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PARTIAL PURIFICATION OF CELLULASE PRODUCED BY *AGROBACTERIUM TUMEFACIENS* ISOLATED FROM PEACH TREES

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Abstract

The study was conducted at The Research Unit of the Department of Biology/College of Education for Pure Sciences/ University of Mosul. Three isolates of the genus *Agrobacterium* were obtained from crown galls formed on the stems of peach trees, rose and *Myrtus Communis* from the forest area and university of Mosul, during the period from October to January. These isolates were cultivated on CMC medium to indicate their ability to produce cellulase enzyme according to the diameter of zone of hydrolysis. Results of both phenotypic and biochemical tests revealed that the isolate Ag2 is belonged to *A. tumefaciens* and the diagnosis was confirmed by using PCR technology to detect the Vir D2 gene using specialized primer. The optimum conditions for enzyme production were determined and the best production of the enzyme was achieved by using carboxy methyl cellulose as a carbon source with a concentration of 1% and sulfate Ammonium as a source of nitrogen and pH 6 after 3 days of incubation at 30°C. Under optimal conditions a partial enzyme purification was performed in steps that included sedimentation with ammonium sulfate at a rate of saturation of 75%, giving a specific efficacy (3.042) units / mg and the number of purification times (8.380), followed by the process of dialysis. The efficacy of the enzyme quality was (27.48) units / mg, and the number of purification times (75.70) with an enzymatic yield 1.361%. The results of the partially purified enzyme properties showed that the optimum pH is ranging 6.5-7.5, respectively, while the optimum temperature for the enzyme's effectiveness has reached 55°C, and the degree of thermal stability has ranged between 40-60°C.
Keywords: cellulose, *Agrobacterium tumefaciens*, peach trees

Introduction

Cellulose is considered the most abundant polymers on earth and the largest component of high-end plants and most used on an industrial field after being treated with many processes of purification and division (Zhao *et al*., 2007). It can be found in nature in the walls of plant cells as well as production in some animals such as organelles and some types of bacteria (Saha, 2004; Liao *et al*., 2011).Cellulose is one of several polysaccharides that composed of glucose units, and consists of repeating the units of bilateral sugar B-cellobios, as thousands of units of glucose sugar are related to each other with clasicide B-1.4, which are linked with each other strongly through the hydrogen bonds to form the cellulose fibers (Percival, 2008; Okade *et al*., 2012). Cellulose structures consist of three enzymes that collectively contribute to the breakdown of insoluble cellulose into glucose. These enzymes are Excoglucanase EC3.2.1.91, Endoglucanase EC3.2.1.4 and B-glycosidase EC3.2.1.21 (Kuhad *et al*., 2011; Peri, 2006).

The production of cellulase enzyme through using microorganisms has attracted great concern as an endless resource. However, microorganisms differ in their ability to produce it (Lynd *et al*., 2002; Cai, 2000). Cellulose has multiple uses, as it is the third-largest group of enzymes used in the textile, detergent, and paper industries and in food and biofuel production (Saravanan *et al*., 2009; Jayant *et al*., 2011; Park *et al*., 2012). In addition, cellulase enzyme is significantly used especially in the analysis of cellulosic residues to fermentable sugars to produce ethanol using microorganisms.

Based on the above mentions, the present study aimed to produce cellulase enzyme from local bacterial isolation and determine the optimum conditions for production and partial purification of the enzyme with some of its properties.

Materials and Methods

The study was conducted at the laboratories of the Research Unit in the Department of Biology / College of Education for Pure Sciences / University of Mosul.

Cultivating media

- Isolation and preservative bacterial medium: which was prepared according to the method mentioned by (Soriful *et al*., 2010). Trypton (5); Mannitol (5);Yeast (2.5); L-glutamic acid (1); KH_2PO_4 (25); NaCl (0.1); $MgSO_4$ (0.1); Bioten (10 ml) 0.1 mg/ml stook; Agar (Charles *et al*., 1995).
- A medium was used to detect the susceptibility of the bacteria to the production of the cellulase enzyme by observing the diameter of zone of hydrolysis. The medium was prepared according to the method (Vincent, 1970), CMC (10); Trypton (2); KH_2PO_4 (4); Na_2HPO_4 (4); $MgSO_4.7H_2O$ (0.2); $CaCl_2.2H_2O$ (0.001); $FeSO_4.7H_2O$ (0.004); Agar (Charles *et al*., 1995).

where pH was adjusted at 7, and dishes were incubated at a degree 30°C for 48 hrs.

Isolation of bacteria from crown gallsformed on the stems of peach, myrtle and rose trees

Coronary tumors formed on the stems of peach, myrtle and rose trees have been eradicated and then collected in sterile flasks. The tumors were sterilized according to method mentioned in (Charles *et al*., 1995) and cut into small pieces using a sterilized scalpel and then crushed in (1) ml of liquid YEM medium using sterile glass rod with alcoholic flame.

Isolation of bacteria

A. tumefaciens bacteria were obtained using the series dilution method. An amount of 0.1 ml was taken of the last dilution (7-10) and spread on the solid YEM medium to

obtain individual colonies. The plates were incubated at a temperature of 28 ± 2°C for 24 hrs, and then each colony was transferred to Agrobacterium Mannitol media and was kept on the last liquid medium in small glass bottles in the refrigerator at 4 °C. until usage (Muragesan *et al*., 2010).

Diagnostic tests

Bacterial colonies grown on YEM were elected on the basis of colonies' shape, strength, color and size on both Agrobacterium mannitol and Mac conkey Agar (Subba-Rao, 1981). Thin smears were prepared and pigmented with Gram stain, and their susceptibility to pigmentation, shape, and arrangement of bacterial cells were observed.

Biochemical tests

All biochemical tests were performed for bacteria isolated from crown galls of peach trees, according to (Moore *et al*., 1988; Holt *et al*.,1994).

Investigating the susceptibility of bacteria for producing cellulase enzyme

The isolates Ag1, Ag2 and Ag3 were used to investigate their susceptibility to the production of cellulase enzyme after the CMC-containing dishes were soaked in Congo red 1% solution, then the dishes were left at room temperature for a quarter of an hour. The solution was poured and the dishes were washed with (1M) NaCl solution for 15 minutes several times, the washing solution was eliminated and the appearance of the zone of hydrolysis around the bacterial colony is evidence of cellulase enzyme production and isolation susceptibility as reported by (Holt *et al*., 2000) using the following equation:

The ability of bacteria to produce cellulase enzyme = diameter of zone of hydrolysis / diameter of the bacterial colony.

Media for production of cellulase enzyme in submerged cultures

Prepare the medium from the following ingredients g/ l: $NaNO_3$-2, K_2HPO_4-1, $MgSO_4$-0.5, KCL-0.5, CMC-10, $CuSO_4.7H_2O$-0.005, $MnSO_4.7H_2O$-0.0016, $ZnSO_4.7H_2O$-0.0014, $COCl_2.6H_2O$-0.002. The medium was distributed in conical flasks of (250) ml capacity (50 ml) for each flask, and sterilized at a pressure of (1) kg/cm^2 at 121°C for 15 minutes. The prepared bacteria suspension of (24) hours were put in the shaker incubator at a temperature of 2 °C ± 28 °C at a rate of (150) round per minute for two days.

Methods of analysis:

- Determination of biomass: After the incubation period has ended, flasks were withdrawn from the incubator and the bacterial cultures were nominated using centrifugation at a speed of 1400 rounds for 10 minutes.
- Estimate the effectiveness of cellulase enzyme: using 3.5-Dinitro Salicylic Acid (DNS) detector prepared according to the method followed by (Miller, 1959). Cellulase enzyme activity was estimated according to method (Mandels *et al*., 1976). The enzymatic unit was defined as the amount of the enzyme that releases 1 micromol of glucose per minute under the conditions of the experiment.

Factors affecting enzyme production

The study examined the effect of various factors such as (incubation period, carbon and nitrogen sources, pH and temperature) on the production of the cellulase enzyme as mentioned in (Yin *et al*., 2010).

Extracting and purifying the genomic DNA of bacteria

Accuprep plasmid mini Extraction kit –k-3030-1 was used to extract the DNA, where 5 ml of liquid YEM was inoculated with young colonies of the studied bacteria, and after the end of the incubation period for 24 hours at a temperature of (28 ± 2) ºC, the extraction steps were performed according to the instructions described by Bioneer Korea to extract the DNA as mentioned by (Garfinkel *et al*., 1981).

Primer and their specificity

Specialized primers were used for the molecular diagnosis of bacteria isolated from the coronary tumors of peach trees represented by two specialized initiators of the VirD2 gene, one forward which is vir D2A with a sequence 5'ATG CCC GAT CGA GCT CAA GT3 'and the other reverse VirD2C with sequence 5'TCG TCT GGC TGA CTT TCG TCA TAA 3' molecular weights 224bp (Jerry *et al.*, 1995).

The PCR reaction was conducted with a final volume 25 µl and applied the 94 ºC reaction program for 3 minutes, followed by 35 cycles of 94 ºC for one minute, then elongation at 50º C, then completing the elongation at 72 ° C.

The DNA bundle was extracted from the agarose gel and added to one of the prefixes, which is the front initiator. The final reaction volume was 12 ltr. The sample was sent to Macrogen company to find the nucleotide sequence. Using Blast program, the bacterial identity was verified.

Purification and characterization of the enzyme

- **Protein estimation:** Estimating the protein concentration in the crude enzymatic extract and the partial purified enzyme from each step of purification according to (Lowry *et al*., 1951) and according to the protein concentration uses the standard curve of a protein in cow serum.
- **Partial purification of the enzymes:** After the end of the incubation period under the optimal conditions for enzyme production, a centrifugation was performed, the filtrate which considered the raw extract of the enzyme was taken and its enzymatic efficacy was estimated, and the protein concentration in preparation for the partial purification procedure which included the first step. The concentration of the raw enzyme with a volume of (92) ml By adding 75% of ammonium sulfate gradually in ice water bath with constant shaking, the mixture was left for the next day (Lee *et al*., 2006), then a centrifugation was performed at a speed of (10,000) r / min, then disposed of the filtrate and collected the precipitate In (10) ml of the sodium acetate ore at (0.05) molar concentration, and pH (5.5), the efficacy of the enzyme and the concentration of the protein were estimated. The second step was conducted using special dialysis bags versus the sodium acetate solution for a period of (24) hours, the solution then was replaced three times during which the efficacy of the enzyme and the protein concentration were estimated thereafter.

- **Enzyme characterization:** by the optimal pH effect of enzyme efficacy and stability, optimum temperature, and thermal stability of the enzyme (Hafsat *et al*., 2017).

Results and Discussion

Isolation

Three isolates were obtained from Agrobacterium from different natural sources, which included coronary tumors formed on peach, rose and *Myrtus communis* trees, in which this type of bacterium was found. The selection of those isolates depended on their ability to grow on a medium contains celluloseas a source of carbon and energy due to their action in decomposing carboxy methylcellulase and forms a clear transparent zone around the growth area. The bacterial colonies were transferred to YEM medium for further purification and in order to prepare them for the initial screening process.

Initial screening

An initial screening was performed to distinguish between the isolates whether producing the enzyme cellulase or non-producing by calculating both the diameter of the growth region G and the region of decomposition Z and extracted the value of Z / G, which was evidence of the susceptibility of the isolates to the production of the cellulase enzyme and its efficiency in the hydrolysis of cellulose. Table (1) shows the isolates, their sources, and the significant variance of these isolates in cellulase hydrolysis, isolate Ag2 was efficient in cellulase hydrolysis (9.50), which was estimated precisely by adding Congo red solution to the medium, which was characterized by its pink transparent color unlike the other parts. Figure (1), therefore, this isolation was chosen to complete the study. This value represents a high activity in the production of cellulose enzyme.

The results obtained were similar to those obtained by (Singh and Singh, 2014). different bacterial isolates from the root nodes of the alfalfa plant and in the production of cellulase enzyme in terms of the diameter of the zone of hydrolysis. It also confirmed the findings of (Abdel-Rahman *et al*., 2015), which indicated that isolated bacteria from different sources produce cellulase enzyme in different quantities using solid media and when shown with iodine dye. This also confirms what were obtained in (Jerry *et al.*, 1995), when isolating 40 samples of bacteria from the soil and testing their ability to produce cellulose using a dye Congo red.

Table 1: Efficiency of different bacterial isolates to produce cellulase enzyme estimated based on the diameter of the cellulose hydrolysis region to the diameter of the zone of hydrolysis

The isolation number	The source	Z/G
Ag_1	*Myrtus communis*	4.26
Ag_2	Peach trees	9.50
Ag_3	Rose	2.54

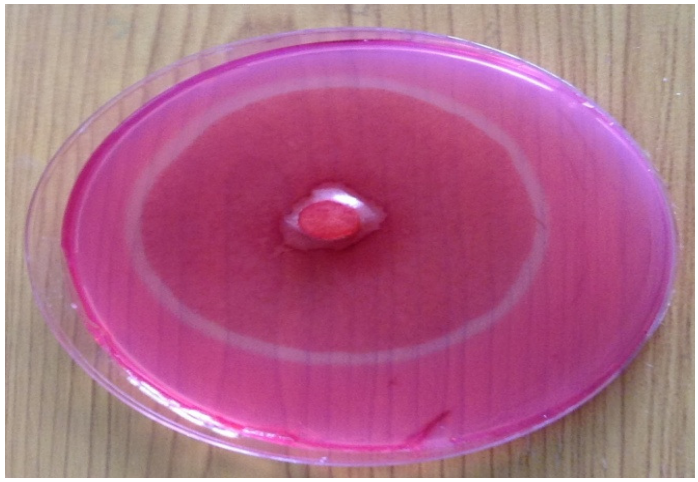

Fig. 1: Diameter of the aura of hydrolysis of the Ag_2 isolation

Secondary screening

A secondary screening process was performed for the isolates in the previous experiment, which showed the ability to grow on the solid medium and reflected its ability to analyze the carboxy methylcellulose and the production of cellulase enzyme, and this step came as an additional confirmation of the real capacity of bacterial isolates and by using the production medium. Table (2) shows the difference in the susceptibility of the bacterial isolates obtained in its production to cellulose, where the isolates Ag2 and Ag3 gave a quantity of cellulase of (3.75 and 1.86) units / ml respectively estimated by CMCase method (0.59 and 0.21) Unit / ml, respectively, estimated by Fpase method. Whereas, the amount of cellulase enzyme reached (1.42) units/ ml in CMCase method and (0.06) units / ml in Fpase method for Ag1 isolate. Depending on the results obtained, the choice was made on the Ag2 isolate, which showed superiority in the production of cellulase enzyme after being subjected to a set of diagnostic tests.

Table 2: Efficiency of different bacterial isolates on the production of cellulase enzyme using liquid medium by estimating the effectiveness of cellulase enzyme in bacterial culture filtrates

The isolation number	Effective Enzyme Unit/ml CMcase	Effective Enzyme Unit/ml Fpase
Ag_1	1.42	0.06
Ag_2	3.75	0.59
Ag_3	1.86	0.21

Diagnosis of isolate

After obtaining individual colonies of bacteria isolated from the crown galls of peach trees, which took symbol (Ag2), the selected isolate from the first and secondary screening stages was subjected to the best cellulase enzyme extraction for a set of phenotypic and agricultural tests Table (3) that showed the isolation belonging to type *A. tumefacienses* according to (Holt *et al*., 1994). The growing bacterial colonies appeared on the solid YEM medium in a creamy white color and were characterized by a convex circular shape and mucous textures, and this is consistent with what was mentioned in many scholars (Holt *et al*., 1994). When preparing swabs from these colonies, they were stained with a dye and examined with a complex optical

microscope using an oily lens. Bacillus bacteria was negative for Gram stain (Kumar *et al*., 2013).

The results showed that the isolated bacteria have the ability to produce the enzyme catalase, which indicates the reduction of hydrogen peroxide while noting the formation of bubbles of gas on the test slide after adding the solution of hydrogen peroxide H_2O_2. A negative evaluation of the jackets is observed, as the medium retained the color of the added reagent and did not change, indicating the inability of bacteria to extract citrate as the only source of carbon. It is noted through the table its ability to consume lactose and mannitol sugar through the change of the color of the medium from red to yellow and the isolation of bacteria was distinguished by its ability to grow at temperatures ranging between (28-35) C. Isolate also showed the ability to grow in different degrees of pH (4-9) in addition to its growth on the YEM medium containing iron ammonium citrate. Comparing the results of these tests with what is available from scholars, it can be said that the isolation under diagnosis is due to *A. tumefaciense.*

Table 3: Biochemical tests of Ag_2 isolation.

Test		The result
Catalase		+
Utilization of carbohydrates	Lactose	+
	Mannitol	+
L-tyrosine utilization		-
Citrate utilization		-
Growth at	28ºC	+
	35ºC	+
	40ºC	-
pH range		4-9
Growth absence	Biotine	+
	Thiamine	+
H_2S Product		-

Diagnosis at the molecular level of the enzyme-producing Agrobacterium isolation using specific-PCR technology

The presence of the VirD2 gene has been investigated by its specific initiative: one forward which is vir D2A with 5'ATG CCC GGA CGA GCT CAA GT3 'and the other reverse VirD2C with 5'TCG TCT GGC TGA CTT TCG TCA TAA 3' with Specific-PCR technology. The results showed the amplification of plasmid DNA of bacteria *A. tumefaciens* (Ag2) previously diagnosed by the generation of individual bundles and clear at the molecular size 224 base pairs, and this appeared from the semi-quantitative determination of the packages according to the CS Analyzer program, and these results came in accordance with what was found from the information (224 basepairs) is concerned with the design of the aforementioned initiatives on the website of the National Center for Bioinformatics Information (NCBI), And with results obtained by (Jerry *et al*., 1995).

The results of the diagnosis of the sequence were based on the nucleotide sequence shown in figure (2). After an alignment using Blast, the phenotypic and biochemical diagnosis of the bacteria was confirmed, as the nucleotide sequence gave a 100% match rate with the isolation of *A. tumefaciens* deposited in the gene bank KJ093488. This result was consistent with the nucleotide sequence determined by (Brun *et al*., 1995).

TTGGAGTACCTGTCCCGTAAGGGAAAGCTGGAA
CTGCAGCGTTCAGCCCGGCATCTCGATTTGGAATATC
TGTCCCGGAAGGGAAAGCTGGAACTGCAGCGTTCAG
CCCGGCATCTCGATATTCCCGTTCCGCCGGATCAAAT
CCGTGAGCTTGCCCAAAGCTGGGTTACGGAGGCCGG
GATTCCCGTTCCGCCGGATCAAATCCGTGAGCTTGCC
CAAAGCTGGGTTACGGAGGCCGGGATTTATGACGAA
AGTCAGTCAGACGATGATAGGCAACAAGACTTAACA
ACACACATTATTATTTATGACGAAAGTCAGTCAGAC
GATGATAGGCAACAAGACTTAACAACACACATTATT
GTAAGCTTCCCCGCAGGTACGTAAGCTTCCCTGCTGG
TAC

Fig. 2: The nucleotide sequence of isolate (Ag_2) *A. tumefaciens* bacteria

Determination of optimal conditions for cellulase production

(i) The effect of different incubation periods on the production of cellulase enzyme:

To find the optimal incubation period for the production of cellulase enzyme and its stabilization in subsequent traders, the isolation of bacteria was developed in a shaker incubator and for different incubation periods. Table (4) showed that the production of the enzyme began from the first day of incubation, but that the production of the highest enzyme was on the third day and amounted to (3.85) units / ml using the CMCase method and (0.77) units / ml using the Fpase method. The increase in the enzyme production was accompanied by an increase in biomass for bacteria, up to a maximum of (2.93) g / l at the time of higher enzyme production. This result is consistent with findings of (Shamugapriya *et al*., 2012) which showed that the optimum incubation period for cellulase is 72 hours when Bacillus sp is grown on CMC medium.

Table 4: The effect of different incubation periods on the production of cellulase enzyme from *Agrobacterium tumefaciens*

Periods of incubation day	Biomass gm/l	Effective Enzyme Unit /ml CMcase	Effective Enzyme Unit /ml Fpase	Final pH
1	2.25	1.36	0.40	6.58
2	2.47	1.75	0.65	6.45
3	2.93	3.85	0.77	6.27
4	1.54	2.72	0.22	5.30
5	1.09	1.20	0.17	5.14

(ii) Carbon source

Table (5) shows the effect of the various carbon sources with a concentration of 1% on the bacterial isolate productivity of the cellulase enzyme on the mean of production within 72 hours of incubation at a temperature of 28 °C. / Ml in CMCase method and 0.66 units / ml in FPase

method, cellulose has the same role in enhancing cellulase enzyme production, as the enzyme efficacy reached 3.40 units / ml in CMCase method and 0.25 units / ml in FPase method. For other carbon sources, Avicell, cotton, and filter papers, their effects were limited on productivity. The emergence of enzyme activity in the presence of carbon sources other than cellulose is one of the basic substances on which the enzyme works. It confirms that the enzyme in these bacteria is one of the induced enzymes, or that it is a permanent enzyme in terms of its secretion to the medium, in the absence of the cellopause that contains the α 1-4, that works on the enzyme.

It is worth noting in other similar studies the use of various sources of carbon to produce the cellulase enzyme, as (Mohamed *et al*., 2015) rice straw was used to produce the cellulase enzyme, whereas cellulose was given at a concentration of 1% higher than the amount of cellulose produced from the isolation of rhizobium isolated from the root nodes of the Trigonella plant (Mateos *et al*., 1992) As for (Andriani and Park, 2010), he obtained the highest cellulase enzyme by using rice straw as a carbon source from Bacillus Licheniformis1-1v isolate.

Table 5: Effect of different carbon sources on the production of cellulase enzyme from *Agrobacterium tumefaciens*

Carbon Source	Biomass gm/l	Effective Enzyme Unit /ml CMcase	Effective Enzyme Unit /ml Fpase	Final pH
Avicell	0.88	1.63	0.05	6.59
CMC	2.56	3.82	0.66	6.27
Filtration papers	1.32	2.29	0.08	6.55
Cotton	1.24	2.57	0.11	6.48
Cellobiose	1.81	3.40	0.25	6.30

(iii) The effect of different nitrogen sources

A number of nitrogen sources were tested to determine their effect on the production of the enzyme cellulase included some organic sources such as peptone, yeast and urea extract as well as some inorganic sources such as ammonium sulfate, sodium nitrate and ammonium chloride, these sources were added separately to the fermentation medium.

Table (6) shows the superiority of the inorganic source of ammonium sulfate in the production of cellulase enzyme compared to other nitrogen sources as its addition had a significant effect on increasing the production of the enzyme, which amounted to (4.28) units / ml and (0.70) units / ml respectively in CMcase and Fpase methods. The use of urea among organic sources also supported the production of cellulase enzyme, as the amount of the enzyme reached (2.76) units / ml and (0.15) units / ml in CMcase and Fpase methods, respectively. While there was no clear effect on the enzyme productivity when adding peptone to the medium, which confirms the bacteria's inability to consume, and thus reflected in its ability to produce cellulase enzyme. As for the biomass, there was an increase in the case of adding nitrogenic sources that gave the highest efficacy to cellulose enzyme and the lowest value of nitrogen sources that gave little enzyme productivity.

As for the final pH, it changed from the initial pH to all the nitrogen sources used. (Andriani and Park, 2010) stated that tryptone was the best among other nitrogen sources that were added to the medium of cellulase enzyme production from *B. subtilis* TD6 as the enzyme production reached 3.99 units/ ml.

The various nitrogen sources, especially the inorganic ones, have different effects on the production medium with the aging of the production period due to the liberation of some groups from them and their diffusion in the medium such as sulfate and nitrate groups which are reflected on the pH of the medium which affects the growth rates of bacteria and their production of the enzyme.

Table 6: Effect of different nitrogen sources on the production of cellulase enzyme from *Agrobacterium tumefaciens*

Nitrogen Source	Biomass gm/l	Effective Enzyme Unit /ml CMcase	Effective Enzyme Unit /ml Fpase	Final pH
Urea	1.46	2.76	0.15	6.10
Yeast Extract	1.20	3.37	0.52	6.37
Pepton	1.07	1.63	0.08	6.24
Ammonium Sulphate	2.68	4.28	0.70	6.09
Ammonium Chloride	1.58	2.57	0.13	6.26
Sodium Nitrate	1.15	3.53	0.62	6.73

(iv) Effect of temperature

The temperature has its impact on determining the activity of different microorganisms, especially the growth and vital activities of the living organisms, and as such is considered an essential way to control all activities of the biological metabolism and demolition of these organisms, especially in the fermentation industries. The ability of bacteria to produce cellulase was tested at different temperatures, ranging from 28°C to 40°C, with a difference of 2each time. The results in Table (7) showed that the production of the enzyme from bacteria reflected a slight increase in temperature from 28 to 30°C. The effectiveness of the enzyme increased from (3.68) units / ml to (4.72) units / ml using CMcase method. The activity of the enzyme tended to decline after this temperature, to reach the efficiency to (0.86) units / ml at a temperature of 36°C. The increase in the production of the enzyme was accompanied by an increase in

the biomass of bacteria and vice versa when there was a decrease in the biomass when the decrease in the productivity of the enzyme confirmed by many From studies in this field (Nandimath *et al*., 2016). The optimum temperature for the production of cellulase enzyme from different strains of bacteria ranges between (28-30).

Table 7: Effect of different temperatures on the production of cellulase enzyme from *Agrobacterium tumefaciens.*

Temperature °C	Biomass gm/l	Effective Enzyme Unit /ml CMcase	Effective Enzyme Unit /ml Fpase	Final pH
28	2.55	3.68	0.61	6.35
30	2.35	4.72	1.11	6.33
32	1.90	1.37	0.63	5.85
34	1.75	1.06	0.35	5.76
36	1.33	0.86	0.19	5.63
38	0.85	0.42	0.07	5.47
40	0.56	0.22	0.03	5.20

(v) The effect of pH

With regard to the effect of pH, it is clear from Table (8) the suitability of pH 6 for the production of cellulase enzyme in terms of obtaining the highest enzymatic efficacy, which amounted to (4.85) units / ml in CMCase method and (2.55) units/ml in Fpase method. As revealed from the results, the effectiveness of the enzyme produced from bacterial isolation was reduced at lower and higher pH levels than optimal pH. As for the biomass of the bacteria, its maximum yield reached 3.27 g/l at the optimum pH and tended to decrease thereafter. The result achieved in this study was consistent with what were found by (Rasul *et al*., 2015) as the optimum pH of cellulase enzyme production from *B. sp.*was 7 with an efficacy of (3.642) units / ml.

Table 8: Effect of different pH values on cellulase enzyme production from *Agrobacterium tumefaciens*

The First pH	Biomass gm/l	Effective Enzyme Unit /ml CMcase	Effective Enzyme Unit /ml Fpase	Final pH
5.0	0.76	1.64	0.08	5.28
5.5	1.25	1.89	0.16	5.15
6.0	3.27	4.85	2.55	6.21
6.5	2.13	3.43	1.65	6.15
7.0	2.35	3.77	0.72	6.10
7.5	1.38	2.45	0.56	6.83

(vi) Enzyme purification

Bacterial cells were separated from the filtrate of the culture by centrifugation. The filtrate was considered a crude enzyme which was deposited with ammonium sulfate with a saturation rate of 75%. Table (9) shows that the specific activity of the enzyme increases from (0.363) units/mg in the raw enzyme to (3.042) units / mg, the number of times the enzyme purification reached to (8,380) times and the enzymatic outcome decreased to 1.127%, and this is what It confirms the effectiveness of ammonium sulfate in the concentration of the enzyme due to its good solubility in water.

The results also showed when conducting the dialysis step, the specific efficacy increased to (27.48) units / mg with an enzymatic yield 1.361% and the number of protein times to (1.33) mg / ml due to the exit of proteins with small weights less than kilo daltons outside the dialysis bags, as attributed The reason also is the entry of quantities of the orbital solution used, which leads to reducing the concentration of the enzyme and thus increasing the number of times the enzyme is purified. It is reported that (Chen *et al*., 2004) obtained a specific activity of (0.386) micromol / min when depositing the enzyme cellulose with ammonium sulfate at saturation levels 40-60% of *Sinrhizobium fredii* CCRC.

Table 9: Summary of partial purification of cellulose from *A. tumefaciens*

Purification step	Vol (ml)	Activity (V/ml)	Protein (mg/ml)	Total activity	Specific activity (V/ml)	Purification fold	Yield
Crude Enzyme	92	4.67	12.85	429.64	0.363	1.00	100
Precipitation by ammonium sulfate (75)%	28	17.31	5.69	484.63	3.042	8.380	1.127
Dialysis	16	36.55	1.33	584.80	27.48	75.70	1.361

(vii) Enzyme characterization

The study included the effect of the optimum pH of the effectiveness and stability of the enzyme and the effect of the optimum temperature and thermal stability of the enzyme

(a) Optimum pH for enzyme activity:

Figure (3) shows that the optimal pH of the partially altered cellulose enzyme in *A. tumefaciens* (6.5), as the enzymatic efficacy reached (47.15) units / ml. It is also noticed that the enzyme activity decreases gradually from the optimal value of the efficacy, and the reason for the decrease is due to the influence of Specific groups within the enzyme

composition with the pH used. In similar studies, (Silvania *et al*., 2014) indicated that the optimal pH of the partially altered cellulose enzyme from Bacillus sp SMIA-2 is between (7 and 8) using (Mcase, Avicelase) as the base material.

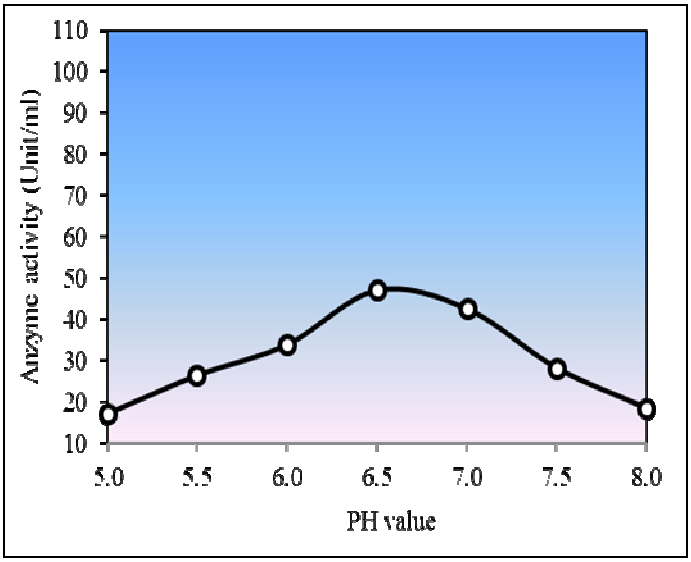

Fig. 3: The optimal pH effect on enzyme activity

(b) Optimum pH to stabilize enzyme effectiveness:

The results of Figure (4) showed that possession of the pH ranged between (6.5-7.5) or the enzyme retained 98% of its efficacy, meaning that the constant of the enzymatic activity was fully achieved, while the effectiveness decreased by half at the pH (9) where the enzyme retained with 50% of its effectiveness. The results agreed with his findings (Nikky *et al*., 2019) that the optimum pH to stabilize the activity of the cellulose enzyme is (7.0) in *Pseudomonas sp.*

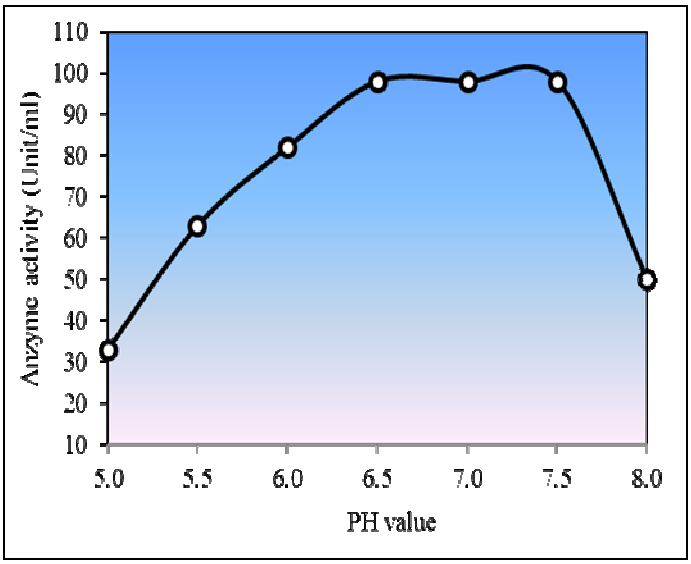

Fig. 4: The optimum pH to stabilize the enzyme's effectiveness

(c) Optimum Enzyme Temperature

At the optimum pH of the enzyme activity, the experiment was conducted to determine the effect of a range of temperatures that ranged between (30-70) °C on the activity of the partially changed cellulose enzyme. The results of the experiment showed in Figure (5) that the enzyme activity increased gradually with increasing temperatures, as the enzymatic activity reached a maximum of (43.75) units / ml at a temperature of (55) °C, while a loss in the activity of the enzyme was observed with a rise from this degree Thermocouple, and its loss reached its temperature at (80) °C, as enzymatic activity recorded a sharp decrease of (19.60) units / ml. This significant decrease in enzyme activity is attributed to the dissolution of the enzyme's active site and a change in the triple structure of the enzyme, which causes it to lose its effectiveness. The obtained results, compared to (Hafsat *et al*., 2017), showed that the optimum temperature for the activity of the partially changed cellulose enzyme in *B. pantothenticus* isolated from the landfill is (60) °C.

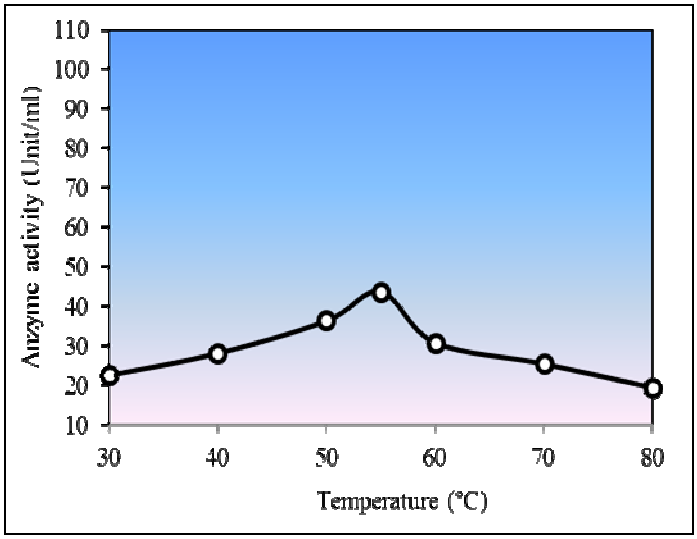

Fig. 5: the optimum temperature for enzyme efficacy

(d) Thermal stability of the enzyme:

The experiment was corrected by incubating the enzyme at different temperatures than the optimal pH of the enzyme's efficacy, and through the results shown in Figure (6), it turns out that the partially changed cellulase enzyme from *A. tumefaciens* had a degree of thermal stability at temperatures ranging between (40-60) °C At this temperature range, the enzyme maintains most of its enzymatic efficacy, which has reached 98-99% for one hour from the start of the enzymatic reaction. At higher temperatures (70)°C, the decrease began to be evident in efficacy, in which the enzyme retention is estimated to be as effective 50%. The above results confirm the influence of temperatures on the activity of the enzyme, the loss of which can explain the triple shape of the protein, which results in the enzyme not reacting at high temperatures (Khalaf,2012).

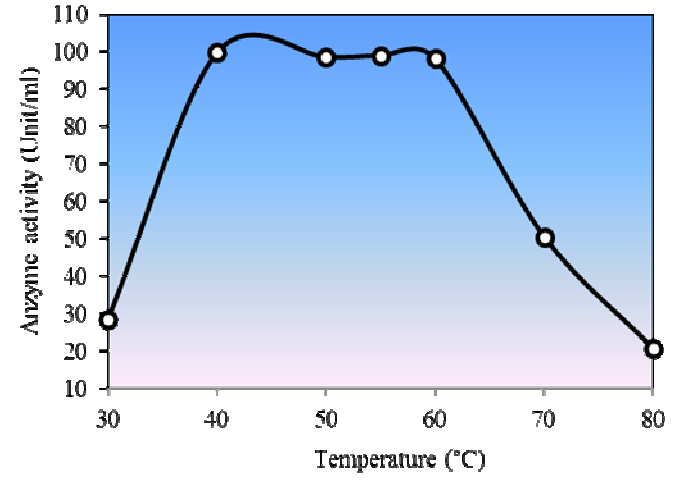

Fig. 6: The optimum temperature to stabilize the enzyme effectiveness.

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