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Purification and characterization of cellulase from *Trichoderma reesei*

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Abstract

This investigation was conducted to study the capability of isolated fungus *Trichoderma reesei* to produce cellulase enzyme, show that the isolation of highly efficient in terms of the analysis of inhibition zone diameter which amounted to (12.46) using Alkounko red stain. Enabled the enzyme purification to the extent of homogeneity in several sequential steps involving ultrafiltration and precipitation with ammonium sulphate by The proportion satisfying 60% and gel filtration with sephadex G-100, the purification folder after these steps was (8.52) once the yield of the enzyme was (23.85%). Enzyme characterization results showed that the optimal pH of enzyme activity was 6.0 and optimum temperature was 52°, and the molecular weight of the enzyme was (68) kDa when determined by gel electrophoresis with SDS. The enzyme was purified to the degree of homogeneity in terms of the emergence of a single packet at migration on gel electrophoresis. Also include diagnosis of the resulting carbohydrate from the decomposition of cellulose by cellulase enzyme, clearly on glucose by using thin layer Chromatography technology.

Keywords: Cellulase, *Trichoderma reesei*, Fungal enzymes, Purification, Characterization.

Introduction

Cellulose is the most abundant compounds all over the world and the most prevalent material and the main compound in plant cells especially cell wall (Rathnan *et al.*, 2012; Devi and Kumar, 2012; Khokhar *et al.*, 2014).It is composed of reiterative units of D- glucose conjugated by β-1,4glucosidic linkage, each unit contains three groups of hydroxyl which make hydrogen bonds that make cellulose amorphous and insoluble material (Rajesh *et al.*, 2012; Ijaz *et al.*, 2014). The microorganisms such as bacteria and fungi are the main sources of cellulase enzyme. Studying cellulase production by Trichoderma fungi has attended interesting for enzyme specific properties especially that produced by *Trichoderma reesei* which is used for commercial production of cellulase because of its high ability for enzyme production and its unique properties (Iqbal *et al.*, 2011; Ahmed *et al.*, 2009). Cellulase is one of enzymes which used in many fields especially in analyzing lignocellulosic wastes to fermentable carbohydrates for ethanol production (Zhu and Pan, 2010; Kumar *et al.*, 2014) and tissue industry for removing starch, moreover it is used in detergents industry and pulp production (Neethu *et al.*, 2012; Elakkiya and Muralikrishnan, 2014). So the aim of this study was to produce and purify cellulase and study some effective factors on its activity.

Materials and Methods

Microorganism: The fungi *Trichoderma reesei* was gained from Biomass Utilization Unit, College of Science, Chulalongkorn University in Bangkok, Thailand. It was stored on potato dextrose slant agar in refrigerator at 4°C and re-cultured every two weeks.

Culture Media: Cellulase activity detection media: this media was used for detect the ability of the fungi to produce cellulase by determine inhibition zone, it was prepared according to (Yoshida *et al.*, 1989) as follows (g/L): K_2HPO_4 (1), KCL (0.5), $NaNO_3$ (2), $MgSO_4.7H_2O$ (0.5), Carboymethyle Cellulose (CMC)(10), Agar(20). pH= 6.0, sterilized at 121°C at pressure 1Kg/cm^2 for 15min. Poured the media in petri dishes and left to solid and kept in refrigerator at 4°C until using.

Enzyme production detection: The detection was made by taking a disc of fungal culture grew on PSA media of 5days age by using cork borer with 6mm diameter, it was transferred by needle to petri dishes containing detection media, incubated at 28°C for 5 days. Using Alkounko red stain to detect enzyme production, which was prepaired by dissolving 1g of the stain in 100ml of distilled water, it was added to the petri dish and left for

10min. then it was poured out and washed by NaCl (1N) solution for 15min. for many times, washing solution was discarded, and the ability of fungal isolate for enzyme production was calculated according to the equation referred by (Sazci *et al.*, 1986): Fungal ability of cellulase production= analysis zone diameter/fungal culture diameter

Detection of fungal growth on filter paper: A media was prepared for detect the ability of fungal isolates to grow on sterilized filter paper Whatman No.1 and using them as a carbon source, it was prepared according to (Doolotkeldieva and Bobusheva, 2011) as follows (g/L): $FeCl_3$ (0.1), NaCl (0.3), $NaNO_3$ (0.01), K_2HPO_4 (2.3), $CaCl_2$ (1), $MgSO_4$ (0.1), Agar (20).

Cellulase production media: This media was prepaired according to (Mandels, 1975) as follows: $NaNO_3$ (2), K_2HPO_4 (1), $MgSO_4$ (0.5), KCl (0.5), CMC (10), $CuSO_4.7H2O$ (0.005), $MnSO_4.7H_2O$ (0.0016), $ZnSO_4.7H_2O$ (0.0014), $COCL_2.6H_2O$ (0.002).pH=6.0, inoculum size was 4%, incubated at 28°C in shaker incubator at 150 RPM for 6 days.

Enzyme assay: The activity of cellulase produced by fungi was determined according to (Mandels *et al.*, 1976) by using 3-5 Dintrosalysilic acid (DNS).

Enzyme purification: A media was used to produce cellulase in a large amount was prepared as follows (Punnapayak *et al.*, 1999) (g/L):

$MgSO_4$(1.0), $CaHPO_4$ (5.0), $(NH_4)_2SO_4$ (4.0), Corn Steep Liquor (7.0), α-Cellulose (30), Tween 80 (2ml), $FeSO_4$ (0.005), $ZnSO_4$ (0.014), $MnSO_4$ (0.016), $CoCl_2$ (0.036). pH=6.0, inoculum size was 4%, incubated at 28°C in shaker incubator at 150 RPM for 6 days. The enzyme was filtered by Buchner funnel under vacuum; the filtered was the crude enzyme. The crude enzyme was purified by following four steps:

Ultrafiltration: It was done by using (UIV Flow 50) where the crude enzyme volume was decreased to the half (Ward and Swiatek, 2009). Precipitation with ammonium sulphate: the enzyme was precipitated by using different concentration of ammonium sulphate (0-70%), it was calculated according to enzyme volume gained from previous step.

Dialysis: the dialysis was carried on by using dialysis tubes against phosphate buffer solution 0.01M at pH 5 for 24hrs with 3 times exchange (Peshin and Mathur, 1999).

Gel filtration: gel filtration was done by using sephadex G-100 in a column (2.5× 30)cm, with flow rate 30ml/hr., the fraction were determined at 280nm and enzyme activity was determined by using CMC as a substrate (El-Zawahry *et al.*, 2010). The protein content was determined in the fractions according to Bradford (1976).

Determination of enzyme molecular weight: the molecular weight of cellulase was determined by using SDS-PAGE technique by using BIO-RAD Electrophoresis according to the method of (Samiallah *et al.*, 2009). Enzyme purity was determined by electrophoresis according the method of (Garfin, 1990).

Optimum pH: Cellulases activity was assumed at different values of pH (4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8).

Optimum temp.: Cellulases activity was determined at different temp. between (20-80)°c.

Identification of produced carbohydrates by cellulases action: The carbohydrates of cellulases action on CMC were identified by using Thin Layer Chromatography (TLC) using (n-butanol: acetic acid: water) (2:1:1) as mobile phase. After separation the plate were dried in oven (60)°C for 15 min., then it was spread with a solution reagent of 5% sulpheric acid in ethanol. Heated at 100°C until spots appear, Rf was determined.

Results and Discussion

Enzyme production detection: According to the ability of many funguses to produce cellulases, it was deduced by forming a clear zone around fungal colony in detection media which was observed after two days of incubation at 28°c which increased in diameter till fifth incubation day (Figure 1) and that gave a proof of CMC analysis by cellulases, which was 12.46mm and this a high activity for cellulose. These results agreed with (Iqbal *et al.*, 2011) who mentioned that *T. ressei* isolate was the best isolate for cellulase production when solid plate method was used, and it agreed with (Shu *et al.*, 2013; Xiong *et al.*, 2013) who said that *T. ressei* had high ability to analyze cellulose when it grew on cellulosic wastes.

Figure (1): Analysis clear zone

Detection of fungal growth on filter paper: Figure (2) showed the qualification of *T. ressei* isolate on cellulose consumption (filter paper) as a carbon and energy source because of its great growth and ability of analyzing cellulose. This result was near to the result of (Damisa *et al.*, 2012) when the grow *T. ressei* MUM97.53 on filter paper and was agree with (Sivaramanan, 2014) when the isolate *T. ressei* produce cellulase and analyze filter paper.

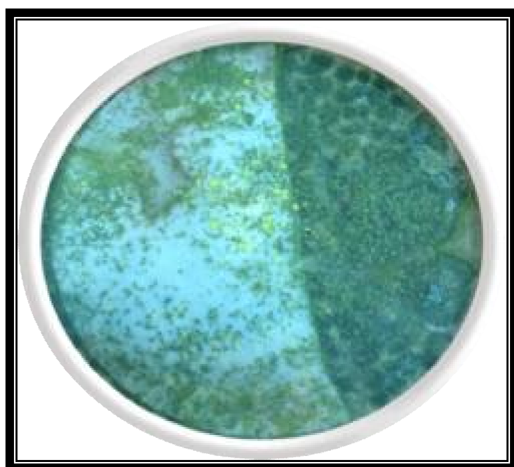

Figure (2): Qualification of *T. ressei* isolate on growth and filter paper consumption

Cellulases purification: The crude enzyme was submitted for purification steps, the first was ultrafiltration were the enzyme activity was 35.86 u/ml as illustrated in Table (1) and the yield was 77.63% and the purification fold was 1.70. Second step was precipitation with ammonium sulphate which achieved 2.41 folds with a yield of 59.51%. Ammonium sulphate was used by (Sahin *et al.*, 2013) for glucanase purification from *T. ouroviride* with 3.3 folds and the yield was 4.3%. On the other hand it was used by (Wood and Mccrae, 1982) for glucanase purification from T. koningii. Third step was dialysis, enzyme volume was 59ml with activity of 51.66u/ml and specific activity24.6 u/mg, and by this step we rid of low molecular weight protein compounds and achieved 4.79folds with yield 48.85%. Cellulase enzyme from *Phlebia gigantea* was purified by (Niranjane, 2006) by dialysis and gained specific activity 12.9u/mg with yield 78% and 2.8 folds.

Figure (3) showed protein peaks gained by gel filtration it is clear that there were three peaks and only second peak was represent cellulase. This peak was pooling and the volume was estimated 23ml and the enzyme activity was 64.72U/ml, the specific activity was 43.72U/mg with 23.85% yield and 8.52 fold. So this peak was selected for further studies and in an experiment for determination enzyme activity by using cellubiose as a substrate, we found that there weren't any activity, so we can predict that the purified enzyme was endoglucanase because it couldn't analyze cellubiose, as mentioned in (Whitaker, 1972) that endoglucanase just work on cellulose not cellubiose. There are many researchers purify the enzyme with different steps and gained different results, for example (Goldbeck *et al.*, 2013) purified cellulase from *Acremonium strictum* with many steps and achieved 35% yield and 2.61 folds, while (Yasmin *et al.*, 2013) gained 4.1% yield and 7.2 folds at gel filtration step when purified cellulase from *T. ouroviride*.

Table (1): Purification steps of cellulase produced by *T. ressei*

Purification Steps	Volume (ml)	Activity (U/ml)	Protein (mg/ml)	Specific activity (U/mg)	Total activity (U)	Fold	Yield %
Crude enzyme	220	28.35	5.52	5.13	6.237	1	100
Ultrafiltration	135	35.87	4.10	8.74	4.842	1.70	77.63
Ammonium sulphate (0-70)%	82	45.28	3.64	12.40	3.712	2.41	59.51
Dialysis	59	51.66	2.10	24.6	3.047	4.79	48.85
Gel filtration	23	64.72	1.48	43.72	1.488	8.52	23.85

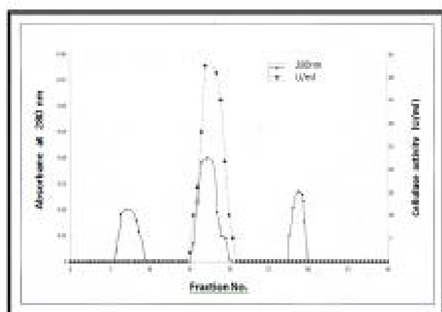

Figure (3): Purification of cellulases enzyme produced by *T. reesei* by gel filtration method Sephadex G-100 (2.5×30) cm equilibrated by phosphate buffer (0.5M), pH (7) with flow rate 30ml/hr.

Determination of enzyme molecular weight: The molecular weight of enzyme was determined by electrophoresis by using poly acrylamide gel with SDS. Figure (4) showed that the molecular weight of purified enzyme was 68 KD with one band and that mean the enzyme is endoglucanase. Using different techniques in determining molecular weights gives different molecular weights and could be due to carbohydrate contents in the protein which increase the weight. A researcher (Dolma *et al.*, 2014) showed that the molecular weight of cellulase produced by *T. viride* was 87KD by electrophoresis. While the result of (Iqbal *et al.*, 2011) for purification of cellulase and xylanase from *Alternaria alternate* showed two bands (64.20) and (40.2) KD for cellulase and four bands (40.2), (45.5), (64.2) and (97.4) KD for xylanase. In another research (Bai *et al.*, 2013a; Bai *et al.*, 2013b) the molecular weight of cellulase produced by *T. ouroviride* was (58) KD.

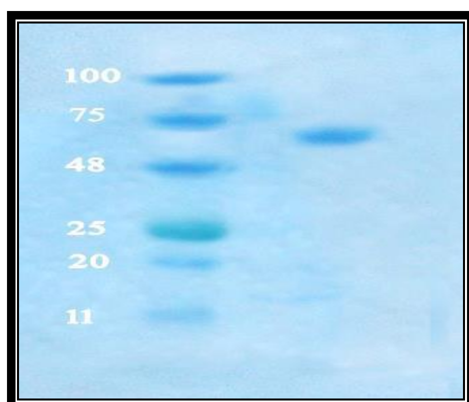

Figure (4): The molecular weight of purified cellulase produced by *T. reesei* by electrophoresis method

Enzyme purity: The enzyme purity step is an important step to identify the quality of purification methods. The electrophoresis was used for this step without SDS, results in Figure (5) showed that there was only one band for each purified enzyme and standard enzyme from *A. niger*, this indicate that the purified enzyme reached homogeneity and the bands at the same level, this result was close to (Pirzadah *et al.*, 2014) who said that there was only one peak of β- glucanase purified from *Penicillium simplicissimum H-11* which indicate for enzyme purity, on other hand the result was close to (Begum and Absar, 2009) during purification of cellulase from *T. reesei* and *T. viride*.

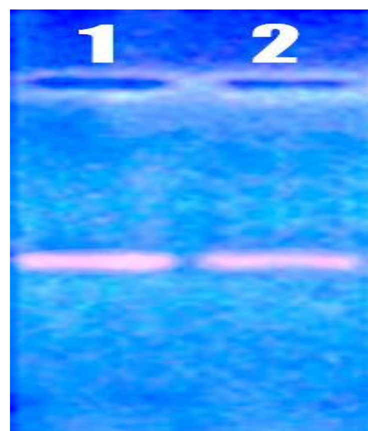

Figure (5): Enzyme purity by electrophoresis

Optimum pH: The optimum pH of cellulase activity was at (6.0) and it decrease by going away higher or lower from this point as shown in Figure (6), this could be because of the effect of pH on some groups inside the composition of the enzyme and the substrate. A researcher (Hasegawa *et al.*, 2012) said that the optimum pH for endoglucanase enzyme activity from *A. niger ANL 301* was (5.5), while (Bilal *et al.*, 2015) found that pH (6.0) was the optimum pH for cellulase activity purified from *Strongybcentrotus intermeodius*. Other researchers (Al-Jubouri, 2014) found that pH optimum of cellulase activity was (5.5) produced from *T. reesei,* this difference could be due to the difference in the resource of enzyme and the difference in their amino acids especially at active site (Shaikh *et al.*, 2013).

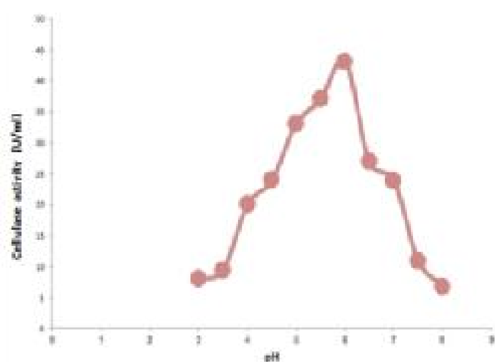

Figure (6): Optimum pH of cellulase produced from *T. reesei*

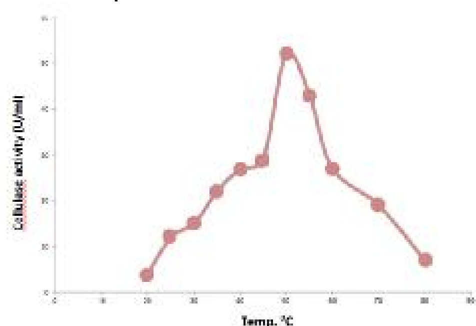

Figure (7): Optimum temperature of cellulase produced from *T. reese*

Optimum Temperature: The effect of temperature on purified enzyme activity was determined with range (20-80)°C. The results showed in Figure (7), the highest activity was at 52°C and decreased by elevating the temperature. The reason of increasing reactions velocity with elevation of temperature due to the increasing in crashing between molecules of enzyme and substrate. The higher temperature causes denaturation for the enzyme, so the activity will decrease. Our result was similar to (Sohail *et al.*, 2011), they found that optimum temp. of endoglucanase produced by *Alternaria sp* was 50°C. while the optimum temp. for glucosidase produced by *P. simplicissimum H-11* was at 60°C.

Identification of produced carbohydrates by cellulases action: Yields of substrate analysis by purified cellulase were determined using TLC method after one hour of enzymatic reaction and in comparison with standard xylose, cellubiose and glucose as shown in fig.8, it clears that there were three spots at the same level with glucose which have the same Rf= 0.53, that means the cellulose was completely analyzed to glucose with the action of cellulase. This result agreed with (Nadeem, 2009; Nayebyazdi, *et al.*, 2012) when they purified cellulase from *T. viride* where the result of substrate analysis was mainly glucose.

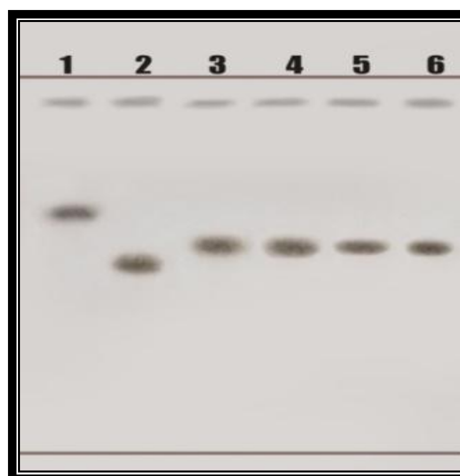

Figure (8): Separation of enzyme reaction results

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