

Screening, Purification, and Characterization of Cellulase from Fungi Isolated from Used Mushroom Substrate

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A large number of microorganism especially filamentous fungi has ability to degrade cellulose. The purpose of this study was to conduct screening, purification, and characterization of cellulase from fungi which was isolated from used paddy straw mushroom substrate. Screening of cellulolytic activity using CMC medium shown that 11 out of 20 isolates of fungi produced a clearing zone surrounding fungal colony. Among them isolate number JMF 12 showed the highest cellulase activity and was further used for purification and characterization. The cellulase was purified to electrophoretical homogeneity by ammonium sulfate precipitation, dialyzed by Novagen D-Maxi Tube™ Dialyzer, and Sephadex G-100 gel filtration chromatography. The recovery and purification fold was 3.82 % and 1.98 respectively, after Sephadex G-100 gel filtration chromatograph. The purified cellulase had an optimal pH and temperature at 6 and 45 °C. The K_m and V_{max} of cellulase was 11.43 mM and 0.006 $\mu\text{mol min}^{-1}$ respectively. The purified cellulase was activated by Na^+ and Zn^{++} but inhibited by Ca^{++} , Co^{++} , Fe^{++} , and Hg^{++} .

Key words: Cellulase, used mushroom substrate

Beberapa jenis mikroba terutama jamur tingkat rendah mempunyai aktivitas selulase yang cukup tinggi. Penelitian ini bertujuan untuk melakukan seleksi beberapa isolat jamur yang mempunyai aktivitas selulase. Isolat jamur tersebut diisolasi dari limbah media budidaya jamur merang. Selanjutnya juga dilakukan pemurnian dan pengungkapan karakter enzim selulase dari isolat terpilih. Hasilnya menunjukkan bahwa isolat dengan kode JMF 12 mempunyai aktivitas selulase paling tinggi diantara 20 isolat lainnya. Enzim tersebut kemudian dimurnikan dengan metode pengendapan amonium sulfat, didialisis dengan *Novagen D-Maxi Tube™ Dialyzes* dan *Sephadex G-100 Gel Chromatography*. Aktivitas selulase mengalami *recovery* dan peningkatan aktivitas sebanyak 3.82% dan 1,98 setelah dimurnikan dengan gel kromatografi. Selulase yang diperoleh mempunyai aktivitas optimal pada pH 6 dan suhu 45°C. Nilai K_m dan V_{max} berturut turut adalah 11.43 mM dan 0.006 $\mu\text{mol min}^{-1}$. Aktivitas selulase meningkat terhadap pengaruh Na^+ dan Zn^{++} dan menurun terhadap pengaruh Ca^{++} , Co^{++} , Fe^{++} dan Hg^{++} .

Kata kunci: limbah media tanam jamur merang, pemurnian enzim, selulase

Cellulose is the most abundant organic polymer and represent about 50% of the cell wall material of plants. It represents about 1.5×10^{12} tons of the total annual biomass production through photosynthesis process especially in the tropical area. It is considered to be an almost inexhaustible source of raw material from different products such as forest products, agriculture, and food processing (Sukumaran *et al.* 2005; Hussain *et al.* 2012). A promising technique for efficient utilization of cellulose resources is the microbial hydrolysis of cellulose material to produce a smaller sugar component. Cellulases are a group of hydrolytic enzymes which has ability to hydrolyze cellulose. Many microorganisms are capable of producing extra-cellular cellulase. Important among

these are the filamentous fungi which digest cellulosic wastes rapidly through their high metabolic rates. The biological degradation of cellulose has been studied for many years, and a number of cellulolytic enzymes, especially cellulases produced by fungi and bacteria have been isolated and characterized (Yin *et al.* 2005; Bakare *et al.* 2005; Khokhar *et al.* 2012).

Bioconversion of cellulosic material to produce edible mushroom can play an important role in managing organic waste. In Indonesia, paddy straw mushroom (*Volvariella volvacea*) is one of the important edible mushrooms which is cultivated in paddy straw (Chang and Miles 1997). The aim of the study was to conduct screening, purification and characterization of cellulase from fungus which was isolated from used paddy straw mushroom substrate.

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MATERIALS AND METHODS

Microorganisms. 20 isolates of fungi isolated from used mushroom substrate of rice straw mushroom (*Volvariella volvaceae*) in Indramayu, Indonesia was deposited on Indonesian Culture Collection (Ina CC), Research Center for Biology, Indonesian Institute of Sciences. Isolates was cultured on Potato Dextrose Agar (PDA) medium.

Screening of Cellulolytic Activity. Cellulolytic activity of the fungal isolates was determined by using plate screening medium of Carboxy Methyl Cellulose (CMC). The medium contained 0.4 g L^{-1} CMC, 0.5 g L^{-1} $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.03 g L^{-1} KNO_3 , 1.0 g L^{-1} K_2HPO_4 , 0.0008 g L^{-1} $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.08 g L^{-1} yeast extract, 2 g L^{-1} NaNO_3 , and 18 g L^{-1} agar. The 5 mm in diameter of agar blocks from one-week old fungal colony grown on PDA plates were inoculated in the center of the CMC media plates. The plates were incubated at room temperature for 2 d. Cellulolytic activity was observed by clearing zone diameter surrounding the fungal colonies. Observation was done by staining the fungal colony with 0.1% Congo red dye, followed by destaining with 1 % NaCl solution. Cellulase activity on medium CMC was determined by the Index of Relative Enzyme Activity (ICMC). It was recorded as the ratio of clearing zone diameter to the diameter of colony (Khokhar *et al.* 2012).

Cellulase Activity Assay. The cellulase activity assay was done by using CMC as a substrate. The crude enzyme solution ($125 \mu\text{L}$) was incubated with $875 \mu\text{L}$ of 0.5 % CMC (w/v) in 0.5 mM sodium phosphate buffer (pH 6.0) at 37°C for appropriate time. After incubation, the enzymatic reaction was added with 1 mL of DNS (3,5- Di Nitro Salicylic Acid) and the reaction was terminated by boiling in water bath for 5 min. Subsequently, the sample was withdrawn and the absorbance was measured at 540 nm (Miller 1959) using a Shimadzu 1700 spectrophotometer. Glucose was used at a concentration of $0.10 - 0.25 \text{ mg mL}^{-1}$ in accordance with the standard curved for cellulase activity measurement. One unit of the enzyme activity was defined as the amount of enzyme which released $1 \mu\text{mol}$ of glucose per minute (U mL^{-1}).

Determination of Protein Concentration. The protein concentration of the crude enzyme as well as that of the purified one was determined by Bradford method (Bradford 1976) using bovine serum albumin (BSA) as protein standard.

Purification of Cellulose. Based on the screening

of cellulolytic activity, fungi with the highest cellulase activity was grown at a temperature of 37°C for 4 d on rotary shaker (120 rpm) in a 500 mL Erlenmeyer flask containing 300 mL of medium Potato Dextrose Broth (PDB). The broth, then was centrifuged at 9500 rpm for 30 min and passed through Whatman filter paper grade 42 to remove cells. The crude cellulase was precipitated by 10-80% saturation of ammonium sulfate. The precipitate was centrifuged at 9500 rpm for 30 min and dialyzed (Novagen D-Maxi Tube™ Dialyzer, MWO 6-8 kDa) against 10 mM phosphate buffer (pH 6.5) overnight. The resulted crude enzymes were eluted by Sephadex G-100 gel filtration chromatography ($2.0 \times 30.0 \text{ cm}$) with 10 mM phosphate buffer (pH 6.5) at flow rate 1.0 mL min^{-1} . Fractions with cellulase activity were collected and used for the characterization and determination of kinetic parameters.

Determination of Kinetic Parameter. The apparent kinetic parameters ($V_{\max}$ and K_m) of the cellulase were determined by varying the concentration of Carboxy Methyl Cellulose (CMC) from 0.05 to 0.30% in 0.5 mM sodium phosphate buffer (pH 6.5). The apparent kinetic parameters were determined from double reciprocal plots (Lineweaver and Burk 1934).

Effect of pH, Temperature, and Cations on Cellulase Activity. In order to reveal the optimum pH value for purified cellulase, the activity of the enzyme was assayed on various pH values from 4.5 to 7.5. Furthermore, the activity was also determined by incubated the enzyme with substrates at different temperatures ranging from 25 to 55°C . The effect of cations on cellulase activity was determined by adding cations: Ca^{++} , Na^+ , Co^{++} , Fe^{++} , Zn^{++} , and Hg^{++} at concentration 10 mM on the reaction mixture. All of cellulase activity assay was carried out by mean of DNS assay.

RESULTS

Screening of Cellulolytic Activity. Clearing zone surrounding fungal colonies on the plate screening medium indicated that the fungus has cellulase activity (Fig 1). Among 20 fungal isolates, there were 11 isolates shown clearing zones. Determination of Indexs of Relative Enzyme Activity on CMC medium (ICMC) was shown in Figure 2. It showed that fungal isolate JMF 11 had the highest ICMC of 0.66, following by JMF 12 and JMF 6 with the same value of 0.50. Furthermore, the fungi of JMF 11, JMF12, and

JMF 6 were grown on PDB medium to produce crude cellulase. Their activity was determined using CMC as a substrate. The results showed that isolate of JMF 12 had the highest activity of 2.78 U mg^{-1} (Fig 3). For that reasons, fungal isolate JMF 12 was used in this study for production of cellulase.

Purification of Fungal Cellulose. The results of purification of cellulase produced by the fungi JMF 12 were summarized in Table 1. Ammonium sulphate precipitation gave purification fold of 1.32. Furthermore, the crude enzyme was dialyzed using Novagen D-Maxi Tube TM Dialyzer and the purification 1.76 fold was achieved. The dialyzed enzyme was subjected to Sephadex G-100 gel filtration chromatography and achieved the purification fold to 1.98. Figure 5 shows the elution profiles (chromatogram) of cellulase from JMF 12. A large protein molecules of cellulase was eluted in a fraction collector no 2 and obtained a single peak at fraction collector no 3. However, a small peak of protein also appeared at fraction collector no. 8. The pattern of total protein in that chromatogram was also similar to that of cellulase activity.

Determination of Kinetic Parameter. The determination of Michaelis-Menten constants, K_m and V_{max} of the purified cellulase for Carboxy Methyl Cellulose (CMC) were calculated from the double reciprocal plot of the data obtained for cellulase activity at various substrate concentrations. The K_m and V_{max} was 11.43 mM and $0.006 \mu\text{mol min}^{-1}$ respectively

Characterization of Cellulase Activity. The optimum pH for cellulase activity of purified enzyme produced by fungi JMF 12 was at pH 6.0. It was $3.39 \text{ Unit mL}^{-1}$. The lowest activity of $0.77 \text{ Unit mL}^{-1}$ was at pH 7.5 (Fig 4). On the other hand, the enzyme activity from fungi JMF 12 was optimum at 45°C of $3.74 \text{ Unit mL}^{-1}$ and at 25°C the enzyme was shown the lowest activity of $2.75 \text{ Unit mL}^{-1}$ (Fig 5). Furthermore, as shown in Figure 6, addition of some cations to the reaction mixture affected the cellulase activity. The results showed that addition of cations Na^+ and Zn^{++} increased cellulase activity of 4.97% and 27.18% respectively. The addition of cations Ca^{++} , Co^{++} , Fe^{++} , and Hg^{++} decreased of the enzyme activity from 2.95 to 23.41%.

DISCUSSION

Most agricultural residues are rich in lignocellulosic compounds whose handling and disposal are often problematic, because of their chemical structure and decomposition properties (Philippoussis *et al.* 2001). Mushrooms are well known for their delicacy, nutritional and economical values but the substrate released after mushroom crop harvest, better known as “used mushroom substrate” is also the subject of great importance. The substrate in mushrooms cultivation is a mixture of cellulosic waste such as agricultural, poultry, or industrial waste and

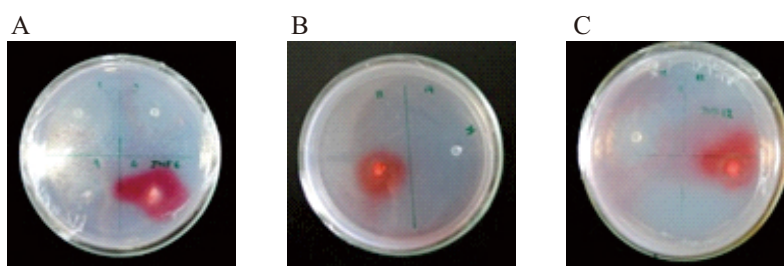


Fig 1 Clearing zone surrounding fungal colonies of JMF 6(a), JMF 11(b), and JMF 12(c).

Table 1 Summary of the purification of cellulase fom JMF 12

Purification steps	Total volume (mL)	Total protein (mg)	Cellulase activity (U mL^{-1})	Total activity (Unit)	Specific activity (U mg^{-1})	Yield (%)	Purification fold
Culture filtrate	180.00	45.56	1.47	264.60	5.81	100.00	1.00
Ammonium sulphate precipitation	21.60	7.64	2.81	60.69	7.94	22.93	1.32
Dialysis Novagen D-Maxi TubeTM Dialyzer	28.08	5.74	2.09	58.68	10.22	22.18	1.76
Sephadex G-100	42.12	0.88	0.24	10.11	11.50	3.82	1.98

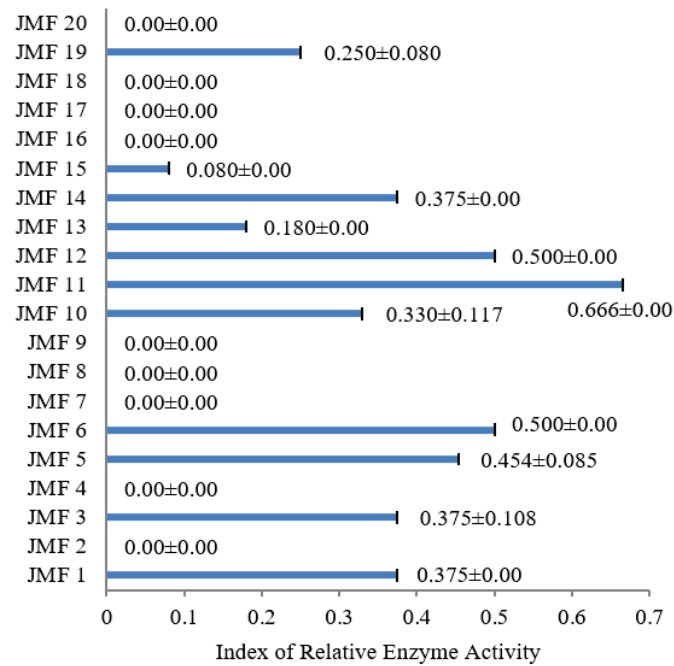


Fig 2 The Index of Relative Enzyme Activity on CMC medium ICMC of twenty numbers of fungal isolates.

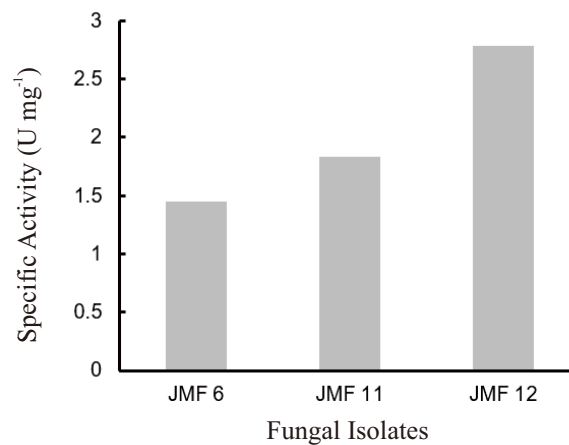


Fig 3 The cellulase activity of fungal isolates JMF 6, JMF 11 and JMF 12 grown on PDB medium.

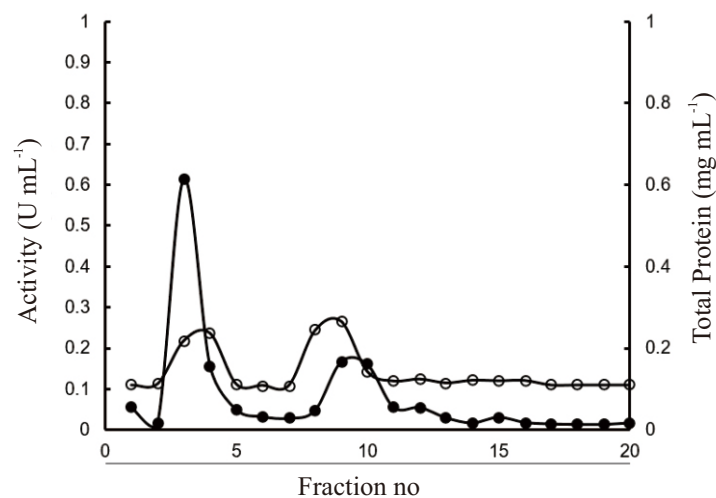


Fig 4 Elution profile of partially purified cellulase from JMF 12 on Sephadex G-100 gel filtration chromatography (●= cellulase activity, U mL⁻¹; ○= protein total, mg mL⁻¹).

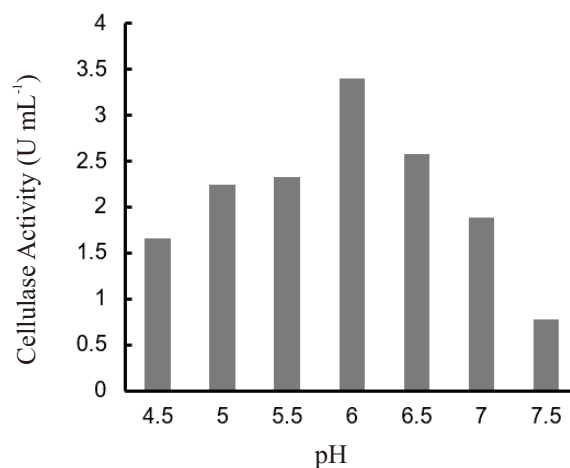


Fig 5 Effect of pH on the activity of cellulase from JM12.

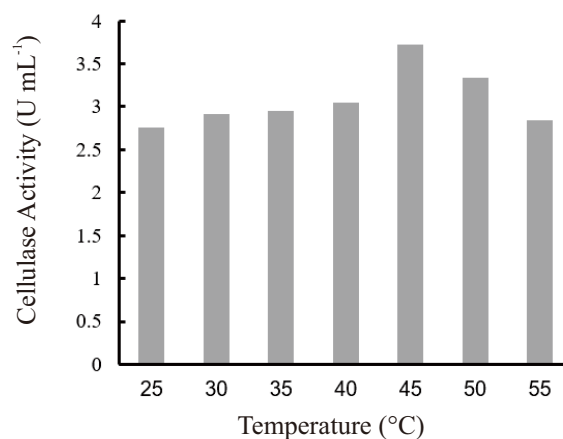


Fig 6 Effect of temperature on the activity of cellulase from JM12.

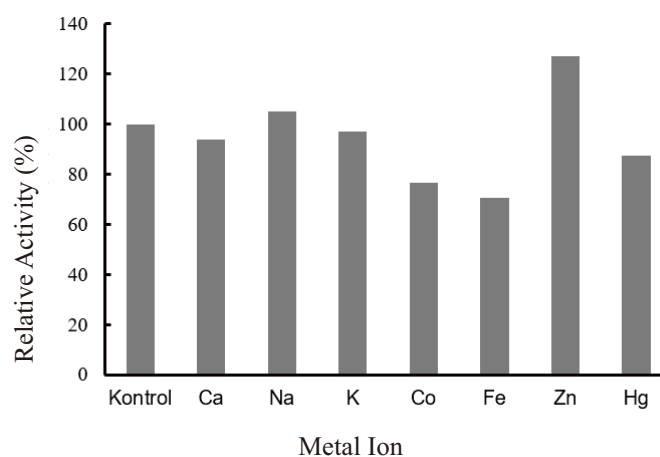


Fig 7 Effect of the addition of cations on the relative activity of cellulase from JM12.

prepared by controlled fermentation process. The used substrate obtained after crop harvest have all essential character of organic manure. However, to obtain more, appropriate condition, the used substrate still needs to be recombined. The recombined processes of used

mushroom substrate requires cellulolytic microorganisms to enhance hydrolysis of the cellulose. In this study, among 20 fungal isolates from used straw mushrooms substrate, 11 fungal isolates were identified having cellulolytic activity. They have been

shown by the clearing zones on the CMC medium where they were grown. The detection of cellulolytic activity using CMC medium is reliable for rapid screening of fungal cellulase (Teather and Wood 1982; Bradner *et al.* 1999; Khokhar *et al.* 2012). Determination of cellulase activity of crude enzyme produced by 11 fungal isolates using CMC as a substrate showed that isolates number JMF 12 had the highest activity and it was used for the purification and characterization of cellulase. Fungi are well known agents of decomposition of organic matter in common and of cellulosic substrate in particular (Lynd *et al.* 2002).

The supernatant of JMF 12 with cellulase activity of 1.47 U mL^{-1} and specific activity of 5.81 U mg^{-1} was used as crude enzyme solution and subjected to partial purification by ammonium sulfate precipitation in 10–80% saturation. The cellulase activity of 2.81 U mL^{-1} , specific activity of 7.94 U mg^{-1} , yield 22.93 unit and purification fold of 1.32 was achieved on ammonium sulfate precipitation of 30% saturation (data not shown). Ammonium sulfate is the most common inorganic salts that can be utilized for the precipitation of proteins. The concentration of ammonium sulfate required for precipitation varies from protein to protein and should be determined empirically (Deutscher and Murray 1990). Dialysis using Novagen D-Maxi Tube TM Dialyzer was applied after ammonium sulfate precipitation. The precipitated protein of cellulase can be separated from small molecules by dialysis through a semipermeable membrane, such as a cellulose membrane with pores. Molecules having dimensions significantly greater than the pore diameter are retained inside the dialysis tube, whereas smaller molecules and ions traverse the pores of such a membrane and emerge in the dialysate outside the tube. This technique is useful for removing salts or other small size molecules, but it will not distinguish proteins effectively.

The dialyzed fraction referred to as partially purified cellulase was then loaded on to Sephadex G-100 gel filtration column. By gel filtration the enzyme was purified to 1.98 fold with a yield and specific activity of 3.82 % and 11.5 U mg^{-1} respectively. The similar method for purification of cellulase from *Trichoderma viride* was done by Iqbal *et al* 2011. Gel filtration separates molecules according to differences in size as they pass through a gel filtration medium packed in a column. On the other hand, small molecules such excess salts during ammonium sulfate precipitation can be easily separated. Purification of

cellulase from JMF 12 using Sephadex G-100 gel filtration showed two single peak of enzyme activity as seen in Figure 4. It happened because cellulase is a complex enzyme that are classified into three types such as Endoglucanase, Exoglucanase and β -glucosidase. Furthermore, the method of cellulase assay used in this study was not able to determine single types of complex cellulase specifically.

The cellulase activity of JMF 12 appeared to depend on pH value. The result that was illustrated by Figure 5 shows that cellulase activity gradually increase and the highest activity was reached maximum at 3.40 U mL^{-1} as the pH value of 6. It was also noted that the activity was decreased at pH value of 6.5 – 7.5. Effect of pH on Cellulase activity of JMF 12 supports the finding of Okoye *et al* (2013) on cellulase activity of *Aspergillus fumigatus*.

Furthermore, temperature is also an important factor that influences the cellulase activity. The temperature profile of the cellulase of JMF 12 showed an increase in activity as the temperature increased from 25 – 40°C. The optimum activity was obtained at 45°C of 3.72 U mL^{-1} . Temperature not only affects the activity of enzyme but also the stability of the enzyme. High temperature will change the protein structures leading to denaturation. Many studies have been reported that the optimal temperature for cellulase production depended on the strain variation of the microorganism (Murao *et al* 1988; Lu *et al* 2003; Sherief *et al* 2010; Solomon *et al* 1997; Wiseman 1995).

Addition of some cations to the reaction mixture affected the cellulase activity. The presence of these cations Na^+ and Zn^{++} increased the enzyme activity of the JMF 12. On the other hand, the addition of cations Ca^{++} , Co^{++} , Fe^{++} , and Hg^{++} decreased the activity. The results partially corresponded to the study of metal ion effect on cellulase activity (Wang *et al* 2012). Another reasons of change in cellulase activity is that ions generally have a noticeable effect on the intensity of DNS color and/or the reducing power of glucose which can affect the cellulase activity determined. So, in the colorimetric determination of cellulase activity in a salt treated solution the percentage inhibition may be related to negative effect of ions on the method of assessment (Sinegani and Emtiazi 2012).

The study of cellulase activity of fungi isolated from used mushroom substrate was carried out and it has potential use in preparation of mushroom substrate during mushroom cultivation. However, further study

in identification of fungi JM12 is needed to explore its character.

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