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Investigation of cellulase activity in some soil borne fungi isolated from agricultural soils

Nazanin Nayebyazdi*[1], Mohammad Salary[1], Mohammad Ali Tajick Ghanbary[2], Morteza Ghorbany[1], Mohammad Ali Bahmanyar[3]

[1]Department of Plant Protection, Faculty of Agriculture, University of Zabol, Islamic Republic of Iran
[2]Department of Mycology & Plant Pathology, Faculty of Agronomic Engineering, Sari Agricultural and Natural Resources University, Islamic Republic of Iran
[3]Department of Soil Science, Faculty of Agronomic Sciences, Sari Agricultural and Natural Resources University, Islamic Republic of Iran

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ABSTRACT

The production of cellulose degrading enzymes by cultivation of fourteen fungal strains which isolated from agricultural soils has been studied. Carboxymethyl cellulose (CMC) and wheat straw (WS) were used as carbon source individually. Statistical analysis among strains showed substantial variation in released sugars, but no substantial variation in released proteins. The Trichoderma reesei S542 gave the highest cellulase activity in CMC and WS media. Rhizoctonia solani (AG-1) showed lowest amount of released sugar while Penicillium citreonigrum had the highest protein production. The best pH and temperature for enzyme activity on T. reesei S542 were 4.5-4 and 55 ˚C in CMC and WS media respectively. Glucose was detected as a degradation product by thin layer chromatography. Electrophoresis of the crude enzymes of T. reesei S542 revealed one major protein having a molecular mass 50 KDa on both of media.

Key words: cellulase activity, soil borne fungi, carboxymethyl cellulose, wheat straw, enzyme.

INTRODUCTION

Cellulose, hemicellulose and lignin are the major structural component of woody plants and non-woody plants such as grass and represent a major source of renewable organic matter [25]. The total amount of cellulose on earth has been estimated at 7×10^{11} tons [8]. Cellulose is polysaccharide with the formula $(C_6H_5O_{10})_n$, where ranges from 500 to 5000, depending on the source of the polymer, consisting of a linear chain of several hundred to over ten thousand β (1→4) linked D-glucose unit [37]. Cellulase refers to a group of enzymes (cellulase complex) which acting together hydrolyze cellulose including exoglucanase (FPase), endoglucanase (CMCase) and β-glucosidase (cellobiase) [27]. Therefore, their structural features have also effects on cellulose degradation [14- 22]. Cellulose can be degraded to glucose with this enzyme in synergistic action. A large number of bacteria, fungi and Actinomycetes are known to degrade cellulose [28] but most bacteria can not utilize crystalline cellulose, which can be done by many filamentous fungi [7] with *Trichoderma* as the leading one. Other fungal species were shown to be interesting cellulose producers, such as *Humicola* [40] or *Aspergillus* [11] but some unstudied strains could reveal some peculiarities of cellullases since much still to be known on this class of hydrolases. Researchers have strong interests in cellulases because of their vast applications in industries including starch processing, grain alcohol fermentation, malting and brewing, extraction of fruit and vegetable juices, pulp and paper industry and textile industry [13- 44]. One of the potential applications of cellulases is the production of fuel ethanol from

lignocellulosic biomass [10] which is a good substitute for gasoline in internal combustion engines. Cellulases are commercially available, but still too expensive for production of fuel ethanol. Another problem is that these enzymes are not developed for hydrolysis of lignocellulose [7], and usually produced using glucose as carbon source [41]. In this investigation cellulase activity and enzyme production of some soil borne fungi isolated from agricultural soils of Iran were studied by using CMC and wheat straw as carbon sources. Furthermore some characteristics like optimum pH and temperature for enzyme activity and electrophoretic studies of the enzymes have been discussed.

MATERIALS AND METHODS

Organisms and subculture

Fourteen isolates consisting four genera, *Rhizoctonia*, *Aspergillus*, *Penicillium* and *Trichoderma* were investigated. The strains were maintained on potato dextrose agar (PDA) slants as stock at 4 °C and subculture every month.

Culture media

A broth medium containing 0.05 gr $FeSO_4$ $7H_2O$, 0.25 gr $MnSO_4$ H_2O, 0.25 gr $CoCl_2$, 0.25 gr $ZnSO_4$, 0.25 gr $(NH4)_2SO_4$, 2 gr KH_2PO_4, 0.25 gr $MgSO_4$ 7 H_2O, 0.4 gr $CaCl_2$, 0.3 gr urea, 0.2 ml Tween 80 and 10 gr CMC as a carbon source (without peptone) was used for 1 L, CMC medium [33]. For WS medium, one gram of wheat straw (particle size average 0.5 cm) was prepared in 100 ml erlenmeyer flasks containing 50 ml of distilled water. Both of media were autoclaved at 120 ºC for 20 min.

Inoculation and sampling

Each erlenmayer containing culture medium was inoculated with three pieces (6mm) of PDA of 48 h/old culture of the fungi and were incubated at 25 °C for one month. Sampling was started two days after inoculation and has been continued every two days for sugar and protein assays.

Measurement of sugar and protein concentration

Five µl of broth medium were sampled for sugar assays. Released sugars concentration was determined by Arsenate Molibdate reagent [20]. And extracellular proteins concentration was measured by Bradford method [6].

Ammonium sulphate precipitation of supernatant

The supernatant of the medium fungi was subjected to fractionation by ammonium sulphate precipitation at 50% saturation to remove some proteins, followed by 95% saturation in a second step to gain most of the cellulase activity. The pellet was resuspended in 50 mM Sodium acetate buffer pH 4 and dialysed against double distilled water overnight [35]. The partially purified cellulase was treated at 4 ºC and used for further studies related to electrophorese for molecular weight determination.

Optimization of temperature and pH for improving cellulase Activity

The optimum temperature of partially purified cellulase was determined at 30 to 80 ºC. Effect of pH on enzyme activity was determined by incubating crude enzyme in buffers of different pH for 1 h. The buffers used were 50 mM sodium citrate buffer, pH 3 to 6 and Tris-Hcl buffer, pH 7 to 9 at room temperature (25 ºC).

TLC analysis

Thin layer chromatography was confirmed using silica gel 60G (Merck) in a solvent system of butan-1-ol/acetic acid/water (2:1:1, by vol.), and sugars were visualized by heat treatment at 120 °C for 10 min after the spraying of 50% (v/v) H_2SO_4 in methanol [38].

Enzyme assay

All detected gel bands were cuted after electrophoresis and then incubated in 1.5 ml CMC and WS media at 4 ºC overnight. Then cellulase activity was determined by Kossem and Nannipieri [20] method. Existence of sugars in tubes containing culture medium was a strong document that the fungi produced cellulase in order to degrade cellulose.

Electrophoresis and staining

To determine molecular weight of cellulase enzymes, a poly acrylamide gel electrophoresis was performed in the absence of SDS in Laemmli [21] system. Poly acrylamide gel electrophoresis with absence of SDS was carried out at room temperature for 2.5 h with a 120 V. The protein bands were observed by staining with coomassie brilliant blue G250 (Sigma). The molecular weight of the cellulase was determined in comparison to marker protein (standard protein marker, 14.4-116 KDa; Fermentas, USA).

Statistical analysis
Analysis of variances were conducted using SAS [v. 9.1] statistical software.

RESULTS

The growth process started a few hours after inoculation. There were differences in measurements of sugar and protein assays of strains in both media (Figure 1, 2). Table 1 showed substantial differences for released sugars and no statistical variations for released proteins among different isolates. Both CMC and WS culture media showed substantial differences for protein and sugar assay.

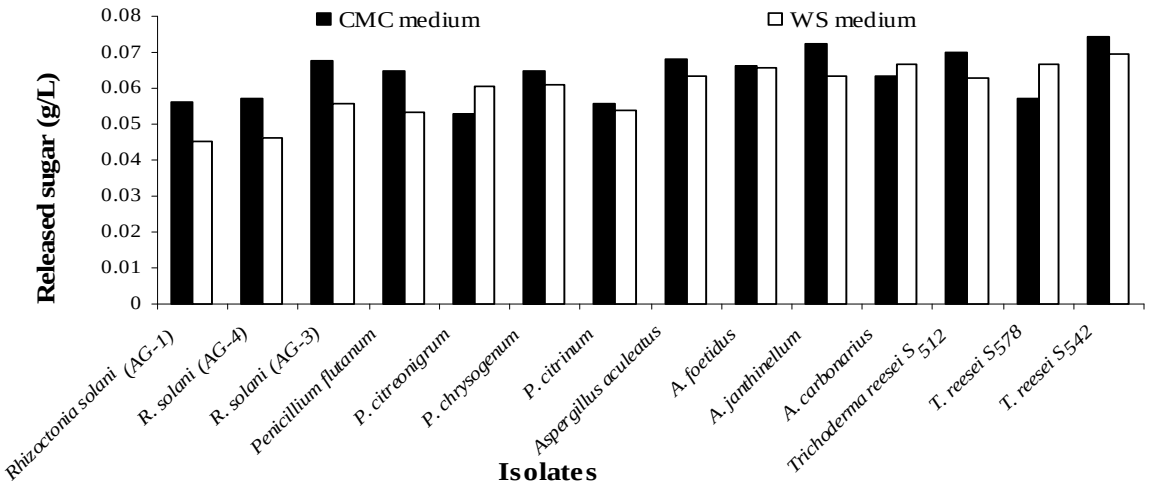

Fig. No. 1: Variations of cellulolitic activities on different strains in CMC and WS media

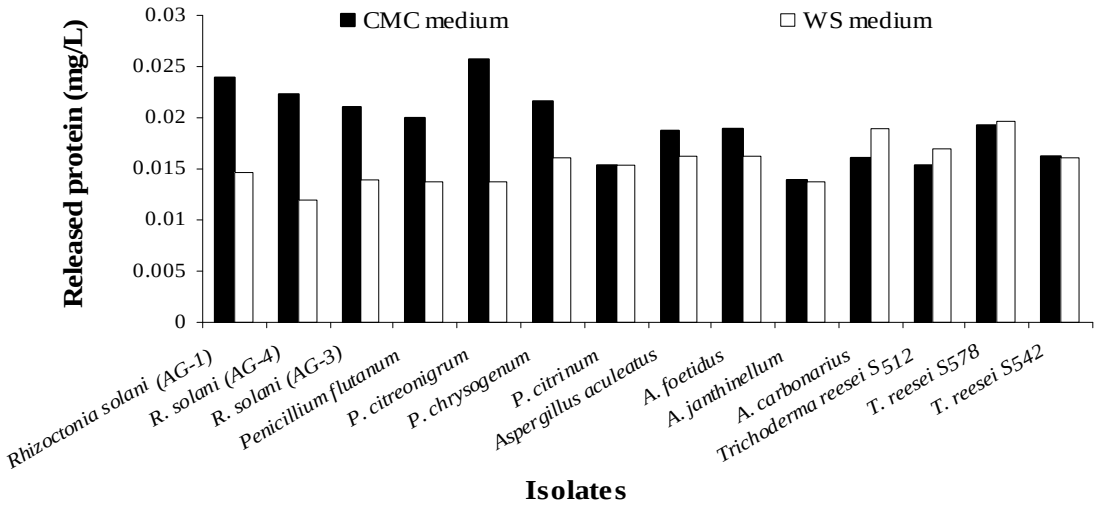

Fig. No. 2: Variations of released proteins on different isolates in CMC and WS media

Sugar and protein assay
Different strains released extracellular enzymes which caused various enhancements in levels of sugar produced from CMC and WS degradation. Although there were substantial differences in released sugars of diverse and similar genera and strains, there is no diversity among isolates resulted to amount of sugar production (Table 1). *T. reesei* S542 indicated the highest while *R. solani* (AG-1) showed the lowest potential of sugar production respectively (Figure 1, Table 2). In the high sugar producer isolate, amount of sugar has been enhanced from 6 to 16 days after inoculation in CMC medium then decreased after days. In WS medium several peaks of released sugars had been observed by *T. reesei* S542 and successively increase and decrease of reducing sugar has been seen (Figure 3). CMC medium sugar production was more than WS medium sugar production (Table 1, 3). Protein assay during experiments showed some gradual changes in released protein concentration. Table 1 and 2 showed no substantial

differences for released proteins among different isolates used intra CMC and WS culture media. However, by comparing between two culture media, there are substantial differences in total released proteins (Table 1, 3). Figure 2 explained various released proteins in CMC and WS culture media for diverse isolates. *T. reesei* S542 with the highest amount of released sugars produced highest released proteins in CMC and WS media in 12 and 10 days after inoculation, respectively (Figure 4).

Table 1 Analysis of variance of protein and sugar assay on CMC and WS culture media

Source of variance	Df	Means	
		Sugar assay (g/L)	Protein assay (mg/L)
Isolate	13	0.00162296**	0.00011985^{ns}
Medium	1	0.00239529**	0.00203659**
Isolate*medium	13	0.00046744*	0.00025192^{ns}
Total error	1232	0.00023637	0.00016073
CV		2.052034	1.762938

* p= 0.05, ** p= 0.01, ^{ns}: non significant

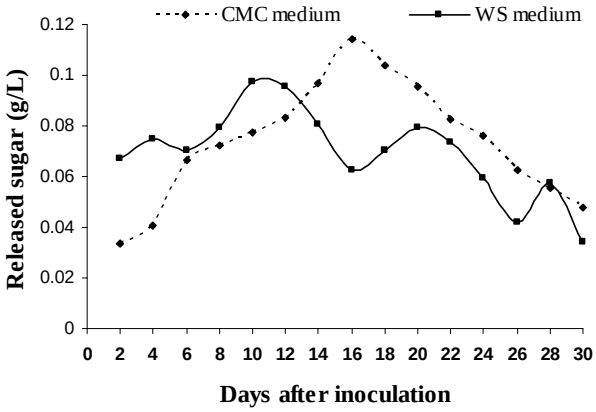

Fig. No. 3: Variations in released sugars from *T. reesei* S542 during sampling days on CMC and WS media

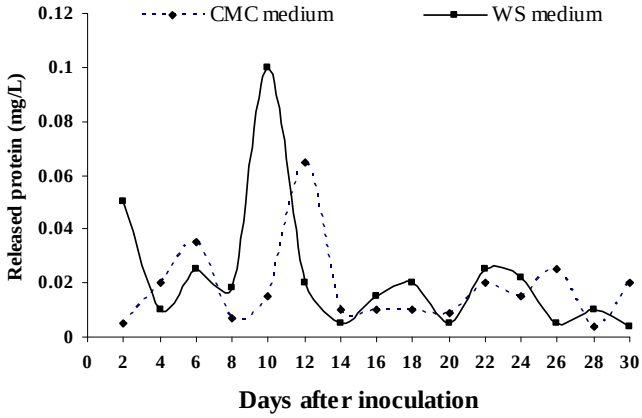

Fig. No. 4: Variations in released proteins from *T. reesei* S542 during sampling period on CMC and WS media

Effect of temperature and pH on enzyme activity

Temperature optimum for enzyme activity was observed at 55 °C on both media. Results from Figure 5 showed that at temperatures higher than 55 °C enzyme starts to losses its activity rapidly as the denaturation of the enzymic protein occurs at elevated temperatures. Results of enzyme assay showed that the cellulase enzymes had an optimum activity at a pH value of 4.5 and 4 in CMC and WS media respectively (Figure 6).

Table 2 Means comparison of protein and sugar assay in different isolates

Isolates	Mean	
	Sugar (g/L)	Protein (mg/L)
Trichoderma reesei S542	0.072041a	0.016167a
Aspergillus carbonarius	0.067757ab	0.017500a
Trichoderma reesei S512	0.066365a-c	0.015833a
Aspergillus foetidus	0.065939a-c	0.017667a
Aspergillus aculeatus	0.065624a-c	0.017500a
Aspergillus janthinellum	0.064934a-c	0.013833a
Penicillium chrysogenum	0.062839b-d	0.018833a
Trichoderma reesei S578	0.062033b-d	0.019500a
Rhizoctonia solani (AG-3)	0.061742b-d	0.017467a
Penicillium flutanum	0.059154c-e	0.016833a
Penicillium citreonigrum	0.056702d-f	0.019667a
Penicillium citrinum	0.054724ef	0.015333a
Rhizoctonia solani (AG-4)	0.051658f	0.017167a
Rhizoctonia solani (AG-1)	0.050654f	0.019333a

Means with the same letter are not significantly different (p= 0.01)

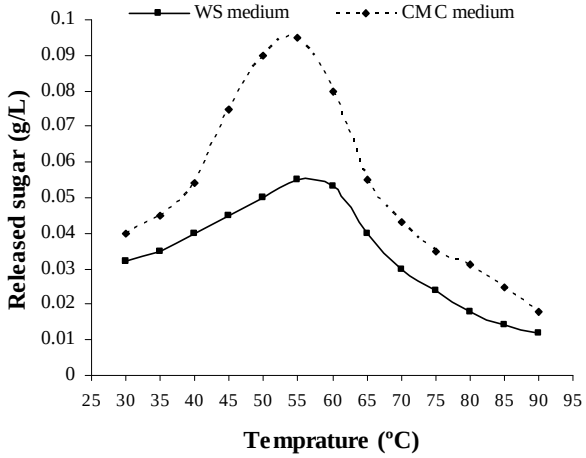

Fig. No. 5: Effect of different temperatures on cellulase activity

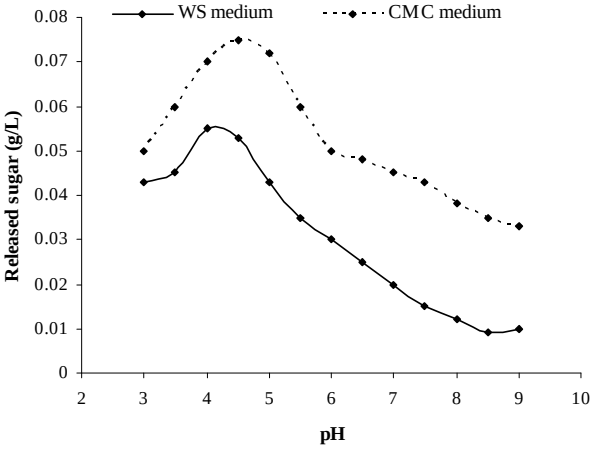

Fig. No. 6: Effect of varying pH values on cellulase activity

TLC

A glucose spot was observed on TLC plate. This result was indicated biochemical paths that carbon source was conversed (Figure 7).

Table 3 Means comparison of protein and sugar assays in different media

Medium	Means	
	Sugar assay (g/L)	Protein assay (mg/L)
Carboxymethyl cellulose (CMC)	0.063627 a	0.019143 a
Wheat straw (WS)	0.059540 b	0.01519 b

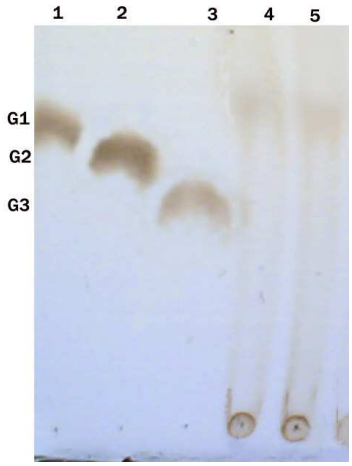

Fig. No. 7: Substrate specificity and degradation products of *Trichoderma reesei* S542 by TLC (A) Lane 1, 2 and 3 standard sugars: glucose (G1), fructose (G2) and cellobiose (G3) Lane 4, *T. reesei* S542 in CMC medium. Lane 5, *T. reesei* S542 in WS medium

Electrophoretic studies

Protein bands with cellulolytic activities were identified using gel electrophoresis and sugar assay method. The band including enzyme activity had 50 KDa molecular mass, whereas the other bands had lower and higher molecular weights (Figure 8).

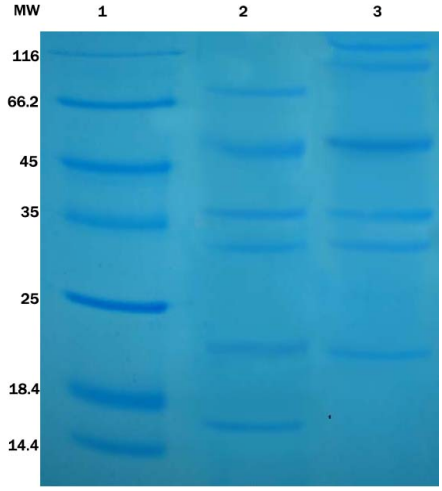

Fig. No. 8: Molecular mass determination of partial purified cellulase by SDS-PAGE, lane MW, molecular weights in KDa of standard marker; lane 1, standard protein markers ß-galactosidase (116 KDa), Bovine serum albumin (66.2 KDa), Ovalbumin (45 KDa), Lactate dehydrogenase (35 KDa), REase Bsp981 (25 KDa), ß-lactoglobulin (18.4 KDa), Lysozyme (14.4 KDa); lane 2, protein profile from CMC medium; lane 3, protein profile from WS medium in *T. reesei* S542

DISCUSSION

The environmental and ecological being pointed at issues in the interest of today's society, like how the cellulase can be successfully used to fulfill cellulose degradation is the main objective of the researchers, while the determination of cellulase activity is the focus of the study [2]. Cellulolytic enzymes play an important role in nature's biodegradation processes where plant lignocellulosic materials are efficiently degraded by cellulolytic fungi and bacteria. In industry, these celluloytic enzymes have found novel applications in the production and processing of chemicals, foods and manufactured goods such as paper, rayon and cellophane. Cellulases, for instance have been extensively utilized for extraction of valuable components from plant cells, improvement of nutritional values of animal feed and the preparation of plant protoplasts in genetic research [26]. The cost of the cellulolytic enzymes is one of the factors determining the economics of a biocatalytic process and it can be reduced finding optimum conditions for their production [24]. Despite the impressive collection of lignocellulolytic microorganisms only a few have been studied extensively [16]. According to this, it is important to determine the hydrolytic capabilities of wood degrading organisms to achieve better digestion of cellulosic materials for industrial purposes [32]. In the present work growth of strains started a few hours after inoculation. It was symptom that fourteen fungi strains could cellulase production and degradation of CMC and WS as carbon sources. This result showed different fungi can

produce extracellular enzymes to made ready their growth requirements and all tested strains in both media had positive cellulase activity.

Sugar and protein assay

According to statistical analysis, the sugar assay showed that there is substantial differences among isolates belong to diverse and similar genera and species in released sugars. The protein assay showed that there are no substantial differences in released proteins among strains. This significant and non significant discrepancies in released sugars and proteins respectively, is probably due to miscellaneous types of enzymes with different biochemical paths which are activated on substrates and insignificant diversities of genetic in similar fungi. Although *P. citreonigrum* generated the highest amount of proteins, but it did not produce the highest released sugars. This event may result from insufficient cellulolytic activity of enzymes of these particular fungi. *T. reesei* S542 showed highest amount of released sugar. Cellulolytic enzymes mostly produced from fungi *Trichoderma* and *Aspergillus* [3]. In *R. solani* (AG- 1) observed lowest level of released sugar (Table 1). This result suggested that the enzyme concentration in total protein was low. In relation to the time course for reducing sugars for CMC and WS culture media in the first stage a continuous increase in reducing sugars concentration was observed. In the second stage significant decrease was seen. Of course in WS culture medium, with difference that increase then decrease were repeated successively. A comparatively high protein production occurred after WS inoculation, probably because of the initial fungal growth [18]. Decrease in protein levels after initial growth could be explained on the basis of catabolic repression. A decrease in the activity of certain catabolic enzymes in the presence of an easily metabolized substrate is called catabolic repression. Commonly this effect is caused by glucose (glucose repression). It has been reported previously that glucose addition greatly repressed enzyme activity [5- 39].

Effect of temperature and pH on cellulase activity

Influence of different incubation temperatures on the production of cellulolytic enzymes was examined. Temperature changes had an effect on the activity of the enzyme. Optimum activity was observed at 55 ^{o}C in both of media. For a variety of industrial applications relatively high thermostability is an attractive and desirable characteristic of an enzyme [12- 15]. Our results are in close agreement with the findings that reported 40-50 °C as an optimum temperate during the characterization of CMCase produced from *Cryptococcus* sp. S-2 [42] whereas 55 °C was found as a best temperature at which the enzyme was most active and stable. Also was reported the same temperature 55 °C as optimum for CMCase activity [34]. For fungi, the optimal temperatures for EG activity are usually between 50 and 60 °C and stable up to 50-55 °C. *T. reesei* is usually used in industrial cellulase production. The optimum temperature of EG from *A. glaucus* was similar to that of *T. reesei* (52 °C) [9- 36], which suggested its potential industrial uses [43]. The enzymes showed optimum activity of pH 4.5 and 4 in CMC and WS media respectively. This shows that acidic conditions favored activity of the cellulases [1] and enzymes produced by *T. reesei* S542 was acidic in nature. Meanwhile the cellulase enzymes were active in a large pH range 4-8 which were little higher than those from *Mucor circinelloides*, 4-7 [19] and *Bacillus circulans*, 4.5-7 [4]. Whereas any further increase in pH from optimum enzyme activity showed decreasing trends in its activity. This little variation in pH optima may be due to the genetic variability among different species [17].

TLC

Glucose spot was observed on TLC plate. This result could be showed cellulase enzymes biochemical paths that carbon source was conversed. Two species *T. ressei* and *T. viride* are popular as it contains high activities of both exoglucanase (FPase) and endoglucanase (CMCase) but low levels of *β*-glucosidase (cellobiase) [23].

Electrophoretic studies

The cellulase activity band identified in this strain (grown on both of media) showed molecular weight 50 KDa which is comparable to those purified from other fungal species *T. viride*, 38-58 KDa [29]. The result regarding the molecular weight of the enzyme is close to CMCase with 54 KDa molecular weight [31]. Furthermore this result showed molecular weight comparable to those reported for endoglucanases from *T. reesei* and *Phanerochaete chrysosporium*, ranging from approximately 25 to 50 KDa. Endoglucanases play a key role in increasing the yield of fruit juices, beer filtration, and oil extraction, improving the nutritive quality of bakery products and animal feed, and enhancing the brightness, smoothness, and over all quality of cellulosic garments [30].

CONCLUSION

Investigation of soil borne fungi to find their capability for cellulase activity is necessary for enzyme industry. A large number of bacteria, fungi and Actinomycetes are known to degrade cellulose, but most bacteria cannot utilize crystalline cellulose, which can be done by many filamentous fungi. Because of it, fungi have made as plausible creature for cellulase production. cellulase production by filamentous fungi differs along with the kind of strain, cultivation conditions like pH, temperature, enzyme concentration and its reaction time, cellulose quality and

composition. So, in this study we tried to screen between isolates and select the best isolates that can produce the most deal of enzyme in especial pH and temperature in both of CMC and WS media. Finally we present *T. reesei* S542 as the best fungi candidate to CMC and WS degradation application in the best pH and temperature that can do it.

Acknowledgement
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REFERENCES

[1]A. O. Adejuwon, A. O. Oni, A. A. Ajayi, P. O. Olutiola, *African Journal of Plant Science* **2009**, *3*, 113-116.
[2]E. T. Aisien, E. R. Elaho, F. A. Aisien, *Advanced Materials Research* **2009**, *62-64*, 258-262.
[3]M. C. Avendano, I. Cornejo, *Biotechnology Letters* **1987**, *9*, 123-124.
[4]Q. K. Beg, R. Gupta, *Enzyme and Microbial Technology* **2003**, *32*, 294-304.
[5]B. Bindu, S. Jitender, R. Kuhad, *World Journal Microbiology* **2006**, *22*, 1281-1287.
[6]M. M. Bradford, *Analytical Biochemistry* **1979**, *72*, 248-254.
[7]P. Cen, L. Xia, *Advances in Biochemical Engineering/Biotechnology* **1999**, *65*, 69–92.
[8]M. Coughlan, *Biochemical Society Transactions* **1985**, *13*, 405-406.
[9]L. M. P. De-Moraes, S. A. Filho, C. J. Ulhaa, *World Journal of Microbiology and Biotechnology* **1999**, *15*, 561-564.
[10]S. J. B. Duff, W. D. Murray, *Bioresource Technology* **1996**, *55*, 1-33.
[11]T. M. Enari, Microbial cellulases. London, Applied Sciences Publishers. **1983**, Pp. 183-223.
[12]M. Fadel, *Online Journal of Biological Sciences* **2000**, *1*, 401-411.
[13]J. Gao, H. Weng, D. Zhu, M. Yuan, F. Guan, Y. Xi, *Bioresource Technology* **2008**, *99*, 7623-7629.
[14]M. P. Geimba, A. Riffle, *Journal of the Science of Food and Agriculture* **1999**, *79*, 1849-1854.
[15]A. Haddar, R. Agrebi, A. Bougatef, N. Hmidet, A. Sellami-Kamoun, A. Nasri, *Bioresource Technology* **2009**, *100*, 3366- 3373.
[16]R. L. Howard, E. Abotsi, E. L. Jansen van Rensburg, S. Howard, *African Journal of Biotechnology* 2003, 2, 602-619.
[17]H. M. N. Iqbal, I. Ahmed, M. A. Zia, M. Irfan, *Advances in Bioscience and Biotechnology* **2011**, *2*, 149-156.
[18]S. Jahangeer, N. Khan, S. Jahangeer, M. Sohail, S. Shahzad, A. Ahmad, S. Ahmed Khan, *Pakistan Journal of Botany* **2005**, *37*, 739-748.
[19]C. H. Kim, *Applied and Environmental Microbiology* **1995**, *61*, 959-965.
[20]A. Kossem, P. Nannipieri, Soil cellulose activity.Methods in Applide Soil Microbiol. Biochem., Academic Prees, San Diego **1995**, 350.
[21]U. K. Laemmli, *Nature* **1970**, *227*, 680-685.
[22]D. Lee, A. Yu, W. Kky, J. N. Saddler, *Applied Biochemistry and Biotechnology* **1994**, *46*, 407-415.
[23]R. S. R. Leite, H. F. Alvez Prado, H. Cabral, F. C. Pagnocca, E. Gomes, R. Da-Silva, *Enzyme and Microbial Technology* **2008**, *43*, 391-395.
[24]L. R. Lynd, P. J. Weimer, W. H. Van Zyl, I. S. Pretorius, *Microbiology and Molecular Biology Reviews* **2002**, *66*, 506-577.
[25]S. Malherbe, T. E. Cloete, *Environtal Science and Biotechnology* **2003**, *1*, 105-114.
[26]M. Mandels, *Biochemical Society Transactions* **1985**, *13*, 414-416.
[27]I. Matsui, Y. Sakai, E. Matsui, H. Kikuchi, Y. Kawarabayasi, K. Honda, *FEBS Letters* **2000**, *467*, 195-200.
[28]M. Nagaraju, G. Narasimha, V. Rangaswamy, *International Biodeterioration and Biodegradation* **2009**, *63*, 1088-1092.
[29]K. Ogawa, Bulletin of the Faculty of Agriculture, Miyazaki University **1990**, *36*, 271-280.
[30]N. J. Parry, D.E. Beever, E. Owen, W. Nerinckx, M. Claeyssens, J. Van Beeumen, M. K. Bhat, *Archives of Biochemistry and Biophysics* **2002**, *404*, 243-253.
[31]Y. Qin, X. Wei, X. Liu, T. Wang, Y. Qu, *Protein Expression and Purification* **2008**, *58*, 162-167.
[32]R. E. Quiroz-Castañeda, E. Balcázar-López, E. D. González, A. Martinez, J. Folch-Mallol, C. Martínez Anaya, *Electronic Journal of Biotechnology* **2009**, *12*, 1-8.
[33]B. L. Rad, R. Yazdanparast, *Biotechnology and Technology* **1988**, *12*, 693-696.
[34]B. C. Saha, *Process Biochemistry* **2004**, *39*, 1871-1876.
[35]W. Schnitzhofer, H. J. Weber, M. Vrsanska, P. Biely, A. Cavako-Paulo, G. M. Guebbitz, *Enzyme and Microbial Technology* **2007**, *40*, 1729-1747.
[36]J. Sharma, A. Singh, R. Kumar, A. Mittal, *the Internet Journal of Microbiology* **2006**, *2*.
[37]K. L. Spence, R. A. Venditti, Y. Habibi, O. J. Rojas, J. J. Pawlak, *Bioresource Technology* **2010**, *101*, 5961-5968.

[38]M. Sugimura, H. Watanabe, N. Lo, H. Saito, *European Journal of Biochemistry* **2003**, *270*, 3455-3460.
[39]H. Suzuki, K. Lgarashi, M. Samejima, *Applied Microbiology and Biotechnology* **2008**, *80*, 99-106.
[40]S. Takashima, A. Nakamura, H. Masaki, T. Uozumi, *Bioscience Biotechnology Biochemistry* **1997**, *61*, 245–250.
[41]A. Thygesen, A. B. Thomsen, A. S. Schmidt, H. Jørgensen, B. K. Ahring, L. Olsson, *Enzyme and Microbial Technology* **2003**, *32*, 606–615.
[42]J. Thongekkaew, H. Ikeda, K. Masaki, H. Lefuji, *Protein Expression and Purification* **2008**, *60*, 140-146.
[43]Y. M. Tao, X. Z. Zhu, J. Z. Haung, S. J. Ma, X. B. Wu, M. N. Long, Q. X. Chen, *Journal of Agricultural and Food Chemistry* **2010**, *58*, 6126–6130.
[44]J. Zhou, Y. H. Wang, J. Chu, Y. P. Zhuang, S. L. Zhang, P. Yin, *Bioresource Technology* **2009,** *99*, 6826-6833.