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Production of cellulase from *Bacillus cereus* by submerged fermentation using corn husks as substrates

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Abstract

The potential of a *Bacillus cereus* strain isolated from local Syrian soils to produce cellulase enzymes from corn husks through submerged fermentation is demonstrated. The effects of temperature, pH, concentration of some metal ions (Mn^{2+}, Fe^{3+},Co^{2+}), surfactant (SDS) and chelating agent (EDTA) on crude enzyme activity were studied. Maximum activity was found to be at a temperature of 30°C, pH 5 and substrate concentration of 1%. The enzyme was activated by Co^{2+}, Mn^{2+} and EDTA, while Fe^{3+} and high concentration of SDS played an inhibiting role.

Introduction

Agro-industrial wastes are renewable form of resources generated round the year all over the world. Wheat and rice bran, sugar cane bagasse, corn cobs, corn husks, citrus and mango peel etc. are important wastes of food industries. Due to the high cellulose content of approximately 63% (Lawal and Ugheoke, 2010), corn husks represent an attractive substrate for cellulase production. The role of micro-organisms in bioconversion of bio-products and bio-waste into value added products has been highlighted in the recent decades, especially in enzyme productions (Pandey *et al.*, 2000; Ahuja *et al.*, 2004). Among such enzymes cellulases are gaining popularity in this regard. These enzymes are generally considered to consist of three enzyme groups for cellulose hydrolysis into glucose monomers, namely exoglucanases, endoglucanases and cellobiases. Synergy between these enzymes is important for the hydrollysis process (Jalak *et al*., 2012). Cellulases have a variety of applications in many different industries such as food, brewery, wine, pulp and paper, textile, detergent, feed and agriculture (Bhat, 2000; Karmakar and Ray, 2011). The application of produced enzymes in industry requires high thermostability along with ability to tolerate a wide pH range. Several microorganisms such as bacteria, yeast and fungi are capable of cellulase production (Kim, 1995; Bhat, 2000; Camassola *et al*., 2004; Haakana *et al*., 2004; Bischoff *et al*., 2006). Bacteria, owing to their high diversity and capability to produce highly thermostable and alkalistable enzyme complement, may serve as highly potent sources of industrially important enzymes (Bhat, 2000; Camassola *et al.*, 2004). Among bacteria, *Bacillus* species can produce many types of extracellular polysaccharide hydrolyzing enzymes (Ozaki and Ito, 1991; Bhat and Bhat, 1997); and many researchers have documented the production of thermostable and alkalistable cellulases from different *bacillus* species (Bhat 2000; Bischoff *et al.*, 2006; Nizamudeen and Bajaj, 2009; Odeniyi *et al*., 2009; Yin *et al*., 2010). In this work the potential of *Bacillus cereus* isolated from local Syrian soils to produce cellulase enzymes from corn husks through submerged fermentation is presented. The effects of temperature, pH, concentration of some metal ions (Mn^{2+}, Fe^{3+},Co^{2+}), surfactant (SDS) and chelating agent (EDTA) on crude enzyme activity were studied.

Materials and Methods

Materials

Agro waste as Corn husks served as substrates on which the bacteria grew for production of Cellulase. Sodium phosphate, sodium citrate, sodium carbonate, manganese chloride, cobalt chloride, iron(II) chloride, Dinitrosalicylic acid (DNS), Carboxymethyl cellulose (CMC), sodium dodecyl sulfate (SDS), Ethylene diamine tetra-acetic acid (EDTA). *Bacillus cereus* strain was isolated from local Syrian soils. All

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the chemicals used in this study were of analytical grad purchased from Sigma Chemicals Ltd.

Isolation of bacteria

One hundred samples of soil were collected for the isolation of cellulose degrading bacteria from different regions of Damascus and Damascus countryside. Soil samples were serially suspended in sterile water and heated for 15 minutes at 80°C, in order to remove thermo intolerant bacteria (Pattnaik *et al*., 2010). After suitable dilutions the samples were incubated in nutrient agar plates for 48 hours at 30°C. Cultures from those samples were grown on CMC agar and stained with 0.1% Congo red (pH 7.0) and counterstained with 1.0 M NaCl for 15-20 min. Cellulase activity was visible as a clear zone around the colonies. Colonies with the highest cellulase activities were selected and identified using API technique. Halos with diameter values in the range of 3.6 cm belong to the strain *Bacillus cereus* (Nema *et al*., 2014). Hence, in this work *Bacillus cereus* strain was used for submerged fermentation using corn husks as substrates.

Enzyme production

Bacterial cellulase was produced from *Bacillus cereus* strain by submerged fermentation using corn husks as substrate. The fermentation medium was prepared according to the method of Li and Gao (1997), with the following composition (w/v%): 0.02 $MgSO_4.7H_2O$; 0.075 KNO_3; 0.05 K_2HPO_4; 0.002 $FeSO_4.7H_2O$; 0.004 $CaCl_2.2H_2O$; 0.2 peptone dissolved in 1 L of distilled water, the fermentation medium was sterilized in an autoclave after addition of corn husks substrate with a concentration of 0.5% (w/v) and a pH of 9.5. Inoculation was performed using an inoculum at the age of 24 hours of 1% of *Bacillus cereus* strain (10^6 CFU/ml), and subsequently incubation was performed at 25ºC for 18 hours with shaking at 150 rpm. The cultured broth was centrifuged at 8000 × g for 20 min and passed through a 0.45 µm membrane to remove the cells. The enzyme solution was concentrated through freeze-drying to 50 ml at a pressure of p = 0.05 bar and temperature of –53ºC. The crude enzyme was stored in the freezer at –20 ºC.

Determination of enzyme activity

Enzyme activity was determined using a modified method of Robson and Chambliss (1984). The modification included use of 1 ml of 1.0% (w/v) carboxymethyl cellulose in 0.05 M sodium citrate buffer (pH 4.8), with addition of 1 ml of the crude enzyme extract. The final assay volume was 2 ml. The mixture was incubated at 50°C for 30 min and the produced reduced sugar was measured by the dinitrosalicylic acid method. Enzyme activity was determined by the measurement of absorption at λ =540 nm and compared with a blank sample containing all components except enzyme solution which was replaced with 1 ml distilled water. One unit of cellulase activity was defined as the amount of the enzyme which catalyses the release of 1 µM equivalent of glucose/ml at 1 min under the specified assay conditions (Miller *et al*., 1960).

Influence of temperature and pH on enzyme activity

Temperature optimum

In order to find the optimal temperature for the crude cellulase activity 100 µl of crude enzyme were added to 900 µl of 1% CMC in 20 mM phosphate buffer (pH 7.0), and incubated at various temperatures (20, 30, 40, 50, 60, 70, 80, 90°C) for 30 min (Yin *et al*., 2010). The activity was then measured according to Miller (Miller *et al*., 1960).

Temperature stability

For enzyme thermal stability enzyme incubation without substrate is usually performed for a certain period at various temperatures, than enzyme activity is measured for each temperature. The thermal stability of cellulase was investigated. For this purpose, 1 ml of crude enzyme in 20 mM phosphate buffer (pH 7.0) was incubated at various temperatures 30, 40, 50, 60, 70, 80, 90°C) for 30 min (Yin *et al*., 2010). The incubation time varied between 30 min and 72 hours for temperatures between 60 and 90°C that might be required for industrial purposes. The residual activity was measured according to Miller (Miller *et al*., 1960). This quantity is defined as the change in enzyme activity due to any treatment, such as thermal or mechanical treatment, in relation to the initial activity before that treatment.

pH optimum

The optimal pH for the crude enzyme was determined by adding 100 µl of crude cellulase to 900 µl of 1.0% CMC at various pH values, and the activity was measured according to Miller (Miller *et al*., 1960). Various pH values were obtained by changing the buffer solution as follows: pH 3.0-7.0: 50 mM citrate buffer;pH 6.0-9.0: 50 mM phosphate buffer and pH 8-11: 50 mM carbonate buffer (Yin *et al*., 2010).

pH stability

One ml of crude cellulase was incubated in

buffers with various pH values at 30°C for 30 min. The pH values varied within the range pH 3.0 to 11. Furthermore, the incubation time varied between 30 min and 72 hours for pH values of 4, 6, 8 and 9 (Yin *et al.*, 2010). The residual activity was measured according to Miller (Miller *et al*., 1960).

Effect of metal ions, SDS and EDTA on enzyme activity

One ml of crude cellulase in 20 mM Tris-HCl buffer (pH 7.0) with each of (Co^{2+}, Mn^{2+}, Fe^{3+}) metal ions or ethylenediamine-tetraacetic acid (EDTA) and sodium dodecyl sulfate, (SDS) with concentrations of 1, 5, 10 mM were incubated at 30°C for 30 min. All metal- SDS and EDTA solutions were not previously autoclaved. After incubation, the residual activity was measured according to Miller (Miller *et al*., 1960).

Effect of substrate concentration on enzyme activity

In order to study the effect of substrate concentration on the enzyme activity CMC was used as substrate with several concentrations (0.1%, 0.5%, 1% and 1.5%). For each concentration the activity was measured according to Miller (Miller *et al.*, 1960).

Statistical analysis

All data represent the mean of three different experiments. Statistical analysis of the results was performed with XLSTAT 2008. The significance of differences was tested at a significance level of P = 0.05.

Results and Discussion

Figure 1 clearly demonstrates that the highest enzyme activity is achieved at 30°C, where at 20°C the enzyme starts to hydrolyze CMC to glucose with an activity of 0.116 IU/ml. Then, the cellulase enzyme activity increases with increasing temperature until 30°C. It gets its maximum of 0.213 IU/ml, after which it decreases with increasing temperature and it gets nearly constant values at temperatures above 70°C. Results of statistical analysis revealed that the differences between activity values at 20°C, 50°C and 60°C were not significant, similarly the differences between activity values at 70°C, 80°C and 90°C were also not significant. While the difference between the activity value at T = 30°C and all other activity values was significant. The achieved optimal temperature in this research work agrees well with literature data, where *Bacillus subtilis* and *Bacillus circulans* recorded optimal cellulolytic activities at 35°C (Otajevwo *et al*., 2011) and a broad range of

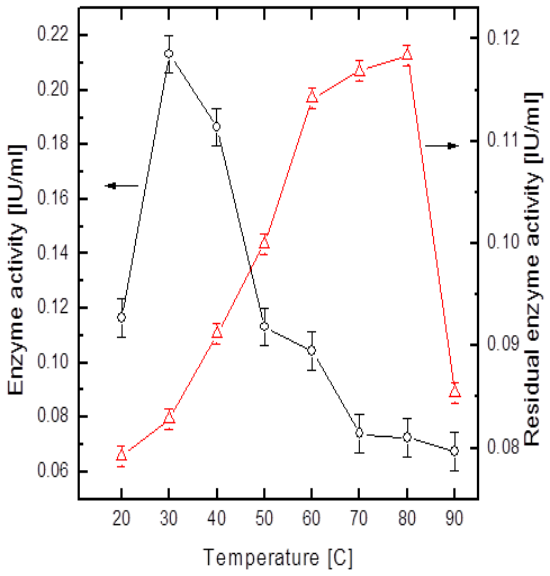

Figure 1. Determination of optimum temperature for maximum cellulase enzyme activity (left). Enzyme solution contains 1% CMC, in 20 mM phosphate buffer (pH 7.0), incubation time 30 min. Effect of incubation temperature on residual cellulase enzyme activity (right). One ml of crude enzyme was solved in 20 mM phosphate buffer (pH 7.0), the incubation time was 30 min.

optimum temperatures (20-40°C) was achieved for Bacillus licheniformis JK7 (Seo *et al.*, 2013). This temperature differs from that obtained for cellulase produced from the same strains by fermentation of palm-fruit industrial residue (Odeniyi *et al*., 2009) and fermentation of rice bran, where the optimal temperature was found to be T =50-60°C (Yin *et al.*, 2010).

Concerning the thermal stability of the enzyme, Figure 1 clearly demonstrates that the enzyme is stable at a temperature range of 60 to 80°C. In this range the value of residual enzyme activity was 0.114-0.118 IU/ml, representing approximately 60% of crude cellulase activity; Statistically the difference between the residual activity value at 70°C and that at 80°C was not significant. Nonetheless, it can be concluded that the residual enzyme activity is nearly constant in that range. Differences between other residual activity values are significant, i.e., there are real differences in the achieved values.

These results agree with data reported by several research groups (Bajaj *et al*., 2009; Nizamudeen and Bajaj 2009; Odeniyi *et al*., 2009), where the enzyme was stable for 15 min in the range between 70 and 90°C (Odeniyi *et al*., 2009), 30 min in the range between 60 and 90°C (Nizamudeen and Bajaj 2009), and 30 min to 1 hour in the range between 50 and 70°C (Bajaj *et al*., 2009).

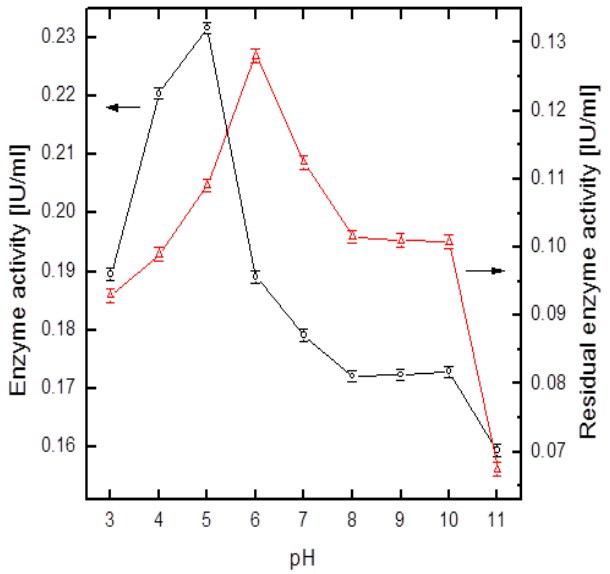

Figure 2. The effect of pH on cellulase enzyme activity (left). pH effect on of residual cellulase enzyme activity (right). Enzyme was incubated at 30°C for 30 min in different buffer solutions at a pH range from 3 to 11.

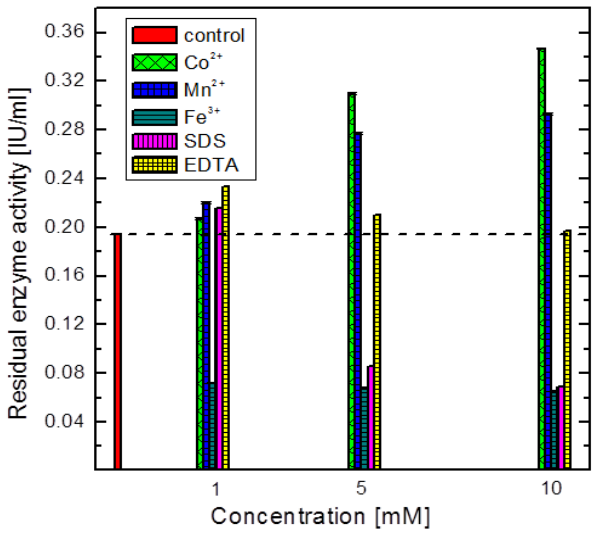

Figure 3. Comparison between cellulase residual activities using metal ions Co^{2+}, Mn^{2+}, Fe^{3+}, SDS and EDTA with final concentrations of 1, 5, 10 mM. "Control" represents the activity of crude untreated enzyme.

Determination of pH optimum and pH stability

Cellulase activity was affected by pH variation in buffer solution with 1% CMC at 30°C where Figure 2 clearly shows that enzyme activity increases with increasing pH till it reaches pH 4-5; then it decreases to get nearly constant values at pH 7-10, and it decreases again at pH 11. Results of statistical analysis revealed that the difference between the enzyme activity values at pH 3 and pH 6 were not significant, and similarly the difference between the enzyme activity values at pH 8 to pH 10 was also not significant. Whereas differences between both activity values at pH 4 and pH 5 and other activity values were significant. These results are comparable with those found by several research groups (Mawadza *et al*., 2000; Otajevwo *et al*., 2011), where an optimum pH of 5.5 was found for cellulase produced from *Bacillus circulans* strains (Otajevwo *et al.*, 2011), and optimum values were found to vary between pH 5-6.5 for cellulase produced from *Bacillus* sp. strains (Mawadza *et al*., 2000).

Concerning the pH stability Figure 2 clearly shows that the maximum residual cellulase enzyme activity was achieved at pH 6, and that the enzyme activity stayed stable at a pH range of pH 8 to 10. Results of statistical analysis indicated that the difference between the residual enzyme activity value at pH 4 and those at pH 8 to pH 10 is not significant, and similarly the difference between the residual enzyme activity value at pH 8 and those at pH 9 and pH 10 is not significant. Whereas differences between both residual activity values at pH 5 and pH 6 and other residual activity values were significant. Variation in pH ranges with regard to cellulase stability was mentioned by several research groups (Tae-Il *et al*., 2000; Nizamudeen and Bajaj 2009), where the pH stability range was found to be at pH 8-9.

Effect of metal ions, SDS and EDTA

The residual activity of cellulase was measured after incubation at T= 30°C for 30 min in 20 mM Tris-HCl buffer (pH 7.0) with metal ions Co^{2+}, $Mn^{2+}$and Fe^{3+}, EDTA and SDS. Figure 3 shows a comparison between the achieved residual activity using those materials with final concentrations of 1, 5, 10 mM. The column "control" represents the activity of crude untreated enzyme. It is clear from this figure that Co^{2+} and Mn^{2+} ions activate the enzyme, whereas Fe^{3+} ion inhibits enzyme activity. Compared to the crude enzyme activity, adding of Co^{2+} led to an increased enzyme activity by 7-79%, whereas adding of Mn^{2+} led to an increased activity by 13-51% with increasing Mn^{2+} concentrations. With adding Fe^{3+} the enzyme was inhibited by 63-66%. The higher the concentration of Co^{2+} and Mn^{2+} the higher the residual enzyme activity, whereas Fe^{3+} inhibits enzyme activity independent of its concentration. Results of statistical analysis revealed that all differences between the residual enzyme activity values are significant. Similar results have been reported for endoglucanase produced from cellulolytic strain *Cellulomonas* sp (Irfan *et al*., 2012), where the enzyme was activated by the ions Co^{2+} and Mn^{2+} by 36% and 20%, respectively, and strongly inhibited by and Fe^{2+} by 80%.

Concerning SDS and EDTA, results clearly

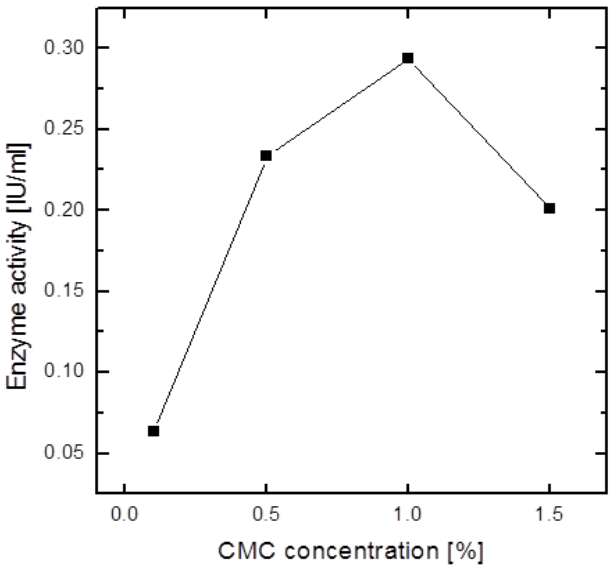

Figure 4. Influence of CMC concentration on cellulase activity

show the decrease in the residual enzyme activity with increasing concentration for both materials. Statistical analysis of these results revealed that all differences between the residual enzyme activity values were significant. In the case of adding EDTA the residual enzyme activity achieved its maximum of 0.234 IU/ml at 1 mM, and decreased very slightly with increasing concentration, still, it was higher than that activity of crude untreated enzyme at all concentrations. Similar results have been found by Pham *et al*. (2012), where EDTA increased cellulase activity for cellulase produced from *Aspergillus niger* VTCC-F021. In contradiction to EDTA the residual enzyme activity using SDS decreased considerably with increasing concentration. At a concentration of 1 mM it was higher than that activity of crude untreated enzyme. With SDS concentration of 10 mM the residual enzyme activity was decreased to 0.06 IU/ml. This indicates that higher concentrations of SDS inhibit enzyme activity. This result agrees well with Lin *et al.* (2012), who found that SDS inhibits enzyme activity.

Effect of substrate concentration

Figure 4 shows the dependence of enzyme activity on CMC concentration. This figure shows that highest activity is achieved at CMC concentration of 1%, which is in agreement with Odeniyi *et al*. (2009). Results of statistical analysis revealed that all differences between the enzyme activity values are significant.

Conclusion

The potential of corn husks for production of cellulase was demonstrated through submerged fermentation using *Bacillus* cereus strains isolated from local syrian soils. Maximum enzyme activity was obtained at 30°C. The enzyme was stable in the temperature range between 60 and 80°C and pH range between 8 and 10. The effect of SDS, EDTA and metal ions on the enzyme activity was studied. In the case of EDTA the residual enzyme activity decreased very slightly with increasing concentration, whereas it decreased considerably with increasing SDS concentration. The ions Co^{2+} and Mn^{2+} activate the enzyme, whereas the ion Fe^{3+} inhibits enzyme activity.

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