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Production and Characterization of Cellulase Enzyme Produced by *Bacillus* Isolates from Different Sources

Elhadi A. I. Elkhalil* and Fatima Y. Gaffar

Department of Botany and Agric. Biotechnology, Faculty of Agriculture, University of Khartoum, PO Box 13314, Shambat, Sudan.

Abstract: This study was conducted to produce and characterize cellulase enzymes from *Bacillus* isolates and to determine the optimum conditions for cellulase activity. Four *Bacillus* isolates were used: iso 9+ from compost and iso13, iso23 and iso20 from soil. The four isolates (9+, 23, 13 and 20) gave average cellulase activities of 2.89, 3.12, 3.48 and 3.53 Unit/ml, respectively. The pH optimum for cellulases from the four isolates was 6, and the temperature optima were 45, 60, 60 and 80°C, respectively. Cellulases from isolates 13 and 23 were more thermostable and retained 40.2% and 21.9% of their initial activities after incubation for 110 min at 90°C. Cellulase was produced under solid state fermentation conditions in maize cobs, sorghum stover or bagasse pretreated with 2% hydrochloric acid from the above four isolates as well as from two more isolates (iso4- and iso2-) which were isolated from cow dung. Maximum cellulase activity was 3.78 Unit/ml from isolate 4- using bagasse and maize cobs as substrate. These results demonstrated that *Bacillus* isolates 13, 23, 20 and 9+ produced active thermostable extracellular cellulase. Bagasse and maize cobs are suitable as substraes for production of the enzyme cellulase under solid state fermentation.

Key words: *Bacillus*; cellulase; pretreated lignocellulosic waste; solid state fermentation

*Corresponding author: E. mail: eaikhalil@yahoo.com

NTRODUCTION

Cellulose, which forms about 40% -50% of plant composition, is the most abundant organic matter on earth. Proper biotechnological utilization of cellulose wastes in the environment reduces pollution and converts them into useful byproducts (Milala *et al*. 2005). Cellulase (a complex multi-enzyme system) acts collectively to hydrolyze cellulose from agricultural wastes to produce simple glucose units (Milala *et al*. 2005). Cellulase is involved in various processes, including food processing, textile laundry, pulp, and paper industries (Singh *et al.* 2004).

There are a few major bottlenecks with the current production of biofuels; one being the lack of biocatalysts that can work efficiently and inexpensively at the high temperatures and/or low pH conditions used in the bioconversion of lignocellulosic materials to bioethanol. Moreover, there is a great need for cost-effective fermentation of derived sugars from cellulose and hemicellulose (Wyman *et al.* 2005). It is therefore important that enzymes be stable and active at high temperatures and/or wide pH ranges (Maki *et al.* 2009).The greatest potential for ethanol production from biomass lies in enzymatic hydrolysis of cellulose using cellulase enzymes. Even after decades of research on cellulases, the costs of these enzymes have remained high. To make biomass processing competitive would require substantial reductions in the current cost of producing cellulase enzymes (Knauf and Moniruzzaman, 2004).

Solid state fermentation may be a more economical method of cellulase production, thereby reducing bio-ethanol production costs. The bio-ethanol will become a viable supplemental fuel source in light of current economic, political, and environmental issues (Zhuang *et al.* 2007). Currently, industrial demand for cellulases is being met by production methods using submerged fermentation processes, employing generally genetically modified strains of *Trichoderma*. The cost of production in submerged fermentation systems is however high and it is uneconomical to use these systems in many of the aforesaid processes. This therefore

necessitates reduction in production cost by deploying alternative methods, for example solid state fermentation systems (Pandey *et al*.1999). The cellulolytic enzymes of *Bacillus* have been the focus of much attention because of their potential use in the conversion of agricultural wastes into useful products including laundry detergents. A preliminary study showed that *Bacillus subtilis* (BTK 106) can produce a considerable amount of cellulase activity (Krishna 1999)

Bacillus spp. produce various extracellular enzymes of industrial importance. Alkalophilic *Bacillus* spp. produce a variety of alkaline hydrolytic enzymes of industrial importance, including cellulases, amylases, pectinases and proteases. However, industrial applications of cellulase have mainly focused on using fungal enzymes **(**Kim *et al.* 2005). The aim of this study was to produce and characterize cellulase enzymes from *Bacillus* isolated from soil, compost and cow dung in liquid and solid state fermentation.

MATERIALS AND METHODS

Isolation and conventional identification of *Bacillus*

Bacillus strains were isolated from compost, soil, and cow dung. Soil samples were taken from rhizosphere of a potato crop field in Shambat, Khartoum North,Sudan. The soil was alkaline (pH 8.5) silty clay. Compost samples were taken from a depth of 20 cm and cow dung samples were obtained from the same region. Strains from different resources were isolated according to Lindquist (2006). Ten g from each sample were dissolved in 90 ml distilled water. Sample suspensions were heated at 80°C for 15 minutes. Five dilutions of samples were made: 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and $10^{-5}$and plates of Nutrient Agar were inoculated (by the spread plate method) with 0.1 ml of heated dilutions 10^{-3}, 10^{-4}, 10^{-5} from each sample and were incubated aerobically at 37°C for 24 hours. The isolates were identified according to Harrigan and McCance (1976) by subjecting to the following biochemical and morphological tests: oxidation/ fermentation, nitrate reduction, Gram's reaction, spore staining, motility test, catalase test, oxidase test, Voges- Proskauer test,

hydrolysis of starch, liquefaction of gelatin, utilization of citrate, production of indole, methyl red test, growth at pH 5.7 and 6.8, and growth in the presence of sodium chloride (2%, 5%, 7% and 10%).

Cellulase production and preparation

Cellulase production was done according to Kotchoni and Shonukan (2002) with slight modifications. A single fresh colony of each isolate was inoculated in Nutrient Broth medium for 24 hours at 37C° with shaking at 150 rpm as preculture. The preculture was inoculated (1:20) in 100 ml medium, containing (per litre) 0.2% glycerol as carbon source, 0.2% carboxylmethyl cellulose as substrates for cellulase synthesis, 10 g peptone, 1 g K_2HPO_4, 0.75 g Mg $SO_4.7H_2O$, 0.75 g NaCl in a 0.1M KH_2PO_4 buffer (pH 6.0) in 250 ml conical flasks. Incubation was continued for 24, 48, 72, 96, 120 or 144 hours to determine the optimum incubation time at 25°C with shaking at 150 rpm. After each period of incubation, the yield of cellulase was estimated in the extracellular fluid after removal of bacterial cells from the culture broth by centrifugation at 500 rpm for 15 min.

Cellulase assay

Cellulase activity was determined by the method of Miller (1959). The assay mixture contained 0.5 ml of 2% carboxy methyl cellulose in 0.05 M Na-citrate buffer of pH 4.8, and 0.5 ml of crude enzyme. The reaction was performed at 50°C for 30 min and stopped by addition of 3 ml of 3.5-dinitrosalicylic (DNS) reagent. The enzyme activity was obtained from a calibration curve prepared by the same procedure, with D-glucose as the standard.

According to the International Union of Biochemistry, one enzyme unit equals 1 micromole of substrate hydrolyzed per min. For cellulase, this was based on bonds hydrolyzed, that is micromoles of glucose released per minute. One micromole of glucose equals 0.180 mg. For a 30 minute assay, 1 mg of glucose equals 0.185 units calculated as follows:

1 mg glucose=

$$\frac{1}{30 \times 0.18}$$

The enzyme unit (U) was calculated from the obtained mgs of glucose.
U= mg glucose ×0.185

Characterization of cellulase

Determination of pH optima of cellulase enzyme: Determination of pH optima of cellulase enzyme was carried out according to Kotchoni and Shonukan (2002) with a slight modification. A substrate solution containing 2% carboxymethyl cellulose was prepared in sodium citrate buffer at the pH values of 4.0, 6.0, 8.0, 10.0 and 12.0. Half millilitres of enzyme preparations were preincubated in a water bath at the assay temperature for 5 min, and the enzyme reactions were initiated by adding 0.5 ml of substrate solution. The mixture was incubated for 30 min at 50°C; reactions were terminated by adding 3 ml DNS colour reagent and the mixture incubated in boiling water for 5 min and the activities of the enzyme were determined as above. .

Determination of temperature optima of cellulase enzyme: The method of Kotchoni and Shonukan (2002) with slight modifications was used for determining the optimum temperature. Preparation of enzymes and substrate solutions as well as mixing ratio was as described earlier. However, the pH of the mixtures corresponded to determined optimum pH of the respective enzymes. The mixtures were incubated for 30 min at each of the following temperatures: 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 and 100°C, then the cellulase activity was measured as described before.

Determination of thermostability of cellulase enzyme: The thermostability was determined in aqueous solutions according to Kotchoni and Shonukan (2002) with a slight modification. The cellulases preparations were preincubated at 40, 50, 60, 70, 80 and 90°C for 10, 20,

30, 40, 50, 60, 70, 80, 90, 100 or 110 min. After preincubation periods, the samples were cooled and reincubated at the optimum temperature determined for the respective enzyme. The residual activities of enzymes were estimated as -mentioned above.

Production of cellulase under solid state fermentation

Lignocellulosic substrate: Lingocellulosic waste (maize cobs, sorghum stover and bagasse) were obtained from the University of Khartoum farm, Shambat. The substrates were crushed into pieces, sun dried and ground to 0.4 mm mesh size and stored in air tight plastic jars.

***Bacillus* cultures and inoculum development:** The pure cultures of *Bacillus* were isolated from different sources: isolates -2 and -4 (from cow dung), isolates 13, 23 and 20 (from soil) and isolate 9^{+} (from compost) were used to prepare the inoculum.

Nutrient Broth was inoculated by cultures aged 24-48 h after sterilizing at 15 Ibs/ $inch^2$ pressure and 121°C in an autoclave for 15 min, and incubated for 1 day for the development of the inoculums (Ahmed *et al.* 2010).

Pretreatment of lignocellulosic waste: Ten powdered grammes of maize cobs, sorghum stover and bagasse were pretreated with 2% HCl in an Erlenmeyer flask (250 ml). The pretreated substrates were kept at room temperature for 2 hours, autoclaved at 121°C and 15 Ib/ $inch^2$ pressure for 15 min. Slurries of substrates were filtered through four layers of muslin cloth, both filtrate and residue were saved. Residue was washed 4 to 5 times with distilled water and filtrate was used for analysis of total sugars and reducing sugars, while the rest was used for production of cellulose (Ahmed *et al.,* 2010).

Fermentation protocol: Cellulases were produced as described by (Ahmed *et al.* 2010) using different agricultural residues and lignocellulosic waste by solid state fermentation. Basal fermentation medium was used to moisten (40% moisture content) the lignocellulosic substrate (10g) in an Erlenmeyer flask (250 ml) for cellulase production

according to Ariffin *et al*. (2006). The major constituents of the medium were $(NH4)_2$ SO_4 (10 g), KH_2PO_4 (4 g), $MgSO_4.7H_2O$ (0.5 g) and $CaCl_2$ 0.5 g per liter. The initial pH value of the medium was adjusted to 6 before sterilization at 121^oC and 15.0 Ibs/ inch^2 pressure for 15 min. The autoclaved medium was inoculated with 5 ml of the prepared culture and incubated at 34±2°C in a temperature-controlled incubator for 8 days under still culture conditions. Control was similarly treated but with no inoculation. The cellulase activity was measured as described before at the optimum temperature of each isolate.

Analytical methods

Estimation of total sugars: In order to investigate the suitability of maize cobs, sorghum stover and bagasse for cellulase production under solid state fermentation conditions estimation of total sugar was done. Phenol sulfuric acid method (Dubois *et al***.**1956) was used to measure the total sugar in the substrates. One milliter sample was mixed with 1 ml 5% phenol and 5 ml conc. H_2SO_4 and kept for 10-15 min. Optical density was checked at 470 nm to measure colour intensity.

Estimation of reducing sugars: For the investigation of the suitability of maize cobs, sorghum stover and bagasse for cellulase production reducing sugars were determined. Reducing sugars in substrates were measured using 3, 5-dinitrosalicylic acid (DNS) by the method of Miller (1959). One millilitre sample was mixed with 3 ml DNS and boiled for 10 minutes. Optical density was checked at 550 nm to measure colour intensity. Reducing sugar content was determined by comparing against a standard curve constructed similarly with known concentrations of glucose.

Cellulase enzyme extraction: The enzyme was extracted by the simple contact method of Krishna and Chandrasekaran, (1996). A 0.05M Citrate buffer of pH4.8 was introduced into each fermented flask in 1:10 (W/V) ratio kept in shaker at 120 rpm for 30 min. and filtered using muslin cloth. The filtrate was centrifuged at 1000 rpm for 15 min to obtain clear supernatant that was used for further enzyme analysis.

RESULTS

Isolation and conventional identification of *Bacillus*

Strains used for cellulase production were isolated from soil, compost and cow dung samples and were selected for their high cellulase expression after initial testing of a large number of isolates in growth medium containing carboxymethyl cellulose. From the biochemical and morphological tests, all isolates proved to be *Bacillus* according to Brenner *et al*. (1986).

Cellulase production and characterization

The results revealed that cellulase activity for each isolate was different; ranging from 2.89U/ml (isolate9+) to 3.53 U/ml (isolate 20). Figure1. shows the cellulase activity during incubation of *Bacillus* isolates in production medium at 25°C. Cellulase activity for the isolates 13, 20, 9+ and 23 were 3.46, 3.53, 2.89 and 3.12 U/mL, respectively. The pH behaviour of cellulase preparations shown in Figure 2. indicated that the pH optimum of the four isolates was acidic (pH 6). Temperature profiles of different cellulases were measured by incubating the enzymes at different temperatures as shown in Figure 3. The measurements of temperature optima gave a value of 60°C for isolates 20 and 23, while for isolates 13 and 9^{+} the values were 80°C and 45°C, respectively. The results of the thermostability measurements (Figures 4-a, 4-b, 4-c and 4-d) indicate that isolate 23 had the highest while isolate 9+ the lowest stability to heat treatment.

Results of the investigation on the suitability of maize cobs, sorghum stover and bagasse for cellulase production under solid state fermentation conditions are shown in Figure 5. The contents of total sugars and reducing sugars were determined before cellulase production. The amounts of reducing and total sugars in the substrates used in cellulase production (maize cobs, sorghum stover and bagasse) were 20.58 and 590.7, 10.50 and 437.5 and 6.830 and 27.49 µm/ ml respectively.

Production of cellulase under solid state fermentation conditions was done using bagasse, maize cobs, and sorghum stover. Bagasse and maize cobs were found to be the most suitable substrates giving enzyme activities of 3.78 and 3.75 U/ml, respectively, while sorghum stover showed no enzyme activity for the tested isolates (Fig 5.).

DISCUSSION

The decrease in cellulase activity may be due to the cumulative effect of cellulobiose. Cellulobiose is a dimer of glucose which is known to inhibit both endo- glucanase and glucosidase. The time for highest cellulase activity depended upon the substrate and microorganism used (Alam *et al*. 2005). But in time, it decreases until the end of the fermentation period. Also, decrease in extracellular enzyme activity was characterized by a decrease in microbial population which resulted in a reduction in the synthesis of new enzymes (Aira *et al.*2007).

The pH optima of the four isolates were acidic (pH 6). Due to the acidic tolerance of this enzyme, it has good potential for industrial use in the hydrolysis of soluble cellulose as well as activity on microcrystalline sources of cellulase (Wang *et al.* 2008). The measured pH optima for the isolates agreed with the data of Hitomi *et al.* (1994), Subramaniyan and Prema **(**2000), and Emtiazi and Nahvi (2004).

There are a few major bottlenecks with the current production of biofuels; one is a lack of biocatalysts that can work efficiently and inexpensively at high temperatures and/or low pH conditions used in the bioconversion of lignocellulosic materials to reducing sugar and then to bioethanol. Moreover, there is a great need for cost- effective fermentation of derived sugars from cellulose and hemicellulose (Wyman *et al*. 1992). Currently, industrial bioconversions of lignocelluloses requires the application of high temperature and acidic or sometimes basic conditions to break down lignin, decrease crystallinity, increase pore volume and solubilize cellulose and hemicelluloses to allow enzymatic hydrolysis of target polysaccharides (Wyman *et al*.1992). This process is both expensive and

inefficient. It is, therefore, important that enzymes be stable and active at high temperature and /or low or high pH conditions. Among the four isolates, isolate 13 had the highest temperature optimum of 80°C, which will make it more suitable for producing cellulase for use in bioethanol production from lignocellulosic biomass.

The measured temperature optima of the preparation are in general agreement with other reports; an alkaline-stable cellulase from *Bacillus* with optimum temperature at 60°C was described by Oshino *et al.* 1986. Celluase from *Acidothermus cellulyticus* has temperature optimum at 80°C as described by Pontanen *et al.*(1987) and Trivedi *et al.* (2011) reported that *Bacillus aquimaris* optimum activity was at 45°C.

Cellulases have been widely studied and are being used for various industrial purposes because of their potential in biomass conversion and in the paper, textile and detergent industries. Some of the applications require heat stable enzymes that can work at elevated temperatures. An increasing number of heat stable cellulases have been described; however, the major factors stabilizing the structure of these enzymes are not well understood (Nemeth *et al.* 2002). The thermostable enzyme from isolate 23 would facilitate development of more efficient and cost effective forms for the simultaneous saccharification and fermentation process to convert lignocellulosic biomass into biofuels.

From the results of the solid state fermentation it was found that bagasse and maize cobs supported the production of cellulases. Adsorption of enzymes and the formation of enzyme-substrate complexes are considered to be critical steps in the enzymatic hydrolysis of cellulose. Cellulose fibers contain both amorphous and crystalline regions; crystalline regions are considered to be more difficult to degrade than the amorphous region (Arifo and Ogel 2000). Therefore, the highest productivity of the cellulase when bagasse and maize cobs were used as substrates may indicate that these enzymes adsorbed efficiently on bagasse and maize cobs. Also, the negative result of cellulase production when sorghum stover was used as substrate may have been due to the considerable amount of crystalline cellulose.

The major factors that affect microbial synthesis of enzymes in a solid state fermentation system include selection of a suitable substrate and microorganism, pre-treatment of the substrate, particle size (inter-particle space and surface area) of the substrate, water content, relative humidity, type and size of the inoculums, control of temperature of fermenting matter, removal of metabolic heat, period of cultivation, maintenance of uniformity in the environment of SSF system, and the gaseous atmosphere, i.e oxygen consumption rate and carbon dioxide evolution rate (Pandey *et al*.1999).

For lignocellulosic materials, sugars are primarily derived from hemicelluloses and cellulose components. Sugar derived from cellulose (glucose), is more easily fermentable, especially for the production of ethanol, than the sugars derived from hemicelluloses, which are more complex and mainly include five-carbon sugars, such as xylose (Sun and Cheng 2005). However, proper pretreatment of the biomass material by diluted acid (2% HCl) was used to improve enzymes accessibility to cellulose by removing hemicellulose, lignin or reducing crystallinity of cellulose. Recently, it has been demonstrated that the dilute acid pre-hydrolysis can achieve high reactions rate in a short time and significantly improve cellulose hydrolysis (Eggeman and Elander 2005). Decrease in cellulase activity under SSF condition may be due to production of aromatic water-soluble products which may repress the cellulolytic action of the enzyme as stated by Hattaka (1983).

CONCLUSION

It could be concluded that an economical process for mass production of cellulase was developed. Further research is needed to optimize the cellulase production under SSF conditions.

ACKNOWLEDGMENT

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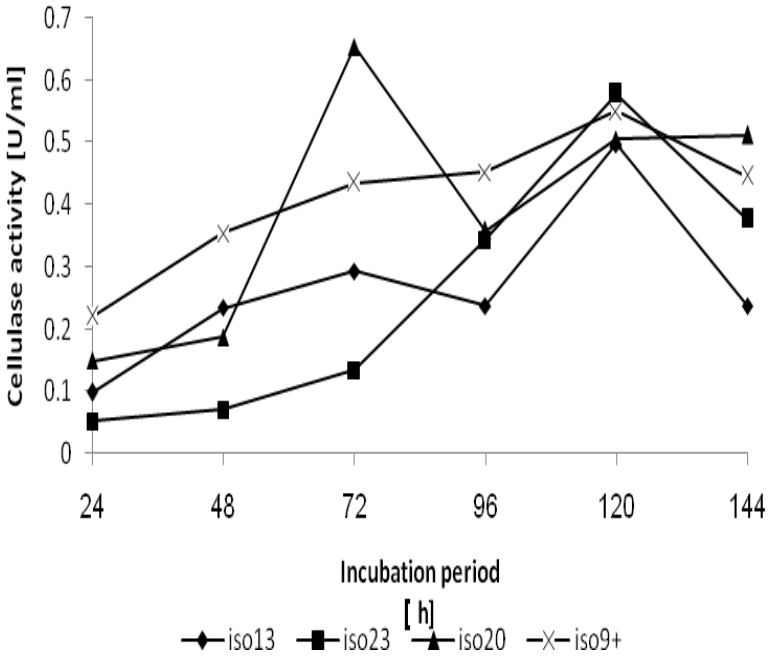

Fig. 1. Cellulase activity during incubation of *Bacillus* isolates in production medium at 25°C.

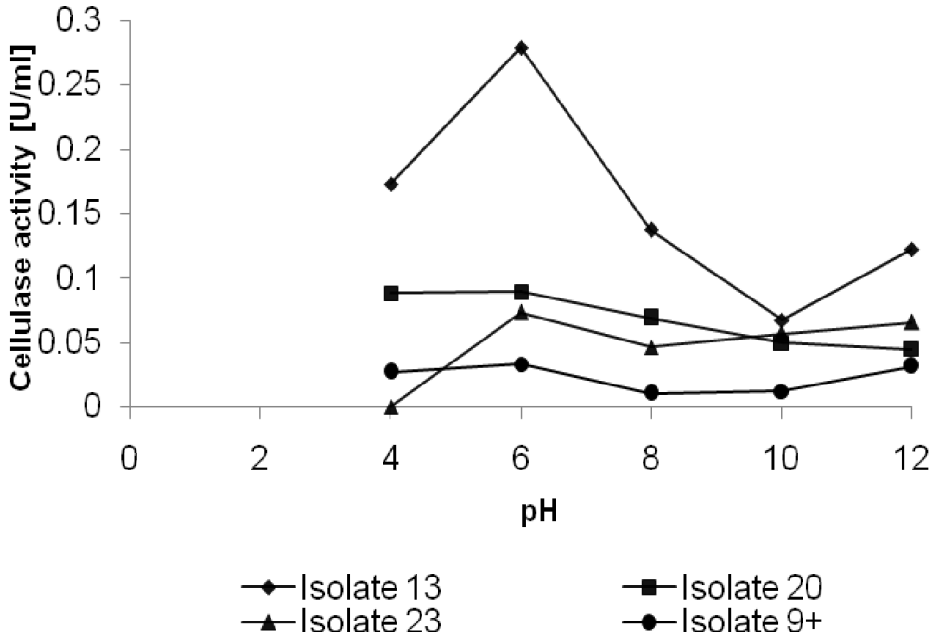

Fig. 2. pH behaviour of cellulase produced from the four isolates 20, 23,13 and 9+.

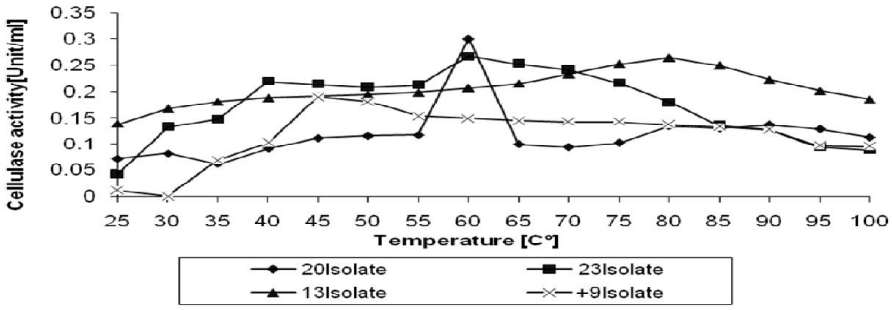

Fig. 3. Temperature profile of cellulase produced from the four Isolates 20, 23, 13 and 9+.

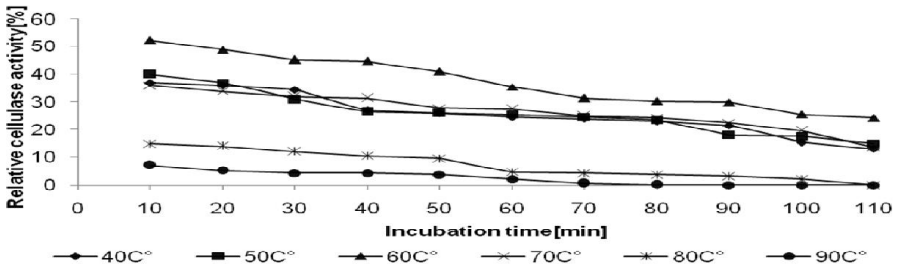

Fig.4-a. Residual activity of cellulase from isolate 20 after exposure for different periods at different temperature. The data are expressed as percentage of activity before heat treatment determined at the optimum temperature for 30 min.

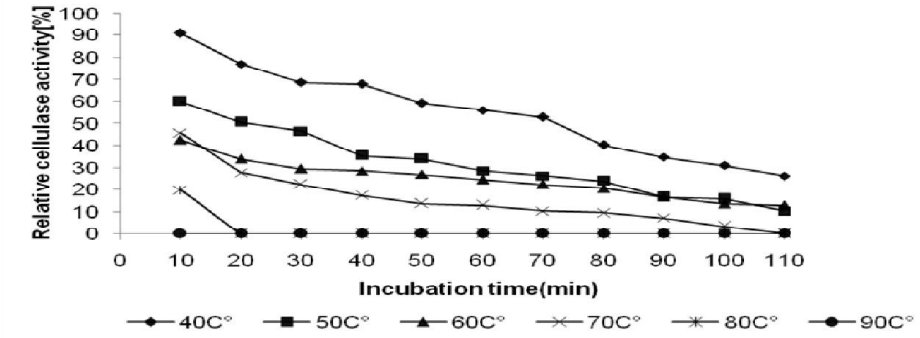

Fig. 4-b. Residual activity of cellulase from isolate 9+ after exposure for different periods at different temperature. The data are expressed as percentage of activity before heat treatment determined at the optimum temperature for 30 min

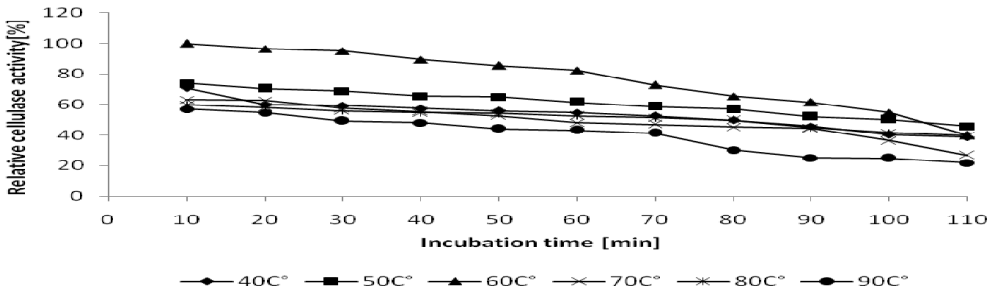

Fig. 4-c. Residual activity of cellulase from isolate 23 after exposure for different periods at different temperatures. The data are expressed as percentage of activity before heat treatment determined at the optimum temperature for 30 min.

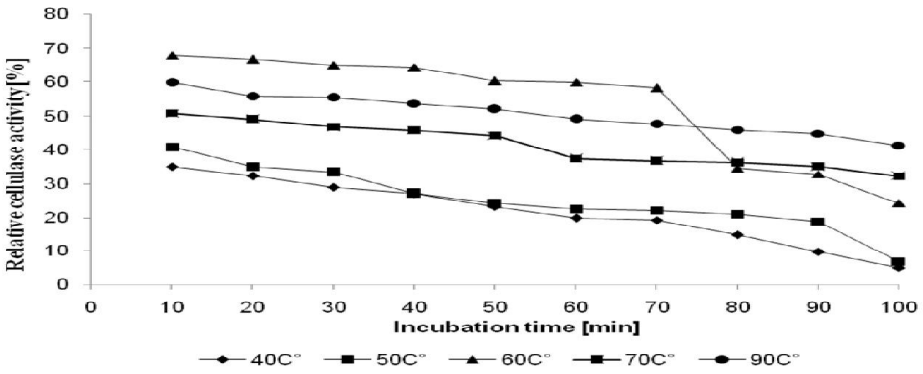

Fig 4-d. Residual activity of cellulase from isolate 13 after exposure for different periods at different temperature. The data are expressed as percentage of activity before heat treatment determined at the optimum temperature for 30 m.

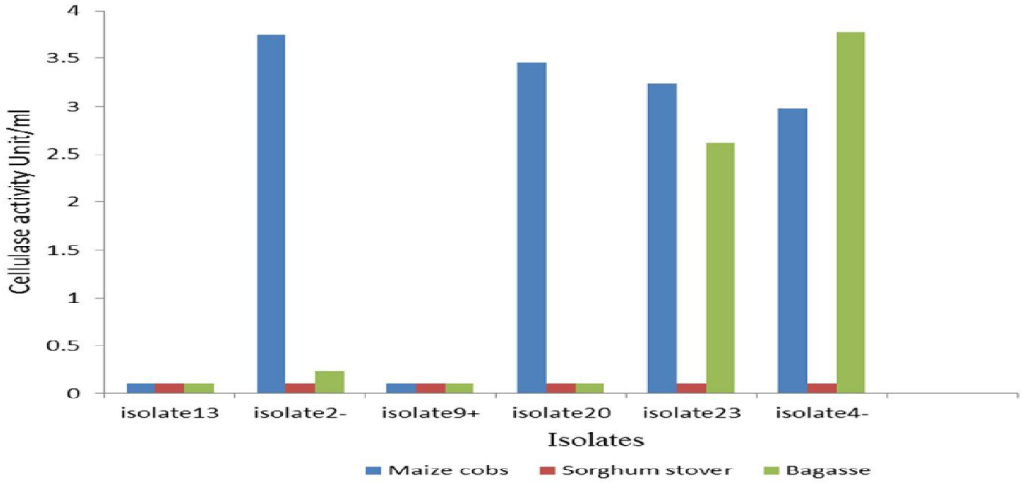

Fig.5. Effect of different substrates on activity of cellulase produced under SSF conditions.

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إنتاج و توصيف إنزيم السليوليز المنتج من عزلات بكتريا الباسلس *Bacillus* sp. من مصادر مختلفة

الهادي علي ابراهيم الخليل وفاطمة يوسف جعفر

قسم النبات والتقانة الحيوية الزراعية – كلية الزراعة، جامعة الخرطوم، شمبات- السودان

المستخلص : أجريت هذة الدراسة لانتاج انزيم السليوليز من عزلات بكتريا *Bacillus* وتحديد الظروف المثلى لنشاطه. استخدمت أربع عزلات بكتيرية: iso9+ من السماد العضوي وiso13 و iso23 و iso20 من التربة. كان متوسط النشاط الانزيمي للعزلات +9 و 23 و 13 و 20 هو 2.89 و 3.48 و 3.12 و 3.53 وحدة/مل علي التوالي. كان الرقم الهايدروجيني الأمثل لانزيم السليوليز المعزول من العزلات الأربع 6، بينما كانت درجات الحرارة المثلى علي النحو الاتي 45 و 60 و 60 و 80°م علي التوالي. وجد أن إنزيم السليوليز من العزلتي iso13 و iso23 أكثر تحملا للحرارة حيث إحتفظا ب 40.2% و 21.9 % من نشاطهما الإبتدائي وذلك بعد تحضين لمدة 110 دقيقة في درجة حرارة 90° م. أنتج إنزيم السليوليز تحت ظروف طريقة التخمير الصلبة في أوساط غذائية تحتوي علي بقايا كيزان الذرة الشامية والذرة الرفيعة بالإضافة للبقاس المعاملة بحامض الهيدروكلوريد المخفف ذو التركيز 2% من العزلات الأربع +9 و 20 و 23 و 13 بالإضافة للعزلتان -iso4 و -iso2 من روث البقر. وجد أن أعلي نشاط لإنزيم السليوليز هو 3.78 وحدة /مل المنتج من العزلة -iso4. دلت النتائج إلي أن العزلات 13 و 23 و 20 و +9 أنتجت إنزيم سليوليز خارج الخلية نشط ومتحمل للحرارة. البقاس وبقايا كيزان الذرة الشامية مناسبة لاستعمالها كمصدر للسليلوز لانتاج إنزيم السليوليز تحت ظروف التخمير الصلبة.