Production and Characterization of Cellulase enzymes of *Xanthomonas campestris* pv. *sesami*

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ABSTRACT

The bacterium *Xanthomonas campestris* pv. *sesami*, is the causal organism of the leaf spot disease (Marad eldum) in sesame plants. Enzymes are protein molecules that catalyze chemical reaction. Cellulase enzymes which were reported to be produced by many plant pathogenic bacteria, were found to play an important role in pathogenesis. The present study was aimed to investigate the capacity of the bacterium *X. campestris* pv. *sesami*, to produce the two cellulase enzymes. (Carboxymethylcellulase, C_x, and Cellobiase). A salt medium supplemented with different cellulose, materials was tested for the production of the enzymes, using viscosity reducing and reducing group methods. The produced enzymes were characterized using different selective tests. The results showed that no cellobiase was produced in any of the substrates and only the (C_x) was produced by the bacterium. Carboxymethyl- cellulose was found as the best substrate giving 49.0 enzyme units (mg/ ml glucose). When the incubation time was tested, the enzyme activity was found maximum at the 7th and 8th days, giving 9.3 and 9.6 viscosity units respectively. The pH optimum of the enzyme activity was found at pH 6.0 which gave 18.0 viscosity units compared to only 5.1 at pH 4.0 and 2.8 at pH 10.0. The results also showed that the optimum degree of temperature for the enzyme activity was at 40.0 ^{0}C. Different metallic ions were tested for their effects on the enzyme activity, Ca^{++}, K^{++} and Mn^{++} were found effective giving enzyme activities of 104.5, 99.5 and 92.2 enzyme unit(mg/ml glucouse), respectively. The C_x enzyme was reported to play an important role in the bacterial leaf spot diseases of plants. The tested bacterium should be investigated for the production of the pectic and hemicellulase enzymes which were also found to have a role in the pathogenesis process.

INTRODUCTION

Enzymes are proteins, composed of amino acids linked together by peptide bonds that act as catalysts, speeding up chemical reactions that would take far too long to occur on their own. Enzymes speed up the vast majority of the chemical reactions that occur in the cells. (Worsfold, 1995). Enzymes are affected by changes in pH, Extremely high or low pH values generally result in complete loss of activity for most enzymes. pH is also an important factor in the stability of enzymes (Holum, 1968). Like most chemical reactions, the rate of an enzyme- catalyzed reaction increases as the temperature is raised. A 10ºC rise in temperature will increase the activity of most enzymes by 50- 100 %.

The reaction rate were found to increase with temperature to a maximum level, then abruptly declines with further increase of temperature. Because most enzymes rapidly become denatured at temperatures above 40ºC (Cheetham, 1985). Certain ions like Ca^{++} are absolutely necessary for the activity of some enzymes Levinskaite (2001). However, Ajayi *et al*. (2006) reported that Mg^{++}, Ca^{++}, Na^{+} and K^{+} were stimulatory to the cellulolytic enzyme activity, while small quantities of $HgCl_2$ and EDTA were inhibitory. Lin *et al.* (2010) reported that most metal ions such as Ca^{++}, Mg^{++}, Cd^{++} and Zn^{++} exhibited slight inhibition effect on the enzyme activities, whereas K^{+} and Mn^{++} enhanced the cellulases to a 10% extent.

The increase in the rate of specific enzyme synthesis from basal to maximum levels caused by the presence of a substrate analog that act as an inducer. The inducer may be a substrate that inactivates a repressor or a chemical in the cell (Gupta and Ayyachamy, 2012). Cellobiose, CMC, lactone and other oxidized products of cellulose hydrolysis can also act as inducers of the cellulase enzyme (Nogawa *et al*., 2001; Nochure *et al*., 2004). It was found that the CMC medium was more favorable for total cellulases production followed by filter paper and cellulose, and that may be due to the nature of the CMC that it is a fully purified and a modified cellulose in salt form, while the cellulose and filter paper still have the complex nature which could make cellulose hydrolysis and glucose utilization more difficult (Kohad and Singh, 1993).

Cellulose is an organic compound with the formula $(C_6H_{10}O_5)_n$. Alinear polysaccharide of glucose residues connected by β-1,4 linkages. Cellulose is used to make hydrophilic and highly absorbent sponges, as well as water- soluble adhesives and binders such as methyl cellulose and carboxy methyl cellulose which are used in wallpaper paste. Microcrystalline cellulose and powdered cellulose are used as inactive fillers in tablets and as thickeners and stabilizers in processed foods. (Klemm *et al*., 2005). Cellulolysis is the process of breaking down cellulose into smaller polysaccharides called cellodextrins or completely into glucose units; this is a hydrolysis reaction that done by cellulose enzymes. The ability to decompose and obtain carbon energy from lignin, cellulose and hemicelluloses is wide spread among fungi and bacteria (Klass, 1983), these microorganisms produces multiple enzymes that are either free or cell associated, and these enzymes degrade natural cellulosic materials that are heterogeneously, intertwined polysaccharide chains with varying degree of being crystalline. These multiple enzyme complexes are known as cellulas enzyme system (Lynd *et al.*, 2002). The biotechnology of cellulases and hemicellulases began in the early 1980s first in animal feed and then in food application (Cheeson, 1987). Cellulases refer to as a group of enzymes which acting together to hydrolyze cellulose (Shin *et al.*, 2000; Ikram *et al*., 2005). Those enzymes degraded β-1, 4 glycoside bond in cellulose compounds and the cellulosic enzyme system consists of three major components, which designated as C_1 , C_x and cellobiase (Rajoka *et al*., 2004).

MATERIALS AND METHODS

Plant Source

Infected sesame leaves showing the leaf spot disease were obtained from the Research Station Wad – Medani.

Source of bacterium

The bacterium *Xanthomonas campestris* pv *sesami* was isolated from the infected leaves The spots was cut and macerated in a sterile distilled water. The solution was streaked on a yeast extract medium which is selective for Xanthomonas. Colonies were streaked on the top of slants of the same medium in small bottles allowed to grow for 48 hours and stored at 4 ^{0}C before being used. For further experiments the bacterium was grown in a nutrient agar or a nutrient both media, at 20-30C^0.

Yeast extract glucose medium

The contains the following components:

Glucose	10 g/l
K_2HPO_4	0.5
$MgSO_4.7H_2O$	0.2
Nacl	0.1
Agar	20.0
Yeast extract(10%)	100.0 ml
Distilled Water	900.0 ml

Medium for the cellulase production

For cellulase enzymes production the bacterium was grown on a basic salt medium with a cellulose substrate containing the following (g1L)

K_2HPO_4	1.0
K_2HPO_4	0.5
$(NH_4)_2SO_4$	1.5
$MgSO_4.7H_2O$	0.5
Cellulose substrate	10.0
Distilled water	100.0

Viscometric method:

Cellulase was assayed by viscosity reducing method. using U – tube viscometers (BS \ U, size G) (Fig 4), containing 5 ml enzyme solution and 5 ml of a substrate (1%) in citrate buffer (0.1 m) at 30°C. Reaction mixtures were as described above. The flow time was recorded when the solution passed through marked points in the tube, using a stop watch after each 4 mint period, up to 24 min from the start of the reaction. The efflux time of substrates alone, without enzyme (zero time), and the efflux time for substrate – plus – enzyme treatment were measured enzyme activity is expressed as 100/t where t is the time in minutes.

Release of reducing groups:

Reducing groups of reaction mixtures were estimated by the Nelson- Somogi method (Nelson, 1944). Copper reagents were mixed on the day of use, 25 parts A to 1 part B. the test sample (0.2 ml) was added to 0.8 ml water and 1.0 ml of the mixed copper reagent and heated for 30 min on a boiling water bath. After cooling 1.0 ml of arsenomolybdate reagent was added and are the blue colour produced was measured at 660 nm against the control treatments in.

Statistical analysis:

The obtained data was statistically analyzed by computer software MSTATS according to analysis of variance (ANOVA), Duncans Maltiple RanngeTest was used for mean separation.

RESULATS

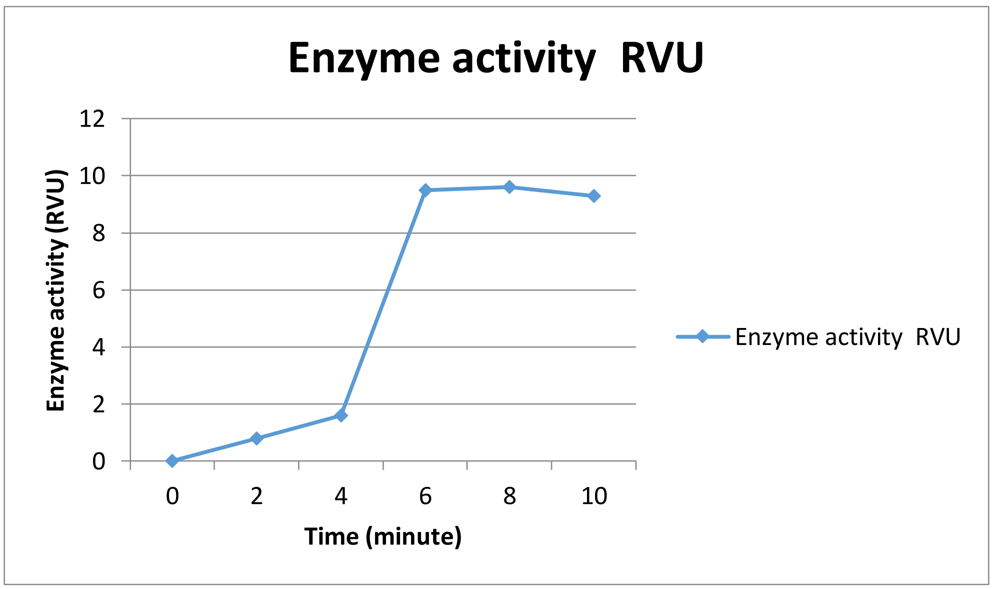
The basal salts medium was supplemented with 1% of each of the cellulose substances as well as some other sugars as carbon sources. The cuffure medium was autoclaved at 121ºC inoculated with 1 ml of the tested bacterium suspension (1×10^{106} cells/ml) and incubated for seven days at 28ºC. The enzyme activity was then measured by the reducing sugar. method using carboxymethylcellulose (CMC) for the carboxymethglcellulase enzyme (C_X) and cellobiose for the cellobiase enzyme activities . Results Table (1) showed that there is a high level of C_X activity with CMC, while a very low activity was detected when pure cellulose and filter paper were used. On the other hand, no cellubiase enzyme was detected in any of the substrates used Table (1).

The medium supplemented with CMC as a carbon source was inoculated and incubated at 28 ºC for 10 days. The enzyme activity was measured every two days. The results showed that the enzyme activity was only detected on the second day. The enzyme activity was increasing with for their incubation time reaching its maximum at the 8th days and decreased at the 10th days Fig(1).

Table(1): Effect of different substrate an production of cellulose enzymes by *X- compestri pv.sesami*

substrate	Cx enzyme activity Mg glucose /ml/br	cellubiase
CMC	49.0	0.0
Pour cellulose	13.0	0.0
Methyl cellulose	10.0	0.0
Filter paper	11.5	0.0

cellubiose	0.0	0.0
glucose	0.0	0.0
Duylan	0.0	0.0
salaction	0.0	0.0



Fig(1): Effect of the incubation time an the production of Cx enzyme by *X- compestri pv. Sesame*

In this test the enzyme production was made by using the same salt medium supplemented with CMC for 7 days at 28 ºC. The enzyme activity was tested in an assay solution adjusted to different pH values using different buffers. The pH values used were ranging between pH 4.0 and 10:0. The viscometry method was used for the enzyme assay. The results Fig(2). indicated that at the lower pH value (4), the activity was at its lowest level (5.1 VRU). However the optimum pH for the Cx enzyme produced by X. campestris pv sesame was found at pH 6.0 (18 VRU). The activity was then decreased with increasing pH value and only it was 2.8 VRU, were detected at pH 10.0.

The enzyme solution of the bacterium which was supplemented with CMC was incubated at different degrees of temperature ranging between 20 – 60 °C, in water baths for 7th days . The reaction mixtures were then assayed for the cellulase enzyme activity, using the release of reducing groups method. The results (Fig(3)**,** indicated that the enzyme activity was increasing with increasing temperature, reaching it maximum at 40 °C70 mg/ml, then the activity decreased and it was only 15 mg/ml at 60 C^0 .

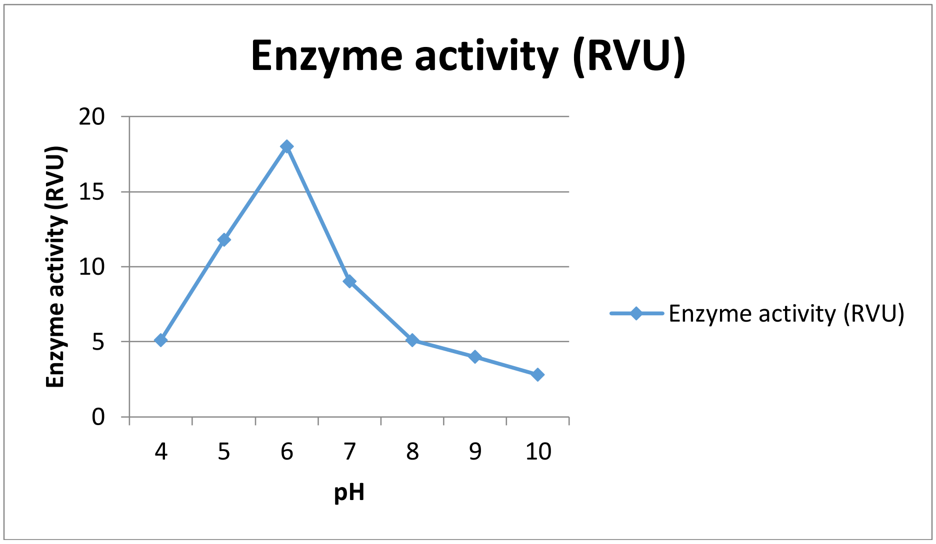


Fig (2): Effect of the pH value an the Cx enzyme activity produced by *X-compestri pv. Sesame*

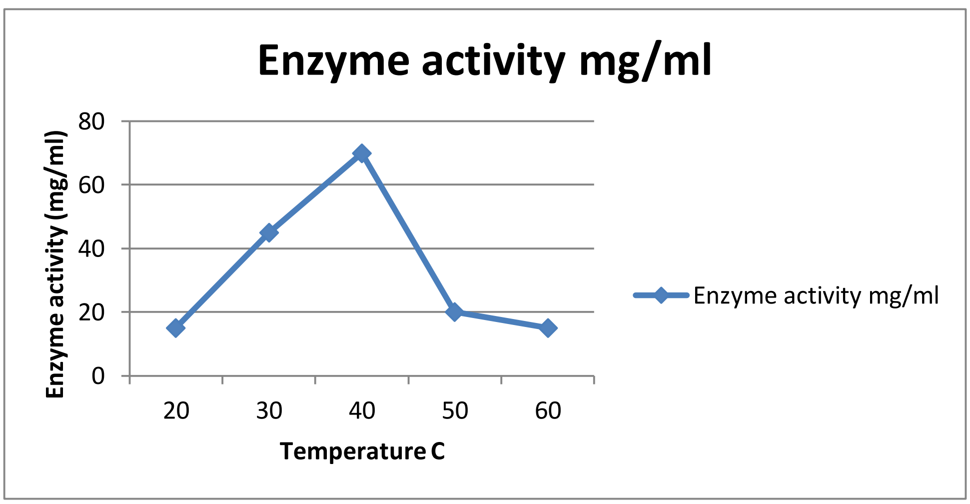


Fig (3): Effect of temperature on the enzyme activity

The enzyme solution of the bacterium, including CMC was supplemented in dividedly with one of the following metal ions (Ca^{++} ,Ba^{++} , K^{+} , Mg^{++}, Mn^{++}, Zn^{++},Cu^{++} and F^{++}). The enzyme solution was then assayed for its activity, using the release of reducing groups method. The results Table(2) showed that the addition of (Ca^{++} ,B^{++} , K^{+} , and Mn^{++} to the enzyme assay solution caused higher increase in the enzyme activity(104.5,92.8 and 99.5 mg/ml, respectively). However, less increase was shown in the enzyme activity by the addition of both Mg^{++} and Zn^{++} metal ions (56.1and 38. On the other hand, the addition of Na^{++} ,Cu^{++} and F^{++} caused a decrease in the enzyme activity.

Table(2): Effect of different method ions on the enzyme activity

Metal ions	Enzyme activity mg/ml
Ca^{++}	104.5
Ba^{++}	92.8
K^{+}	99.5
Mn^{++}	92.2
Mg^{++}	56.1
Zn^{++}	38.5
Na^{++}	10.1
Cu^{++}	9.5
F^{++}	7.8
control	20.8

DISCUSSION

The bacterium *X.compestris pv.sesami* was grown on the salt medium to which different cellulose compounds were added separately. The maximum yield of the cellulase enzyme (C_x) was found when CMC was used as an inducer substrate. However no cellobiase was produced when pure cellulose or cellobiase was used. This indicated that the bacterium was able to produce only one cellulase enzyme, the carboxymethyle cellulase (C_x).It was reported that CMC was the best among different substrates, used for the production of cellulase enzyme by the different very *Bacillus spp*.(Shanmugapriya *et al*., 2012; Li *et al*., 2010; Devendra *et al*., 2012).

In the present study, it was found that the bacterium *Xanthomonas campestris pv. sesami* was producing higher amounts of the cellualase enzyme at the 6th day. the level was decreasing with further incubation time. Simlar result were also found by Swelim *et al*. (2010), Gautam *et al.* (2010), Ahmed *et al.* (2009) and Christina and Sunil (2012). However, Ali *et al*. (2011) found that both *T.vinde* and *A. niger* gave a maximum cellulase after 10 days. The differences could be related to the nature and the different isolates of the organisms or different isozyme of the same enzyme.

Regarding the effect of the pH level on the activity of the cellulase enzyme produced by *X. campestris pv.sesami, the* optimum was found at the pH 6. This was in agreement with the results reported by Azzaz (2012) .who found that the highest pH maximum for the cellulase of both *A. niger T. virde* was pH 6.0 . However, the pH maximum for the cellulase of *P. digitatum* was reported by Baig *et al*. (2014) at two levels pH 6.0 and 7.0 . They also found that the pH maximum for the activity of the cellulase enzyme of *T. hariziamum* was 5.5. On the other hand, Ahmed *et al.* (2009) was found the pH maximum for the cellulase enzyme of *A. niger* was pH 4.0.

The optimum temperature of the cellulose enzyme activity produced by *Xanthomonas campestris pv. sesami* was fond at 40 ºC. This value was similar to that reported for the cellulose enzyme produced by the fungus *Aspergillus niger* (Gokhan *et al*., 2001). Cellulase enzymes from different microorganisms showed different values for their maximum temperature degrees as shown by Ahmed *et al*. (2009) and kim *et al*. (2009).

In the present study the addition of Ca^{++}, Ba^{++}, K^{+} and Mg^{++}, metal ions to the cellulase enzyme solutions of *Xanthomonas campestris pv. Sesami* was found to cause a highly increase activity. Similar results wear also obtained for different enzymes produced by different microorganisms (Amid *et al*., 2012; Dong *et al*., 2012, Bakarr *et al*., 2005). However, Ponnuswamy and prakash (2012) reported that the cellulase enzyme of *Bacillus sp*. was highly active in the presence of Mn^{++} and was strongly inhibited by Hg^{++}. Li *et al*. (2010) found that the puritied cellulase enzyme of *Bacillus subtilis* was activated by Mn^{++} and Co^{++} but was inhibited by Hg^{++}, Cu^{++}, and F^{++}.

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تأثير السرعة الأمامية ونوع جهاز نقل الحركة على أداء الجرارات الزراعية تحت ظروف التربة الطينية

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الخلاصة

تم اجراء التجربة بالمزرعة التجريبية بجامعة القضارف في موسم 2016 وكانت نسبة الرطوبة 7.24% والكثافة الظاهرية 1.40جرام/سم^3. استخدمت ثلاثة أنواع من الجرارات بنظم نقل حركة مختلفة وهي النظام التقليدي والنظام الناقل بالقدرة والنظام المزدوج. تم قياس قدرة الجر ومعدل استهلاك الوقود ونسبة انزلاق والسعة الحقلية ونسبة انزلاق العجل عند السرعات 5 و 6 و7 كم/الساعة. استخدم نظام القطاعات العشوائية الكامل بأربعة تكرارات. من التجربة وجد أن هنالك فرق معنوي في قدرة الجر بين النظام التقليدي والنظامين الآخرين عند سرعة 5 كم/الساعة حيث أعطى النظام التقليدي 20 كيلوات أقل. لا يوجد فرق معنوي بين هذه النظم عند السرعات 6 و7كم/الساعة . بالنسبة لاستهلاك الوقود لا يوجد فرق معنوي ($p \geq 0.05$) بين النظام التقليدي والنظام الناقل بالقدرة وأعطى النظام المزدوج أعلى معدل استهلاك ولكن عند السرعة 6 كم/الساعة كان أقل استهلاكا للوقود. لا يوجد فرق معنوي بين النظم الثلاثة عند السرعة 7 كم/الساعة. بالنسبة للسعة الحقلية لا يوجد فرق معنوي بين النظام الناقل بالقدرة والمزدوج عند السرعة 5كم/الساعة ولا يوجد فرق معنوي بين النظم الثلاثة عند السرعة 6 كم/ الساعة أما عند السرعة 7 كم في الساعة فوجد فرق معنوي بين النظام المزدوج والنظامين الآخرين حيث أعطي النظام المزدوج أعلى سعة حقلية. بالنسبة لانزلاق العجل فلا يوجد فرق معنوي بين النظم الثلاثة عند السرعات 5 و 6 كم/الساعة، كما لا يوجد فرق معنوي ($p \geq 0.05$) بين النظام الناقل بالقدرة والنظام المزدوج عند السرعة 7 كم/الساعة بينما يوجد فرق معنوي بين النظام التقليدي والنظامين الآخرين حيث أعطى النظام التقليدي أعلى نسبة انزلاق للعجل.