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*Original Article*

# Screening of some soil *Fusaria* for cellulose activity and partial purification of cellulose

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## ABSTRACT

Several soil borne *Fusaria* species were screened for cellulase activity, partial purification and characterization of cellulase from superior isolates. Carboxy Methyl Cellulose (CMC) and Wheat Straw (WS) were used as two sole carbon sources separately in a minimal culture medium. Released proteins and sugars were assayed three days after inoculation with their related reagents and then these actions were repeated three days intervals. Statistical analysis among tested *Fusarium* species showed significant variation in released sugars, but no significant variation in released proteins. The highest and lowest released sugars were produced by *F. solani* and *F. oxysporium*, respectively. The highest released sugars in *F. solani* isolates were observed 9 and 12 days post inoculation for WS and CMC media, respectively. Released proteins in *F. solani* indicated the highest increased in 12 and 6 days post inoculation on WS and CMC media, respectively. Optimal conditions for cellulase partial purification of *F. solani* grown in CMC and WS supplemented media were pH 6 and temperature between 40-50°C. The study on protein bands showed that, only one with molecular weight of 24 kDa, out of all tested protein bands on CMC medium, indicated the cellulase activity. Our observations showed that different *Fusaria* species have dissimilar behaviors and variable speeds in cellulose degradation. Also, WS medium showed high ability for producing cellulase enzyme that can be effectively used as a cheap organic waste medium. Characterization of cellulase *F. solani* showed that this strain produced a acidophilic, and thermostable cellulase.

**Key-words:** *Fusarium, cellulase activity, Carboxy Methyl Cellulose (CMC), Wheat Straw (WS), Released protein, Released sugar, partial purification*

## INTRODUCTION

Organic wastes from renewable forest and agricultural residues are rich sources of cellulose, hemicellulose and lignin, in an average ratio of 4:3:3 [5]. The exact percentage of these components varies among sources [29]. Cellulose as the most abundant degradable organic compounds produced by plants, consisting about half of impure product of photosynthesis worldwide. It contains a chain of C6H10O5 units with $\beta$-1, 4-glycosidic bands. Other than plants, cellulose is also a part of growing alga, fungi and some protozoan cysts [3-14]. Postharvest return of cultivated plant residue to soil, increase populations of saprophytic fungi and bacteria, then their role expands in breakdown of plants debris [2-33]. In comparison with bacteria, fungi play more important role in plant residue degradation, thus, it can analyses more organic compounds than bacteria. Different fungi like *Zygomycetes, Ascomycetes, Basidiomycetes* and *Deutromycetes* can efficiently degraded cellulose and hemicelluloses [25-27]. Biological decline of cellulose in soil happens gradually. Therefore, it takes place step by step hydrolysis of extracellular enzymes. A complex of cellulase acts synergistically and break $\beta$-1,4 linkages of cellulose chain. At least three cellulase enzymes named Betaglucosidase [EC 3.2.1.21], endoglucanase [EC 3.2.1.4] and exoglucanase [EC 3.2.1.91] are taken part in cellulose degradation [6-32], so, their structural features have also effects on cellulose degradation [7-17]. Present work were undertaken to test a number of saprophytic soil borne fungi for monitoring their roles in nature. From more than 70 *Fusarium* species identified in soils worldwide, all are saprophytes, but some have facultative parasitic activity on plants [9-10]. Soil biologists believed that many biochemical processes take place in cultural soils that thousands of microorganisms play a major role [34]. the present study was targeted to: 1] Screen the indigenous fungal isolates of some genera of *Fusaria* for determine the cellulolytic enzyme activity on Carboxy methyl cellulose [CMC] and wheat straw [WS] as substrates. 2] Effect of different culture media on cellulase production on tested isolates. 3] Appointment of optimum ph and temperature for type of using enzyme in different industrial. 4] Study on electrophoretic characteristics of obtaining information like molecular weight, number of enzymes involved in cellulose degradation in both isolates and 5] Study on similarity of produced enzymes in both media.

## MATERIAL & METHODS

### Fungal isolates and maintenance

Eleven *Fusarium* isolates from cultural soils of Iran belong to *F. oxysporum*, *F. solani*, *F. sporotrichoides*, *F. moniliform*, *F. camptocoras* and *F. tabacinum* and *Fusarium* sp. were selected for current experiments. The isolates were grown on PDA [Potato Dextrose Agar] slants and stored at 4ºC.

### Culture medium

A basic liquid mineral medium with the following composition 0.05 g FeSO4 7H2O , 0.25 g MnSO4 H2O, 0.25 gr CoCl2, 0.25 g ZnSO4, 0.25 gr [NH4]2SO4, 2 gr KH2PO4, 0.25 gr MgSO4 7 H2O, 0.4 g CaCl2, 0.3 g urea, 0.2 ml Tween 80 was used for both carboxy methyl cellulose [CMC] and wheat straw [WS] media. One gram of wheat straw [after cut to 1 cm length pieces] and one gram CMC were prepared in 100ml erlenmeyer flasks containing 50 ml of basal liquid mineral medium and then autoclaved at 120 ºC for 20 min.

### Inoculation and sampling

Each flask was inoculated with one ml of 5000 ml-1 fungal spore suspension and incubated for 31 days at 25ºC based on a completely randomized design with three replications. The flasks inoculated with fungal isolates and incubated for 31 days at. Sampling [500 µl broth medium] was started four days after inoculation and repeated each two days interval for protein and sugar assays.

### Protein and Sugar assay

Five hundred µl of broth medium in each clean test tube were subjected to released proteins and sugars assays. Concentrations of released fungal extracellular proteins and sugars were determined using Bradford method and Arsenate-Molybdate reagent, respectively [4-16].

### Ammonium sulphate precipitation

The proteins in culture medium were precipitated by the addition of solid ammonium sulphate to 80% saturation. The precipitate was allowed to format 4 ºC for 10 h, and was collected by centrifugation of 7000 rpm in a cold centrifuge at 4 ºC for 40 min. The precipitate was dissolved in a minimal amount of 50 mM acetate buffer [pH 5] containing 1 mM EDTA, and then dialyzed for 24 h by three times changes.

**Effects of temperature and pH on cellulase activity**

The optimum temperature of partially purified cellulase was determined at 25 to 55 ºC. The optimum pH of cellulase activity was determined using 50 mM sodium acetate buffer, pH 4 to 6 and Tris-Hcl buffer, pH 7 to 9 at 40 ºC.

**Electrophoresis**

To determine molecular weight of cellulase enzymes, a Polyacrylamide gel electrophoresis was performed in the absence of SDS in laemmli [1970] system. Polyacrylamide gel electrophoresis with absence of SDS was carried out on 12% separating gel and 5% stacking gel. Protein bands were visualized by staining with coomassie R-250 brilliant blue and their molecular weight was determined using Fermentas molecular mass pattern, consisting of ß-galactosidase [116 kDa], Bovine serum albumin [66 kDa], Ovalbumin [45 kDa], Lactate dehydrogenase [35 kDa], REase Bsp981 [25 kDa], ß-lactoglobulin [18.4 kDa], Lysozyme [14.4 kDa].

**Enzyme assay**

All detected gel bands were cut after electrophoresis and then incubated in 1.5 ml CMC medium at 37 ºC for 12 h. Therefore, total amount of released sugars and thus, cellulase activity was determined using Kossem and Nannipieri [16] method. Observation of released sugars in tubes containing culture medium is a reason to prove the existence of active enzyme.

**Statistical analysis**

Analyses of variances were conducted using SAS [v. 9.1] statistical software.

## RESULTS & DISCUSSION

The growth process was started twelve hours after inoculation. Increasing mycelial mats showed substrate breakdown takes place and fungal isolates can produce extracellular enzymes to prepare their growth requirements. Table 1 showed significant differences for released sugars and no statistical variations for released proteins among different isolates. Both CMC and WS culture media showed significant differences for protein and sugar assay.

**Effects of cellulase production on sugar released**

Released extracellular enzymes of different species caused various increase in sugar levels produced from CMC degradation. Although there are significant differences in released sugars of various species, there are no variations among isolates of one species for amount of sugar production [Table 1]. Species *F. solani* indicated the highest and *F. oxysporum* illustrated the lowest potential of sugar production, respectively [Fig. 1-Table 2]. In high sugar producer isolates, sugar level have been increased from 6 to 12 days post inoculation in CMC medium and decreased. In WS medium the peak of released sugars by *F. solani* was produced in day 9th and then decreased in the other days [Figure 2]. Amount of sugar production in CMC medium was higher than WS medium [Table 1-3].

**Effects of cellulase production on protein released**

Protein assays during experiments showed some gradual changes in released protein concentration. Table 1 and 2 showed no significant statistical variation for released proteins among different isolates used intra CMC and WS culture media, however, in comparison between two culture media, there are significant differences in total released proteins [Table 1 -3]. Figure 3 demonstrated various released proteins in CMC and WS culture media for different *Fusarium* isolates. Species *F. solani* with the highest amount of released sugars produced highest released proteins in CMC and WS media in 9 and 6 days post inoculation, respectively [Fig. 4]. However, the additive trend of released proteins was reduced in the next remained days.

**Effect of pH on enzyme activity**

The optimum pH of extracted cellulase of CMC and WS media inoculated by *F. solani* was observed at pH 6 [Fig. 5].

**Effect of temperature on enzyme activity**

The optimum temperature of extracted cellulase from CMC and WS media inoculated by *F. solani* was observed at 40 and 50°C, respectively [Fig. 6].

**Electrophoretic studies**

Figure 7 represents the protein profile of cellulase produced by *F. solani* using CMC and WS culture media. The protein fractions have been assigned numbers in order to increase mobility towards the anode pole. In case of *F. solani* grown on both CMC and WS culture media, only one band with molecular weight of 24 kDa, out of all tested protein bands indicated the cellulase activity.

Cellulase complex enzymes have a series of industrial applications that increases their

commercial importance. The fungi are described as the best sources of cellulase. Screening of different genera, species and even isolates is the first step for finding acceptable enzyme producer isolates. Macris [19] in his work on some genera like *Trichoderma, Fusarium, Aspergillus, Phanerocheate, Chrysosporium* and *Sclerotium* showed some differences in their cellulase activity. [26] reported that *T. harzianum* and *A. niger* showed highest and *Trichothecium roseum*, *T. reesei*, *A. ochraceus* and *Penicillium italicum* using CMC as their carbon source exhibited lower activity. Jahangeer et al. [15] measured cellulase activity of 115 isolates of fungal strains and reported that *Trichoderma*, *Aspergillus* and *Fusarium* showed the highest cellulase activity. Cellulase activity of sharp saprophytic soil inhabitant fungi and some allied species and cellulase characteristics of superior isolates were examined in current work. Youshida et al. [37] from 520 strains of plant pathogenic fungi that produce higher levels of cellulolytic enzymes showed that *F. oxysporum* strain SUF850 was the best producer.

The results of sugar assay showed that there is no significant differences among isolates belong to one species in released sugars, however, significant statistical variations are detectable among different *Fusaria* species.

The protein assay showed that there are no significant differences in released proteins among different *Fusaria*. Existence of significant and non-significant differences in released sugars and proteins respectively, is probably due to various types of enzymes which are activated on substrates. Although *F. oxysporium* and F. sp. generated the highest amount of proteins, produced the lowest amount of sugars. This phenomenon may result from poor cellulolytic activity of secreted enzymes of these fungi.

A high protein production is takes place after CMC inoculation, probably because of fungal initial growth [30]. Decrease in protein level is probably due to the feedback effects of CMC degradation and protease secretion [23-36]. In current work, both protein and sugar assays were indicated the highest amount in *F. solani* on CMC and WS media 6 and 9 days post inoculation, respectively. However, Alconada and martinez [1] suggested that the highest celluloytic activity was occurred in 15 days post incubation by *F. oxysporum*. Similar to results obtained in present work were reported in *Fusarium oxysporum* [31], *Alternaria alternata* [11], *Neurospora sitophila* [20], *Aspergillus terreus* [12] and *Sclerotinia sclerotiorum* [24].

## CONCLUSION

Statistical analysis showed that released sugars in CMC were higher than WS, however, released proteins in WS were higher than CMC. Because of high level cellulose existed in CMC than WS, more sugars were produced in CMC, while in WS, in contrast, due to having low level of cellulose, fungus enforced to produce higher amount of enzymes. Despite CMC is more suitable medium for cellulase production [21], WS as an abundant and cheaper material in nature, can also be used for this aim.Factors affecting enzyme properties are temperature, pH, enzyme concentration and its reaction time, cellulose quality and composition [8-22]. Each enzyme has its maximum activity in an optimum temperature and pH, however, its activity will be reduced by higher or lower temperature and pH [18]. In present work, optimum pH for extracted cellulases from both media using *F. solani* was defined as 6, however, optimum temperature for CMC and WS media were 40 and 50°C, respectively. Wood [35] reported that optimum CMCase activity in *F. solani* was in pH 5.4 and optimum temperature was 65°C. The optimum activity of endoglucanase and ß-glucosidase extracted from *F. oxysporium* was happened at pH 4.5- 4.8 and temperature 60°C [28].

From electrophoretic analysis conducted for crude enzyme of *F. solani* in both culture media, only one cellulose band with 24 kDa was detected in protein profile. Youshida et al [37] found a similar protein band with 24 and 23 kDa for CMCase in *F. oxysporium* and Immanuel et al [13] also reported 23 and 21 kDa for *A. niger* and *A. fumigatus* on coir waste medium, respectively.

Ultimately, current study resulted that different *Fusaria* species demonstrated various analytical behavior for cellulose degradation. Other microorganisms which are known as sugar consumer, especially soil bacteria can also change the in vitro results for cellulose degradation. Studies on interaction between different soil inhabitant microorganisms are an interesting subject for future study. In present study a member of strong soil saprophytic flora was tested for monitoring how they act in their natural habit. More than 70 *Fusaria* species are identified from soils worldwide some with saprophytic behavior and some with facultative parasites on plants.

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**Table1.** Analysis of variance of protein and sugar assay on CMC and WS culture media

| Source of Variations | DF | Mean Square | |
|---|---|---|---|
| | | Protein assay | Sugar assay |
| Isolate | 10 | $0.00097^{ns}$ | $0.0017^{**}$ |
| Medium | 1 | $0.0051^{**}$ | $0.32^{**}$ |
| Isolate*medium | 10 | $0.0011^{ns}$ | $0.0018^{**}$ |
| total Error | 638 | 0.000077 | 0.00022 |
| CV | | 2.6 | 1.35 |

***p=0.01*

**Table 2.** Means comparison of protein and sugar assays in different isolates

| Isolate | Protein assay (mg/lit) | Sugar assay (g/lit) |
|---|---|---|
| *Fusarium oxysporum* | 0.0271a | 0.0306c |
| *F. camptoceras* | 0.0194a | 0.0427b |
| *F. solani* | 0.0245a | 0.0587a |
| *F. solani* | 0.0236a | 0.0562a |
| *F. sporotrichides* | 0.0264a | 0.0467b |
| *F. moniliform* | 0.0235a | 0.0463b |
| *F. moniliform* | 0.0182a | 0.0454b |
| *F. moniliform* | 0.016a | 0.0454b |
| *F. tabacinum* | 0.0245a | 0.0439b |
| *F. tabacinum* | 0.0165a | 0.0431b |
| *Fusarium* sp. | 0.0185a | 0.0412c |
| LSD | 0.02 | 0.006 |

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

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

| medium | Protein assay (mg/lit) | Sugar assay (g/lit) |
|---|---|---|
| Wheat straw (WS) | 0.0244a | 0.0234b |
| Carboxy methyl cellulose (CMC) | 0.0188b | 0.0675a |

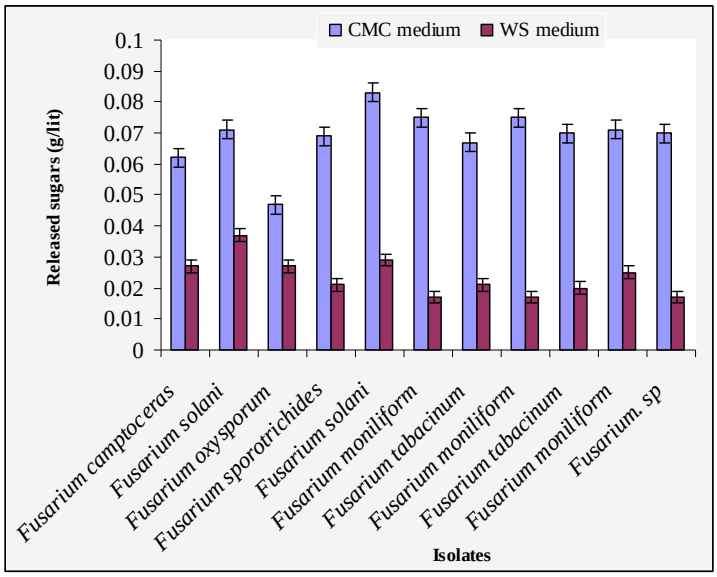


**Fig1**. Variations in released sugars from different isolates

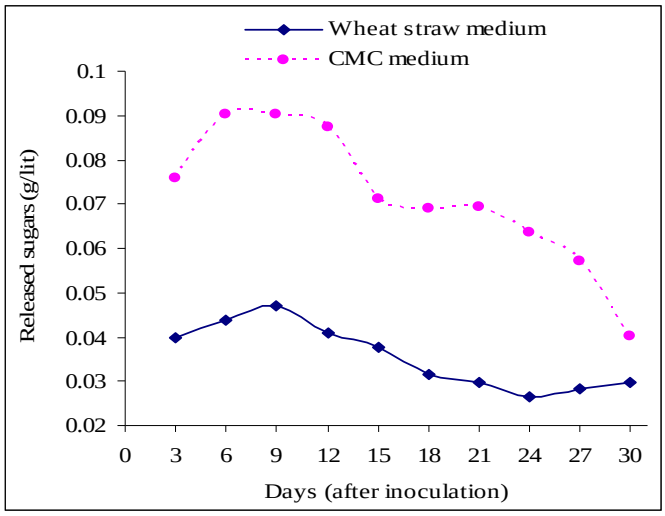


**Fig2**. Variations in released sugars from *F. SOLANI* during sampling days in CMC and WS medium

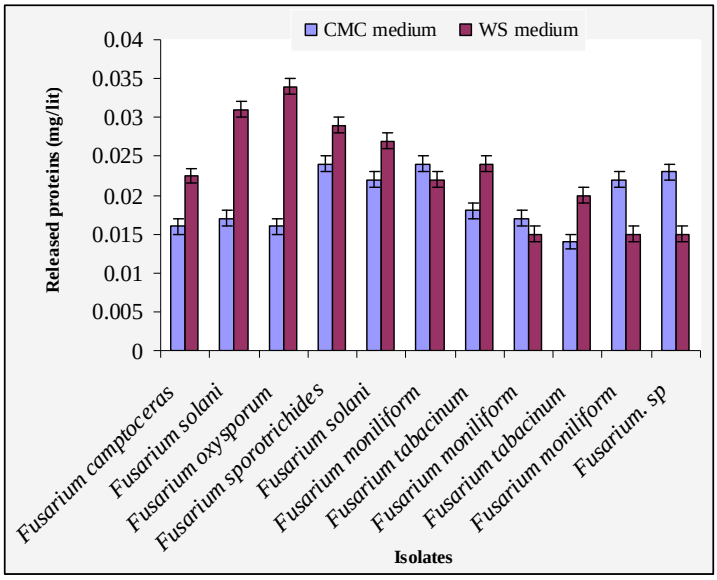


**Fig3**. Variations in released proteins from different isolates

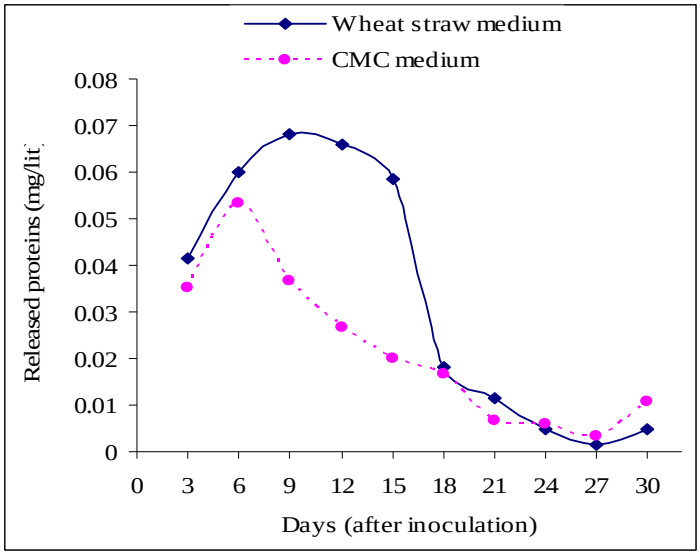


**Fig4**. Variations in released proteins from *F. SOLANI* during sampling period in CMC and WS medium.

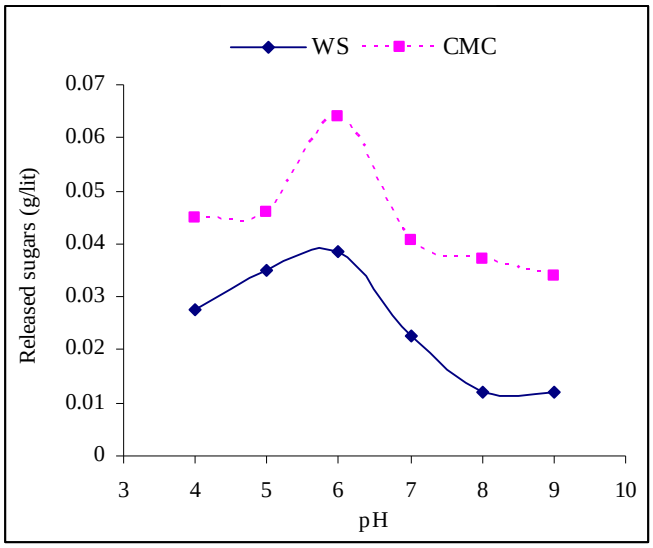


**Fig 5**. Effect of pH on activity extracted cellulase of WS and CMC from *F. solani*

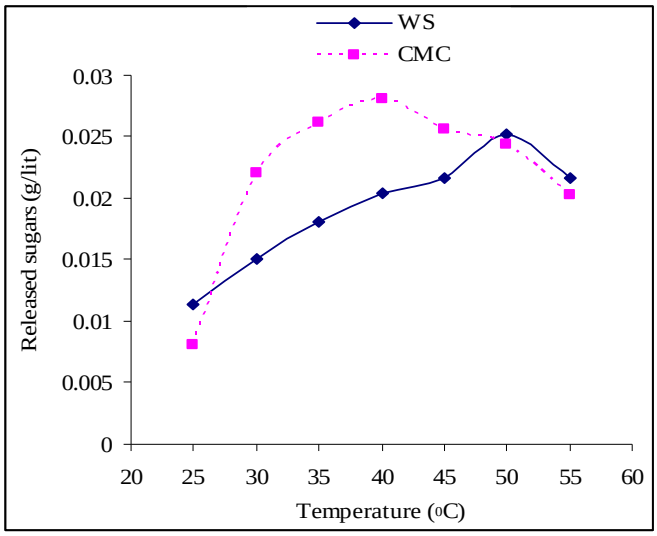


**Fig6**. Effect of temperature on activity extracted cellulase of WS and CMC from *F. solani*

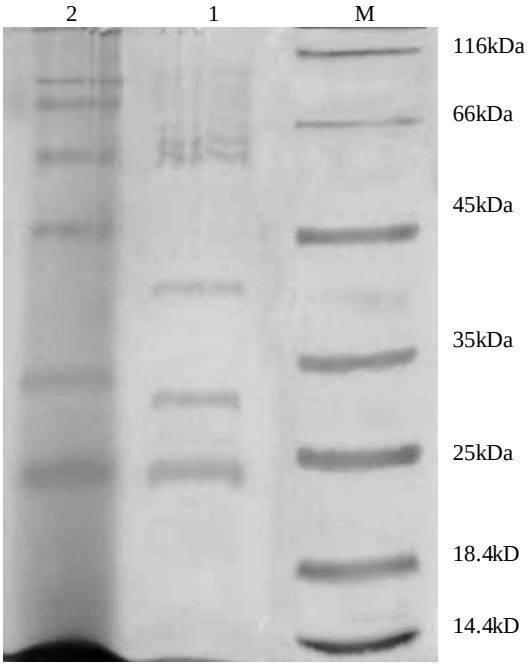


**Fig7**. PAGE of crude protein concentrated by ammonium sulfate. 12% separating gel was used, stained with coomassie R-250 brilliant blue. A. protein profile produced by *F. solani* at WS (lane 1) and CMC (lane 2). M. protein size marker.