et al*. (2014) who reported that inorganic nitrogen source sodium nitrate was found to enhance mean activities. Also similar results were reported by different workers with *Alternaria* spp. including *Alternaria helianthi*, *Alternaria triticina* and *Alternaria sesame* Jha and Gupta, (1988). But the finding is in disagreement with the work of Pothiraj and Eyini (2007) who reported that good cellulase production can be obtained with the

organic nitrogen sources, such as yeast extract and peptone.

Table 4. Yield of cellulase enzyme U/ml under different nitrogen source

Nitrogen source	Test Microorganisms		
	A. niger	*F. solani*	*T. viride*
Yeast extract	0.43	1.8	1.7
Urea	0.99	0.55	2
$NaNO_3$	2.64	2.4	1.74

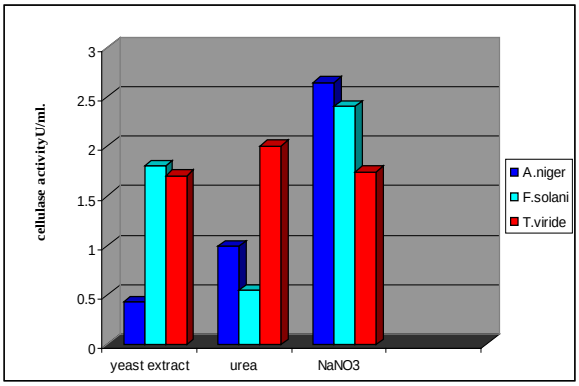

Figure 9. Effect of Nitrogen sources on enzyme production, production of cellulase by *T. viride* was not affected by the different sources of nitrogen and the amount almost similar. *A. niger* produces cellulase significantly when grown in $NaNO_3$ and less significant when use urea and yeast extract as nitrogen sources. *F. solani*, significantly produces cellulases when grown in $NaNo_3$ and yeast extract while produces less amount when grown in medium use urea as nitrogen source

4. Conclusion

The results of the present study showed that all isolated fungi which were confirmed as (A) *Apergillus niger* (B) *Fusarium solani* and (C) *Trichoderma viride* have cellulolytic enzymatic activity. The best result obtained from the fermentations showed that *Aspergillus niger* produced the highest amount of cellulase among the test microorganisms (2.9 IU/ml) at pH 5 and temperature of 50ºC on Day 5 of fermentation.

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