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Isolation, Optimization and Characterization of Cellulases and Hemicellulases from *Bacillus Cereus* LAZ 518 Isolated from Cow Dung Using Corn Cobs as Lignocellulosic Waste

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Abstract: As a result of the magnitude of the problem of the accumulation of agricultural wastes and increase the environment and economic problems caused of it . It was necessary to find a scientific solution to face this problem and convert these wastes to high-value products , by using microbial organisms such as bacteria .Corn cobs have been chosen from shandawil research station in sohag governorate as an example of agriculture wastes and has been treated by physical and chemical methods to remove lignin and retain cellulose and hemicellulose free of any impurities. One hundred bacterial isolates, isolated from Water, Soil and cow dung in Sohag governorate, 6 isolates from cow dung were tested for their ability to produce extracellular Cellulases and hemicellulases, of all these 6 isolates isolate number (41 had the highest potential for celluolytic and hemicelluolytic activity was chosen. From various morphological, biochemical and 16S rRNA, the isolate was identified to be *Bacillus cereus* LAZ 518. Physiological studies were conducted to determine the optimum cultural conditions for maximum cellulases and hemicellulases production by *Bacillus Cereus* LAZ 518. The highest enzyme yield was obtained after 48 hours incubation at 50 °C and pH 7.0 when corn cobs was used as sole carbon source, respectively Carboxymethyl cellulose (CMC) was found to be a good inducer for cellulases and hemicellulases production . High level of enzyme production was obtained with the addition of yeast extract as a nitrogen source at concentration of 0.1 %. Tween-80 as an addition to medium inceases enzymes production. When examining the properties of cellulases and hemicellulases it was found that enzymes had a high degree of constancy at a temperature 70° C and pH 5 and improved performance when added with elements such as cobalt, calcium, sodium, potassium, and substances such as urea ,EDTA , β-mercaptoethanol , Tween-60 ,Tween- 80 and oxidizing agents such as hydrogen peroxide and sodium peroxide , it has been found that enzymes were strongly inhibited when added with mercury or high concentrations of oxidizing agents and surfactants.

Keywords: Cellulase, Hemicellulase, *Bacillus cereus* LAZ 518, Cow dung, Corn cobs, Optimization, Characterization.

1 Introduction

Cellulose is the most abundant biomass on Earth [17]. It is the primary product of photosynthesis in terrestrial environments and the most abundant renewable bioresource produced in the biosphere [7]. Cellulose is commonly degraded by an enzyme called cellulase. This enzyme is produced by several microorganisms, commonly by bacteria and fungi[8]. Cellulose is the principal constituent of the cell wall of most terrestrial plants. The source of cellulose is in plants and it is found as microfibrils ("2 – 20nm" in diameter and "100 – 40,000 nm" long). These form the structurally strong framework in the cell walls.

Despite a worldwide and enormous utilization of natural cellulosic sources, there are still abundant quantities of cellulosic sources and there are still abundant quantities of cellulose containing raw materials and waste products that are not exploited or which could be used more efficiently. The problem in this respect is, however, to develop processes that are economically profitable. Complete hydrolysis of the enzyme requires synergistic action of 3 types of enzymes, namely, Cellobiohydrolase, Endoglucanase or Carboxymethyl cellulase (CMC-ase), and Beta-glycosidase [12].

Bacteria which have high growth rate as compared with fungi have good potential to be used in cellulase

production. However, the application of bacteria in producing cellulase is not widely used. The cellulolytic property of some bacterial genera such as *Cellulomonas*, *Cellulovibrio*, *Pseudomonas* sp. *Bacillus*, and *Micrococcus* **[**15**]** was also reported. Enzyme production is closely controlled in microorganisms and for improving its productivity these controls can be ameliorated. Cellulase yields appear to depend upon a complex relationship involving a variety of factors like inoculums size, pH value, temperature, presence of inducers, medium additives, aeration, growth time, and so forth **[**36**].**

Cellulose, a polymer of glucose residues connected by beta 1,4 linkages, being the primary structural material of plant cell wall, is the most abundant carbohydrate in nature **[**49**&**50**],** therefore, it has become of considerable economic interest to develop processes for effective treatment and utilization of cellulosic wastes as inexpensive carbon sources. Cellulase is the enzyme that hydrolyses the beta 1, 4 glycosidic bonds in the polymer to release glucose units **[**2**].**

Cellulose containing wastes may be agricultural, urban, or industrial in origin sewage sludge might also be considered a source of cellulose since its cellulosic content provides the carbon needed for methane production in the anaerobic digestion of sludge **[**26**].**

Cellulases are used in the textile industry for cotton softening and denim finishing; in laundry detergents for colour care, cleaning; in the food industry for mashing; in the pulp and paper industries for drainage improvement and fiber modification, and they are even used for pharmaceutical applications **[**15**]**

Corn Cobs (CC) is one of the most abundant lignocellulosic crop residues in the world. Its annual production is about 731 million tons which is distributed in Africa, Asia, Europe and America. This amount of CC can potentially produce 205 billion liters of bio-ethanol per year. Egypt produce about 13.5 million tons of corn cobs distributed in upper and Lower Egypt and mostly in Lower Egypt according to **Fao state organization, (2014).**

2 Materials and Methods

2.1 Source of raw material

Corn cobs waste was collected from Shandawil research station in Sohag governorate, Zea maize is cultivated with a ratio of 3.75 million/ tones in Sohag governorate and 13.5 million/ tones in Egypt **(Ministry of Egyptian agriculture, 2015).**

2.2 Pretreatment of lignocellulosic material (Corn cobs)

Corn cobs was washed with tap water and leaved to dry, then cut to small pieces, then grinded to particle sizes (0.4 mm), 50 g of grinded corn cobs soaked in NaOH (4 %) solution (w/ v) of ratio 1:20 (substrate : solution) for 24 hrs at room temperature 25°C (50 g/ 1L NaOH 4% solution) in conical flask then, Corn cobs was filtered and washed by running tap water until neutralization (pH 7.0) free from sodium hydroxide then washed by distilled water, dried in the air to be used as a carbon source in the medium **[**39].

2.3 Isolation and screening of cellulase producing bacteria from cow dung, water and soil.

Cellulase-producing bacteria were isolated from cow dung by the dilution pour plate method using carboxymethyl cellulose (CMC) agar media. The plates were incubated at 37^{o}C for 24 hours. To visualize the hydrolysis zone, the plates were flooded with an aqueous solution of 1% Congo red for 15 min and washed with 1M NaCl. To indicate the cellulase activity of the bacteria .The diameter of the clear zone around colonies on CMC agar was measured. Besides, a more quantitative assay method was used to determine the cellulase activity of the selected bacterial isolate in liquid medium. The cellulase activity of each culture was measured by determining the amount of reducing sugars liberated by using miller **[32]** dinitro salicylic acid (DNS) method. A bacterial isolate with the highest activity was selected for optimization of cellulase production.

2.4 Identification of bacteria

The bacterial isolates were presumptively identified by means of morphological examination,biochemical characterizations and 16S rRNA gene sequence. The results were c ompared with Bergey's Manual of Determinative Bacteria [9] .

2.5 Media for enzyme production.

Cellulase enzyme was produced using basal medium with following composition: 0.01% MgSO4, 0.1% yeast extract, 0.2% KH2PO4, 0.7% K2HPO4, 0.05% Sodium citrate, supplemented with 1% carboxymethyl cellulose (CMC) as carbon source **(7).** Initial pH of the basal medium was adjusted to (7.0). 250 ml Erlenmeyer's flask with 50 ml of autoclaved production medium inoculated with 1 ml of *Bacillus cereus* LAZ 518 was incubated in rotary shaker at 200 r.p.m at 37°C for 72 hours.

2.6 Enzyme Assay

The activity of Cellulase was assayed using the method of Miller (3, 5- dinitrosalysilic acid) [32**]**. The enzyme crude extract was prepared as:

10 ml of culture was centrifuged at 1000 rpm for 15 minutes. The cell free extract was subjected to enzyme assay. DNS assay was carried out as follows. 0.5 ml of culture filtrate was mixed with 1.0 ml of 1.0 % (CMC -

Cellobiose and Hemicellulose) in 0.2M acetate buffer (pH 5) in a test tube and incubated in a shaking water bath at 50°C for 30 minutes (CMC-ase, Cellobiase and Hemicellulase) and for 60 minutes for FP-ase. The reaction was terminated by adding 3 ml of DNS reagent. The colour was then developed by boiling the mixture for 5 min, followed by cooling in cold water path. The optical density was taken at 640 nm against blank containing all the reagents minus the crude enzyme. One unit of the enzyme activity refers to the amount of enzyme that released 1 μM of glucose.

2.7 Optimization for maximum cellulases and hemicellulases production using corn cobs as a carbon source [7]

Cellulases and hemicellulases enzymes were produced using basal medium with following composition: 0.01% MgSO4, 0.1% yeast extract, 0.2% KH2PO4, 0.7% K2HPO4, 0.05% Sodium citrate, supplemented with 1% Corn cobs as a carbon source. Initial pH of the basal medium was adjusted to 7. Erlenmeyer's flask with 50 ml of autoclaved medium inoculated with 1 ml of culture was incubated in rotary shaker at 200 r.p.m at 37°C for 72 hours.

2.8 Factors affecting cellulases and hemicellulases production

2.8.1 Temperature

Production medium at pH 7 was inoculated with overnight grown *Bacillus cereus* LAZ 518. The broth was incubated at different temperatures from 20, 30, 40, 50, 60, and 70∘C for 72 h. At the end of incubation period, the cell-free culture filtrate is obtained and used as enzyme source.

2.8.2 pH

Flasks with broth containing corn cobs as a carbon source are taken and at 50°C and the pH of the broth is adjusted to 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 in different flasks using 1N HCl and 1N NaOH and sterilized.

2.8.3 Incubation Time

The effect of incubation on production by *Bacillus cereus* LAZ 518 was determined by inoculating the bacteria in the production media. The experiment was carried out individually at 50°C, pH 7 and various incubation times (24, 48, 72, 96 and 120 hours). The enzyme assay was carried out after every incubation period.

2.8.4 Inoculum Level

Different concentration of inoculum level 1, 2.5, 5, 7.5, and 10% (v/ v) were tested for their ability to induce cellulases and hemicellulases production in the production medium at 50°C, pH 7 and 48 hours incubation.

2.8.5 Carbon Sources

The effect of various carbon sources carboxymethyl cellulose, glucose, sucrose, lactose, and fructose at the concentration of 1 % was examined in the production medium at 50°C, pH 7, 48 hours incubation and 5% inoculume level .

2.8.6 Nitrogen Sources

Various nitrogen sources like yeast extract, peptone, gelatine, glycine, casein and malt extract were examined for their effect on enzyme production by replacing 0.1% yeast extract in the production medium at 50°C, pH 7 , 48 hours incubation and 5% innoculume level.

2.8.7 Surfactants

To identify the surfactants facilitating cellulases and hemicellulase production, four different surfactants were used for experimentation. They were SDS (Sodium Dodecyl Sulphate) Tween-20, Tween-40 and Tween-80. The selected surfactants were tested individually at the concentration of 0.1% in the optimized production medium.

2.9 Enzyme characterization

2.9.1 Factors affecting cellulases and hemicellulases activity

Temperature

The optimum temperature of powder enzymes was determined by carrying out the assay reaction in a shaking water bath at different temperatures (10 - 80°C) for one hour, then the activities were determined by DNS method and total protein was determined by the method of Lowry.

Thermal stability

In this experiment 2 ml of the enzyme powder incubated separately at different degrees of temperature (50, 60, 70 and 80°C) for different incubation periods (15, 30, 45, 60, 75 and 90 min.). Then 1.0 ml of 1% CMC and 1 ml of 1 % hemicellulose were incubated with 1 ml of the preheated enzyme buffer (0.2M acetate buffer pH 5.0) for 1 hour. The residual activities % were plotted against different temperature values for each incubation time.

pH

pH stability were studied by incubating 1 ml enzyme to 1ml of 1% CMC and hemicellulose prepared in a suitable buffer of a particular pH values range from (3 – 7) for 1 hour, different buffers such as 50 mM of sodium citrate (pH 4.0 and 5.0), potassium phosphate (pH 6.0-7.0) and Tris-HCL (pH 8.0-9.0). Finally the enzymes activities determined.

Reaction progress with time (Time curve)

For determine the time curve of the reaction process the

enzyme 1 ml incubated with the substrate 0.5 g (CMCase and hemicellulose) at 70ºC under shaking conditions for different times (10, 20, 30, 40, 50, 60, 70 and 80 min) after each incubation time the activity was determined .

Metal salts

To study the effect of metal salt to the enzyme we take 1 ml enzyme (10mg/ ml buffer) and 0.5 gm (CMCase and hemicellose) and 0.1 ml of concentration of (5×10^{-3} M) from the following metals (NaCl,,KCl ,$CaCl_2$,$HgCl_2$,$CoSO_4.7H_2o$,$CuSO_4.5H_2O$ and $ZnSO_4.7H_2O$). At pH 5.0 and 70ºC under shaking conditions for 1 hour. Finally the activities were determined.

Inhibitors

The effects of ethylene diamine tetra acetic acid (EDTA) ,β-mercaptoethanol , p-chloromercuribenzoate (pCMP), Iodo acetic acid (IAA), Dithiothretiol (DTT) and urea as inhibitors on cellulose and hemicellulose activities were investigated at a concentration of 5 mM and 10 mM in order to characterize enzyme. Crude enzyme was pre-incubated with the above mentioned reagents for 1 h at 70 ºC and residual activity (%) was determined under standard assay conditions.

Surfactants

The enzyme was incubated with surfactants as Triton-x-100, Tween -40, Tween-60 , Tween-80 , SDS (0.1 and 1.0 % , v/ v), for 1 hour at 70º C and then the residual activity (%) was tested under standard assay conditions .

2.9.2 Oxidizing agents

The enzyme was incubated oxidizing agents as H_2O_2 (0.1 and 1.0 % v/ v) , sodium perborate and sodium hypochlorite (0.1, 0.5 and 1.0 % v/ v) for 1 hour at 70ºC and then the residual activity (%) was tested under standard assay conditions.

2.10 Application of Cellulases and Hemicellulases

Determination of Monosaccharides by HPLC [46]. To apply this work and make sure of cellulases and hemicellulases by using HPLC (1200 series agilent technologies) in the Ragiona Center of Foods and Feeds of Agriculture Research Center ,Giza, Egypt .cellulases and hemicellulases resulted from adding 5 ml hydrolysate on 1 gm of Corn cobs at 70°C and pH 5 for 1 hour.

3 Results and Discussion

The present study deals with the cellulases and hemicellulases producing bacteria isolated from cow dung

3.1 Isolation, purification , screening and identification of bacteria from cow dung sample

Cow dung is one of the most abundant and unexploited resource for cellulolytic enzyme production . Table (1) indicate cow dung sample contains 36.12 % cellulose, 31.36 % hemicellulose, 15.3 % ash, and 1.58 % nitrogen and many essential nutrients such as Na, K, Ca, Zn, Fe, Cu, and Cd.The carbon and nitrogen ratio in cow dung manure is an indication that it could be a promising feed stock for culturing the microbs .

3.2 Corn cobs composition

Table (2) shows that the fibre composition of the raw material was determined in regard to the proportion of cellulose, hemicellulose and lignin. It was investigated that corn cobs show varying compositions.The fiber compositions before the pre-treatment of corn cobs (from Shandawil in Sohag governorate) were determined as (cellulose: 36.7% hemicellulose: 42.5 %, lignin: 12.4 %, Fats: 4.36 %, starch :0.91 % and ashes: 0.57 %) .

Around 100 bacterial isolates were isolated from the cow dung of which 50 isolates were found to have cellulolytic activity Among them 6 isolates gave maximum diameter zone were chosen and subjected to secondary screening to choose the best cellulolytic isolate, it was found that the best cellulolytic isolate was bacterial isolate (41) .Cellulase and Hemicellulase producing isolate (41) isolated from cow dung was identified based on the morphological , biochemical characteristics and 16S rRNA gene sequence as *Bacillus cereus* LAZ 518 results are illustrated in tables (4&5) and figures (1&2).

3.3 Optimization for maximum cellulases and hemicellulases production using corn cobs as a carbon source

Among the various temperatures tested, the maximum cellulases and hemicellulases production was obtained at 50°C followed by this, at 40°C was the second best temperature on cellulases production as illustrated in figure (3). On the other hand, the minimum amount of cellulase production was observed at temperature 70°C. By increasing the temperature above 50°C, a gradual decrease in enzymes formation occurred up to 70°C .at 80°C, no bacterial growth was found and subsequently no enzyme formation could be noticed at that degree. This can be interpreted by the alteration of cell membrane composition and stimulation of protein catabolism. These results agree with [29**]** who reported that the best temperature for cellulase and hemicellulase is from 45-50 ºC with the bacterial isolate *Achromobacter xylosoxidans* isolated from marine environment with *Bacillus subtilis* 45º[36**]**.

As indicated in figure (4) maximum cellulase and hemicellulase production was recorded at pH 7.0. The minimum cellulases and hemicellulases production was recorded at pH 10.0. This is probably as the bacteria is

neutrophilic. These results agree with [36] who reported that the optimum pH for CMC-ase of *Bacillus subtilis* isolated from soil contaminated with paper industry effluents at pH 7 was the optimum pH for production of cellulases in *Bacillus*, *Pseudomonas* and *Serratia* [17] and disagree with [29] who reported that maximum recording for enzyme cellulase and xylanase at pH 6.0 .

Figure (5) discuss the maximum amount of cellulase and hemicellulase production was observed in 48 hours incubation time. The minimum amount of cellulase production was obtained in 120 hours of incubation time. after the optimum time we notice that gradual decrease and rapid decline in the enzyme yields level at 72 hours was observed this is probably due to the depletion of nutrients in the medium which stressed the bacterial physiology resulting in the inactivation of secretary machinery of the enzymes [3] or the drop of the growth and release of proteases into the medium during the later growth phase of the bacrterial strain .This results agree with [36] who reported that Bacillus subtilis detected the maximum production was found between 48-72 hours .

The maximum enzymes specific activity were registered at the 5 % of inoculum level as seen in figure (6). On the other hand, the minimum amount of cellulase production was observed at 10% of inoculum level. Results not agree with [36] who declared that the maximum production for CMC-ase and FP-ase at 2% inoculum in case of *Bacillus subtilis* isolated from soil in media supplemented with paper waste as a sole carbon source and [19] who indicated that maximal production of CMC-ase occurred with 15% inoculume size (v/ w), based on dry weight of banana fruit stalk, but further increase resulted in reduced enzyme yields, as the higher inoculum sizes increased the moisture content to a significant extent. This fact was also confirmed by [33] who reported that larger inoculume size is detrimental to growth and production, apart from adding to the fermentation cost .

Here the maximum enzymes production was recorded in Carboxymethyl cellulose (CMC) supplemented medium. The minimum enzymes production was recorded in glucose and fructose added medium as illustrated in figure (7). Reduction in cellulase and hemicellulase production was seen in glucose and fructose amended medium which might be due to feedback inhibition of enzyme production as the glucose and fructose are the end product [23]. While enhancing cellulase production by *Bacillus* species on various cellulosic materials reported that increasing substrate concentration aided cellulase production. Since effect of CMC concentration on cellulase production by *B.cereus* has not been determinded before, so the current finding can be of significance .

The effect of different kinds of organic nitrogen sources on cellulases and hemicellulases production after 48 hours of incubation period at 50°C figure (8) showed maximum amount of enzyme production in yeast extract supplemented medium and minimum amount of enzymes production in Casein supplemented medium . Yeast extract has been showen by numerous researchers to be the best nitrogen source for optimal cellulase production by various bacteria [4&1&21]. These date is not agree with the results of [36] who reported that malt extract gave the maximum enzyme production with *Bacillus subtilis* . This enhancement of cellulase production incurred from addition of organic nitrogen sources is due to the fact that growth rate of the bacteria is faster in medium supplemented with organic nitrogen as they are rich in vitamins and other growth precursors. Carboxymethyl cellulase is produced by *B.pumilus* during growth phase and hence the promotion of bacterial growth by organic nitrogen source, during the growth phase influences its production [5] .

Among the tested surfactants, figure (9) discussed that maximum amount of enzyme production was recorded in Tween-80 added medium. The minimum amount of cellulase enzyme production was recorded in SDS supplemented medium. The stimulatory effect of surfactants may be a consequence of its action on cell membranes causing increased permeability by promoting the release of cell-bound enzymes. In enzyme hydrolysis of cellulose, surfactants might increase cellulase stability and prevent denaturation of enzyme by desorbing it from cellulose substrate [14,52]. In accordance with our results, Tween 80 at a concentration of (0.2–0.4 % v/ v) promote CMC-ase production by *Aspergillus glaucus* [10].

In contrast, [31] reported that the presence of tween-80 did not influence the production or secretion of CMC-ase of *Postia placenta* . .

3.4 Enzyme characterization.

The activity of cellulase and hemicellulase of *B.cereus* LAZ 518 was completely stable in the broad temperature range of 10 - 70°C during 1 h incubation. However, with further increase in every 10°C temperature, there was a gradual decrease in enzyme stability over 70°C.As showen in figure (10) the cellulase activity of *B.cereus* LAZ 518 is more thermostable than cellulase studied by several other researchers. Most of workers have reported that thermostable cellulase stable up to 60-100 °C but retained only 50% activity at 100 °C [26,6]. Most other thermotolerant *Bacillus* cellulases reported to so far, Cellulases exhibited higher temperature optimum for activity and showed good thermal stability [30,48]. These are the properties considered to be very important for industrial cellulose saccharification. Hence it is evident that the cellulase of *B.cereus* LAZ 518 is more thermostable , and may be applied to several biotechnological and industrial purposes .

As a function of time. The enzyme was fully stable up to 50 , 60° C with out apparent loss of activity for one hour, and the residual activity was regularly decreased as a function of both time of exposure and temperature. As table (5) illustrates, results obtained indicate that our CMC-ase seemed to be more thermostable than other CMC-ase from

other microorganisms such as *Sinorhizobium fredii* [11] where the purified CMC-ase retained 96% of its activity at 40°C and Mollusca, *Ampullaria crossean* where Eg 45 show less than 10% activity was retained at 50° C [25] .

It was observed in figure (11) that maximum cellulase activity was established at pH 5.0, The cellulase and hemicellulase from *Bacillus cereus* LAZ 518 were stable in a range of pH (3.0-7.0) approximately 80% of its activity was retained this agree with [18,48] .They declared that cellulases are generally stable over a wide range of pH from 5 to 10 .

The results in figure (12) indicate that the enzymes activities were linear with reaction time only up to 60 min, after which the reaction linearity gradually decreased and disappeared upon extended incubation time. According to these results the use of 60 min at 70°C was found to be suitable for enzyme assay .

Cellulases and Hemicellulases of *Bacillus cereus* LAZ 518 were activated by $Ca^{2+,}$ K^{+} , and Na^{+} and not inhibited by all other metal ions to avariable extent. Results suggest that cellulase and hemicellulases showed maximum relative activity in the precence of calcium (Ca^{2+}), potassium (K^{+}) and sodium ions (Na^{+}). Cellulases and hemicellulases were strongly inhibited in the presence of Hg^{2+} as indicated in figure (13) . Similar results were reported for *Bacillus* strain [28,38] and *Bacillus amyoliquefaciens* DL-3 [23]. It has been reported that the inhibition of cellulase activity by Hg^{+} ion might be related to its binding with thiol groups, tryptophan residue, or the carboxyl group of amino acid residues in the enzyme [28]. The inhibition of hemicellulases by $Zn^{2+,}$ and Cu^{2+} ions could be due to competition between the exogenous cations and the protein-associated cations, resulting in decreased metallo-enzyme activity.

As figure (14) illustrates cellulase enzyme activity was retained at 110%, 70%, 50%, 134%, 114%, and 167% of the original activity at 10 mM for (EDTA , IAA, p-CMB, DTT , urea and β-mercaptoethanol). and 123%, 105% , 65% 145% , 117% and 170% for hemicellulases Similarly, IAA and p-CMB inhibits cellulase activity because they can bind with the SH group with different degree interaction and subsequently inhibit the activity. However, the β-mercaptoethanol and DTT can reduce the disulfide bonds and re-nature their activity, if the oxidation or aggregation of these enzyme proteins occurs during purification and storage. These phenomenons suggested that the active site of the enzyme contains SH group [44].

Figure (15) proved that the enzyme was appreciably stable in the presence of non- ionic surfactants like tween-40, tween-60, tween-80 and triton-100 and SDS and oxidizing agents like Sodium perborate, Sodium hypochlorite and H_2O_2. Among the oxidizing agents tested the cellulase activities enhanced in presence of sodium hypochlorite and H_2O_2, whereas higher concentrations decrease the stability except sodium hypochlorite as discussed in figure (16)..

Our results agree with [51,52] who reported that cellulase from *B. cepacia* was highly stable in the presence of hydrogen peroxide, sodium hypochlorite and sodium perborate after 1h. The stability profile of the cellulase in the presence of surfactants and oxidizing agents prove its potential application in the detergent formulations as these agents are the active components of house hold detergents.

3.5 Application of cellulases and cemicellulases by HPLC test

HPLC test for determination of monosaccaridesugers was done in the Ragiona center of foods and feeds (RCFF) in agricultural research center (ARC), Giza, Egypt.

From our results in figure (17) we prove that *Bacillus cereus* LAZ 518 can produce both of enzymes cellulases and hemicellulases which can analyze both of cellulose and hemicellulose (poly saccharides) exists in corn cobs waste into glucose,

From the analysis of poly saccharides (cellulose + hemicellulose) into oligo saccharides (glucose + arabinose + xylose) by enzymes cellulases and hemicellulases from *Bacillus cereus* LAZ 518 .

Cellulose $\longrightarrow$ glucose

Hemicellulose $\longrightarrow$ xylose + arabinose

Table (1): Composition of cow dung sample

Parameters	**Content**
pH	**7.3**
Electric conductivity(ds/m)	**1.38**
Total organic carbon(g/kg)	**498**
Total nitrogen(g/kg)	**5.87**
Total available phosphorus (g/kg)	**6.4**
Total potassium (g/kg)	**2.14**
Total sodium (g/kg)	**1.95**
Total calcium (g/kg)	**1.15**
Iron (mg/kg))	**1982**
Copper (mg/kg))	**35**
Zinc (mg/kg))	**123**
Cadmium (mg/kg))	**1.54**

Table (2): Composition of corn cobs

Composition	**Content %**
Cellulose	**36.7**
Hemicellulose	**42.5**
Lignin	**12.4**
protein	**2.56**
Fat	**4.36**
Starch	**0.91**
Ashes	**0.57**

Table (3): Screening of bacterial isolates for cellulase production and some other characters (gram staining and shape of bacterial cell).

No of isolate	Gram staining	Shape of bacterial cell	Clear zone (cm)	No of isolate	Gram staining	Shape of bacterial cell	Clear zone
1	+ve	Bacilli	0.7	51	-ve	Rod shape	-
2	-ve	Rod shape	-	52	+ve	Bacilli	0.2
3	-ve	Rod shape	-	53	+ve	Bacilli	1.6
4	-ve	Rod shape	-	54	+ve	Bacilli	0.8
5	+ve	Cocci	0.9	55	+ve	Bacilli	0.6
6	+ve	Bacilli	2.4	56	+ve	Bacilli	0.7
7	+ve	Bacilli	2.1	57	-ve	Rod shape	-
8	+ve	Bacilli	0.4	58	-ve	Rod shape	-
9	-ve	Rod shape	-	59	+ve	Bacilli	1.3
10	+ve	Bacilli	1.2	60	+ve	Bacilli	2.8
11	-ve	Rod shape	-	61	+ve	Bacilli	0.4
12	-ve	Rod shape	-	62	-ve	Rod shape	-
13	-ve	Rod shape	-	63	-ve	Rod shape	-
14	+ve	Bacilli	1.5	64	+ve	Bacilli	3.7
15	-ve	Rod shape	-	65	+ve	Bacilli	0.8
16	-ve	Rod shape	-	66	+ve	Bacilli	0.3
17	-ve	Rod shape	-	67	+ve	Bacilli	0.9
18	+ve	Cocci	0.6	68	+ve	Bacilli	1.6
19	-ve	Rod shape	-	69	+ve	Bacilli	1.4
20	-ve	Rod shape	-	70	-ve	Rod shape	-
21	-ve	Rod shape	-	71	-ve	Rod shape	-
22	-ve	Rod shape	-	72	-ve	Rod shape	-
23	+ve	Bacilli	2.3	73	+ve	Bacilli	1.7
24	+ve	Bacilli	0.2	74	-ve	Rod shape	-
25	-ve	Rod shape	-	75	-ve	Rod shape	-
26	+ve	Bacilli	0.5	76	-ve	Rod shape	-
27	-ve	Rod shape	-	77	+ve	Bacilli	2.4
28	-ve	Rod shape	-	78	+ve	Bacilli	2.1
29	-ve	Rod shape	-	79	+ve	Bacilli	2.8
30	-ve	Rod shape	-	80	+ve	Cocci	0.1
31	+ve	Bacilli	0.8	81	+ve	Cocci	0.6
32	-ve	Rod shape	-	82	+ve	Cocci	0.3
33	+ve	Bacilli	1.5	83	-ve	Rod shape	-
34	+ve	Bacilli	1.1	84	-ve	Rod shape	-
35	+ve	Bacilli	3.6	85	-ve	Rod shape	-
36	-ve	Rod shape	-	86	-ve	Rod shape	-
37	-ve	Rod shape	-	87	+ve	Bacilli	3.7
38	-ve	Rod shape	-	88	+ve	Bacilli	3.7
39	-ve	Rod shape	-	89	+ve	Bacilli	0.4
40	+ve	Cocci	1.8	90	+ve	Bacilli	1.7
41	+ve	Bacilli	3.8	91	-ve	Rod shape	-
42	+ve	Bacilli	0.8	92	-ve	Rod shape	-
43	-ve	Rod shape	-	93	+ve	Bacilli	0.5
44	-ve	Rod shape	-	94	+ve	Bacilli	1.7
45	-ve	Rod shape	-	95	-ve	Rod shape	-
46	-ve	Rod shape	-	96	-ve	Rod shape	-
47	+ve	Bacilli	3.8	97	+ve	Cocci	0.7
48	-ve	Rod shape	-	98	-ve	Rod shape	-
49	+ve	Bacilli	1.2	99	-ve	Rod shape	-
50	+ve	Cocci	0.4	100	+ve	Bacilli	1.5

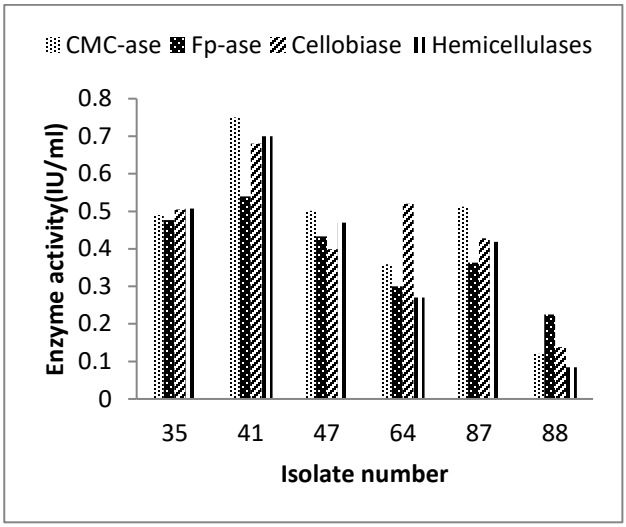

Fig (1): Enzyme activity for 6 bacterial isolates showing maximum clear zone on corn cobs broth medium.

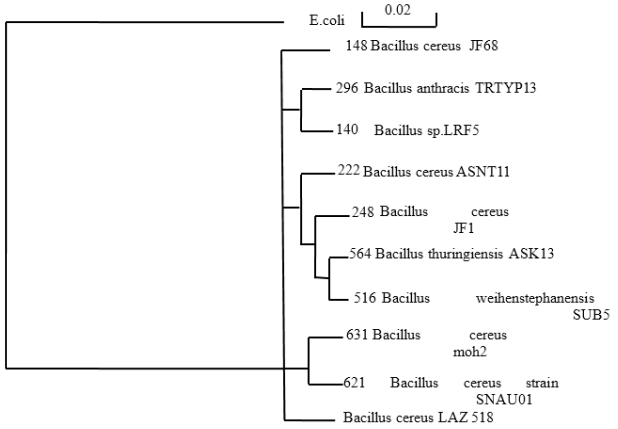

Figure (2): Phylogenetic tree of *Bacillus cereus* LAZ 518 and related bacteria based on 16S rRNA gene sequence . Boot values were based one 1000 replicates. Scale shows percentage of substitution per nucleotide position.

Table (4): Morphological characters for 6 bacterial isolates showing maximum clear zone on CMC agar medium.

Morphological characters	Isolates					
	35	41	47	64	87	88
colony appearance	Circular	Circular	Circular	Circular	Circular	Circular
Translucency and opacity	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque
Elavation of colony	Flat	Flat	Convex	Convex	Flat	Flat
Margin of colony	Undulate	Entire	Entire	Undulate	Entire	Entire
Surface of colony	Smooth	Smooth	Rough	Smooth	Smooth	Rough
Pigmentation	Endo	Endo	Endo	Endo	Endo	Endo
Colour of colony	White	Pale white	White	White	Orange	Orange
Cell shape	Bacilli	Bacilli	Bacilli	Bacilli	Bacilli	Bacilli
Gram staining	+ve	+ve	+ve	+ve	+ve	+ve
Spore staining	Spore forming	Spore forming	Spore forming	Spore forming	Spore forming	Spore forming
Results	Bacillus sp.	Bacillussp.	Bacillus sp.	Bacillussp.	Bacillus sp.	Bacillus sp.

From table (2) the cellulose present in corn cobs is 36.7 % and hemicellulose is 42.5 % and from The resulting enzymatic hydrolysates of CC contain both pentose (xylose + arabinose) and hexose sugers (glucose) . Figure (17) the content of glucose resulted from the analysis of cellulose is 41.42 % and the content of xylose and arabinose resulted from analysis of hemicellulose is 41.5 % and 17 % of unidentified sugars and this agood prove that *Bacillus cereus* LAZ 518 can produce both enzymes cellulases and hemicellulases equally , this regardes with our expectations that hemicellulases will be more cellulases as the content of hemicellose is bigger than the content of cellulose corn cobs. Similarly, ahigher ethanol production from amixture of hexose and pentose sugars by co-culture of P.stipites and S. cerevisiae **[** 40**&** 16**].**

The results presented in this study reveal that CC can be considered as a promising feedstock for bioethanol production . this process could reduce ethanol production costs and be used for the bioconversion of all carbohydrates present in various lignocellulosic biomasses. Successful conversion of such waste biomasses into ethanol would not only help to solve pollution and economic proplems but also create a new revenue source.

Table (5): Physiochemical characteristics of the isolate (41):

Test	Isolate (41)
Gram staining	**(+)**
Motility	**(+)**
Shape	Rod shape
Spore forming	Spore forming
Starch hydrolysis	**(+)**
Casein hydrolysis	**(+)**
Growth at 65º C	**(+)**
Reduction of nitrate	**(+)**
Acid and gas from glucose	**(-)**
Growth at 50º C	**(+)**
Growth on 7% NaCL	**(+)**
Growth on 12% NaCL	**(+)**
Anaerobic growth	**(+)**
V-P reaction	**(-)**
MR Test	**(+)**
Catalase Test	**(+)**
Oxidase Test	**(-)**
Citrate Test	**(+)**
Urease Test	**(-)**
Gelatine hydrolysis	**(+)**
Lipase Test	**(+)**
Growth at 20º C	**(+)**
Growth at 45º C	**(+)**
Growth at 65º C	**(+)**
Oxidation-Fermentation (O-F)	Facultative anaerobe

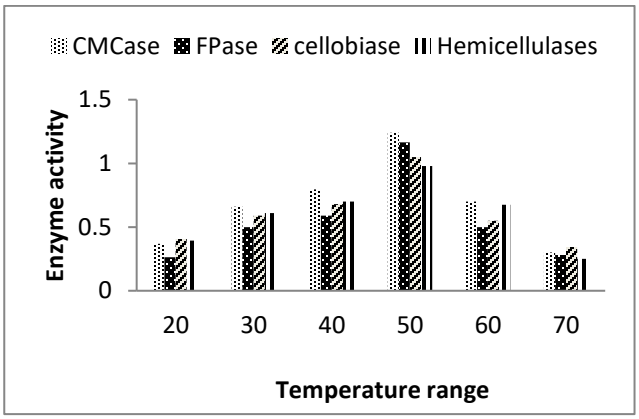

Figure (3): Effect of Temperature conditions on Cellulases and hemicellulases production by *B. cereus* LAZ 518.

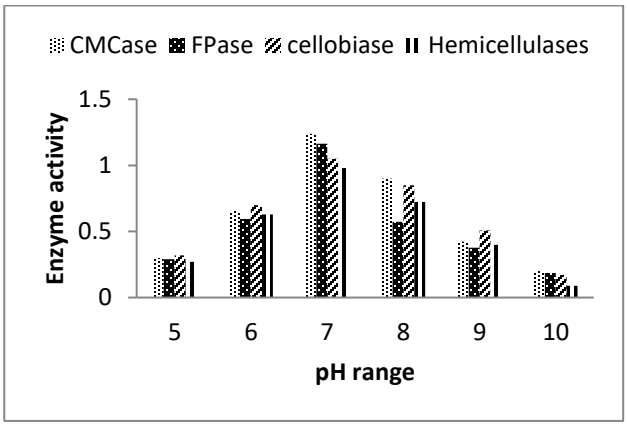

Figure (4): Effect of different pH values on Cellulases and hemicellulases production by *B. cereus* LAZ 518.

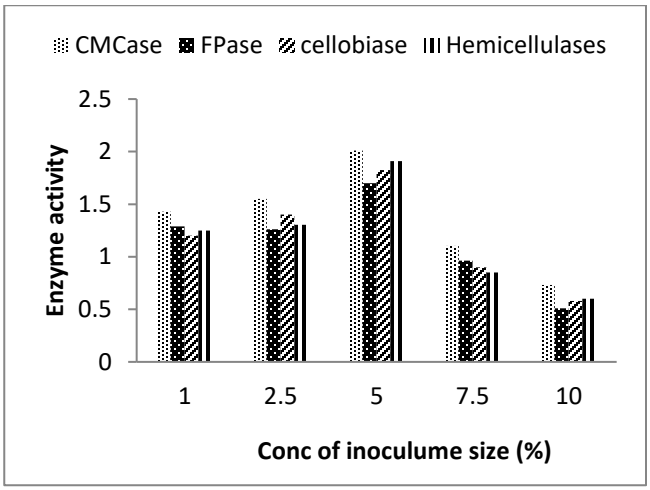

Figure (5): Effect of different concentration of inoculume size on Cellulases and hemicellulases production by *Bacillus cereus* LAZ 518.

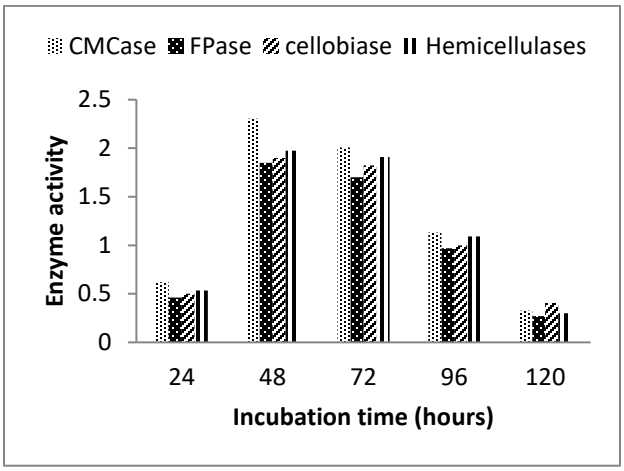

Fig (6): Effect of incubation period on Cellulases and Hemicellulases production by *B. cereus* LAZ 518.

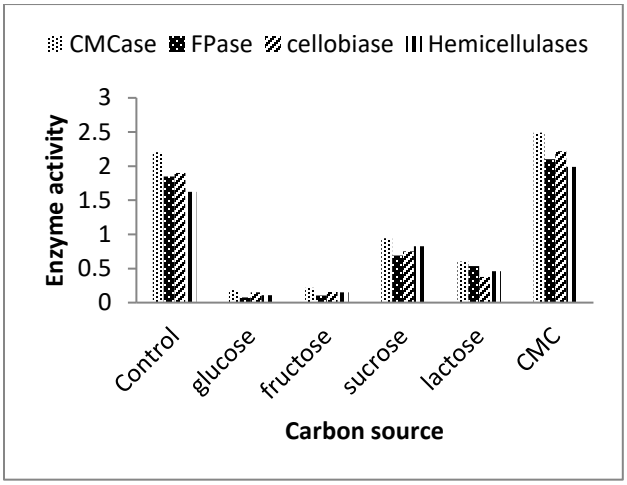

Fig (7): Effect of addition of some carbon sources on Cellulases and Hemicellulases production by *B. cereus* LAZ 518.

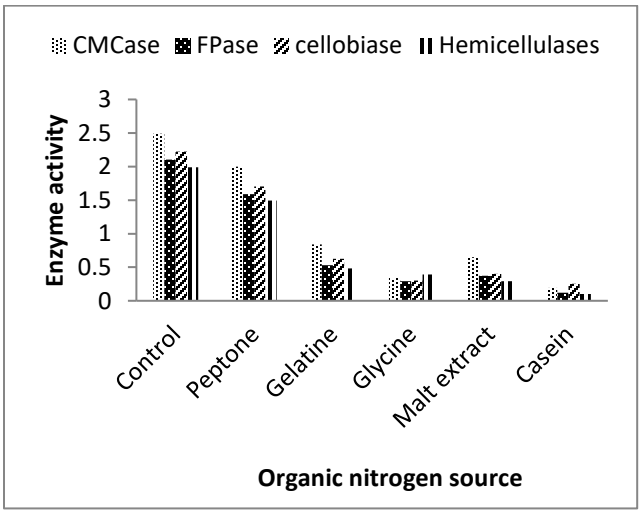

Fig (8): Effect of adding organic nitrogen sources on Cellulases and hemicellulases production by *B. cereus* LAZ 518.

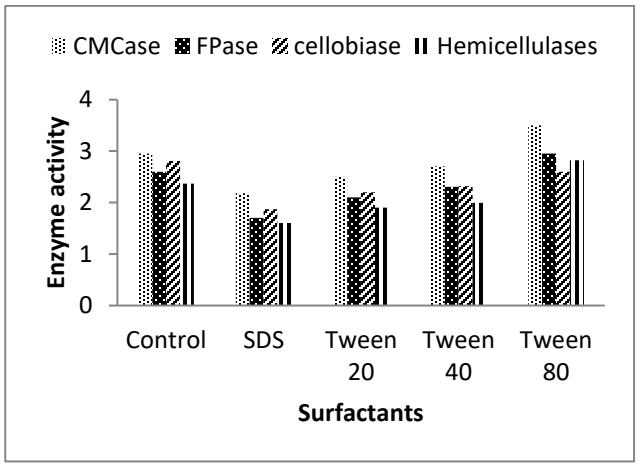

Fig (9): Effect of adding different surfactants to production media on Cellulases and Hemicellulases production by *B. cereus* LAZ 518.

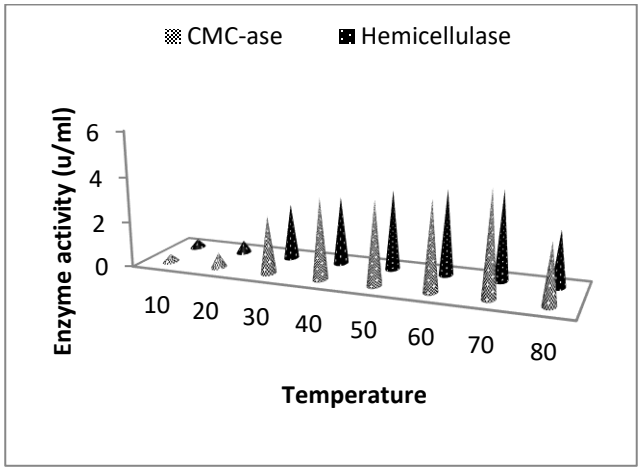

Figure (10): Effect of Temperature on the activity of powder CMC-ase and hemicellulase of *B. cereus* LAZ 518.

Table (6): Effect of thermal stability behaviour on the activity of purified CMCase and hemicellulase of *Bacillus cereus* LAZ 518.

Incubation time (min)	Residual activity (%)							
	CMC-ase				Hemicellulase			
	50	60	70	80	50	60	70	80
15	100	100	74.99	11.13	100	100	69.04	9.31
30	100	91.35	39.32	9.31	100	93.76	61.90	9.08
45	100	84.30	32.79	5.35	100	90.54	56.22	5.42
60	100	84.00	26.97	4.29	100	87.12	33.04	5.40
75	100	80.50	18.66	0.00	100	87.10	13.44	1.95
90	100	77.05	9.31	0.00	100	85.60	10.50	0.00

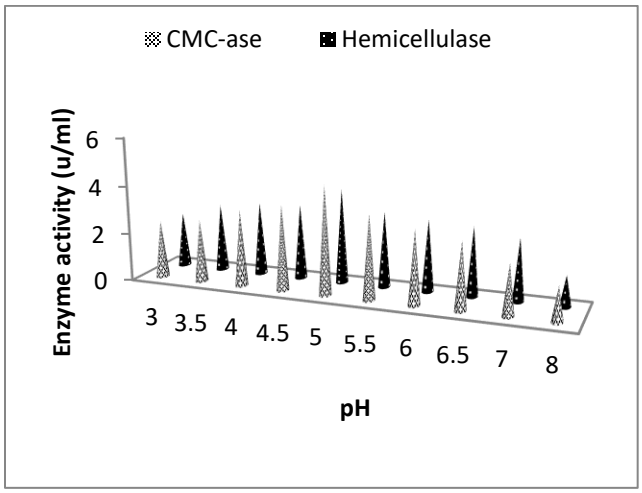

Figure (11) : Effect of reaction pH values on the activity of purified CMC-ase and hemicellulase of *Bacillus cereus* LAZ 518.

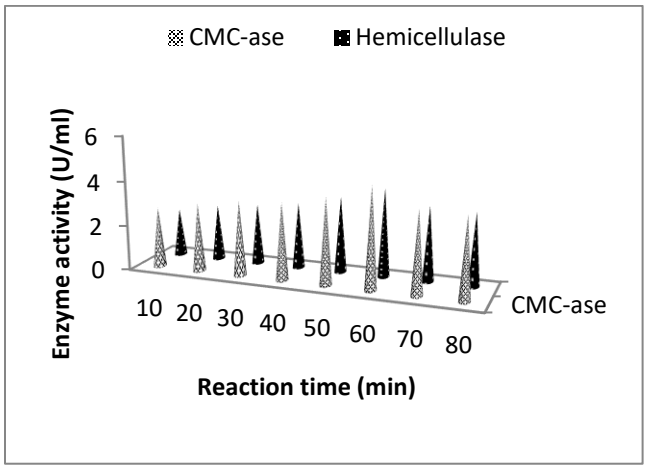

Figure (12): Effect of Reaction progress with time (Time curve) on purified CMC-ase and Hemicellulase of B. *cereus* LAZ 518.

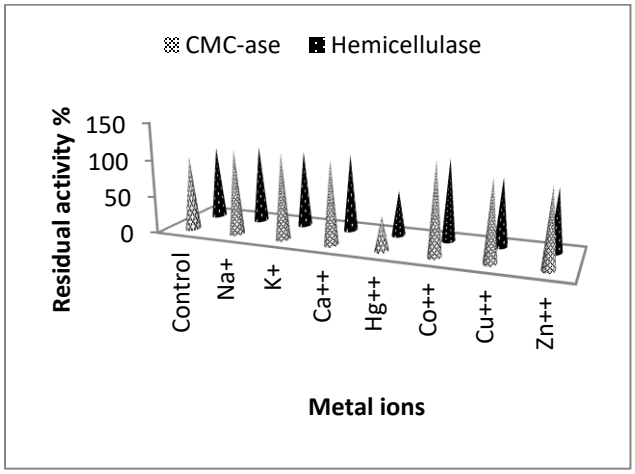

Figure (13): Effect of addition of some metal salts to purified enzyme on CMC-ase and Hemicellulase of *B. cereus* LAZ 518.

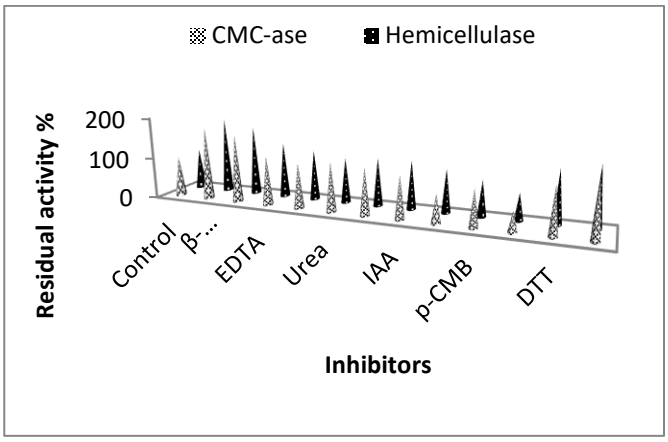

Figure (14): Effect of addition of some different inhibitors to purified powder enzyme on CMC-ase and Hemicellulase of *B. cereus* LAZ 518

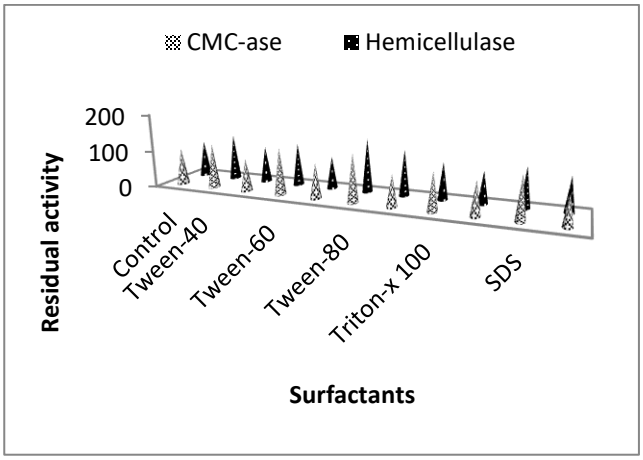

Figure (15): Effect of addition of some surfactants to purified powder enzyme CMC-ase and hemicellulase of *B. cereus* LAZ 518 stability.

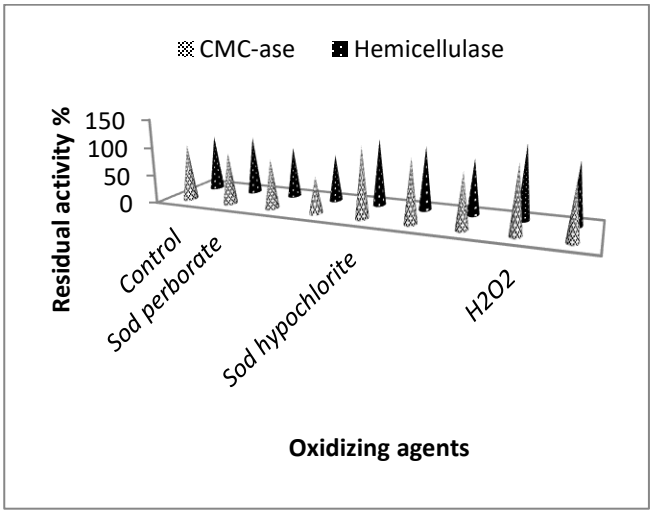

Figure (16): Effect of addition of some oxidizing agents to purified powder enzyme CMC-ase and hemicellulase of *B. cereus* LAZ 518.

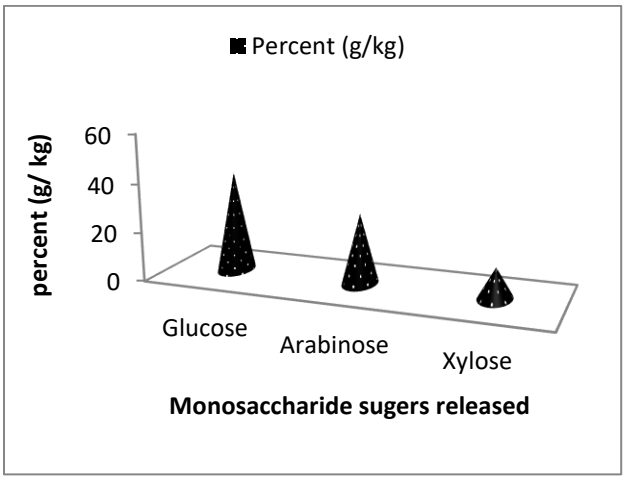

Figure (17): Glucose, Arabinose and xylose (g) resulted from analysis of 1 Kg of corn cobs waste by cellulases and hemicellulases from *Bacillus cereus* LAZ 518

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