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# Avicelase Production by a Thermophilic *Geobacillus stearothermophilus* Isolated from Soil using Sugarcane Bagasse

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***Abstract*—**Studies were carried out on the comparative study of the production of Avicelase enzyme using sugarcane bagasse-SCB in two different statuses (i.e. treated and untreated SCB) by thermophilic *Geobacillus stearothermophilus* at 50ºC. Only four thermophilic bacterial isolates were isolated and assayed for Avicelase production using UntSCB and TSCB. Only one isolate selected as most potent and identified as *G. stearothermophilus* used in this study. A specific endo-β-1,4-D-glucanase (Avicelase EC 3.2.1.91) was partially purified from a thermophilic bacterial strain was isolated from different soil samples when grown on cellulose enrichment SCB substrate as the sole carbon source. Results shown that *G. stearothermophilus* was the better Avicelase producer strain. Avicelase had an optimum pH and temperature 7.0 and 50ºC for both UntSCB and TSCB and exhibited good pH stability between "5-8" and "4-9", however, good temperature stability between (30-80ºC) for UntSCB and TSCB, respectively. Other factors affecting the production of Avicelase were compared (i.e. SCB concentration, inoculum size and different incubation periods), all results observed and obtained were revealed that the TSCB was exhibited maximal enzyme activity in comparison with the results obtained from UntSCB, so, the TSCB was enhancing the Avicelase production.

***Keywords*—**Geobacillus stearothermophilus; Avicelase; Sugarcane bagasse.

## I. Introduction

MICROORGANISMS mediate decomposition by utilizing a wide variety of organic compounds under diverse environmental conditions, extracting energy from organic compounds by fermentation, anaerobic and aerobic respiration [10]. Microbial microorganisms release enzymes into the surrounding environment in order to degrade macromolecular and insoluble organic matter prior to cell uptake and metabolism [9]. The biological hydrolysis of cellulose depends upon endoglucanase, exoglucanase and β-glucosidase, acting synergistically on structural polysaccharides [4], generating low-molecular weight reducing sugars [25]. Interest in cellulase arises because it is an important enzyme related to the decomposition of lignocellulosic material. We hypothesized that abiotic (*i.e.* temperature) and biotic factors (type of C-source of detritus) also governed cellulase activity by microheterotrophs organisms once the decay rates of lignocellulosic material are mainly affected by these factors [10]. A cellulosic enzyme system consists of three major components: exo-1,4-β-D-glucan cellobiohydrolases, which cleave cellobiosyl units from the ends of cellulose chains; endo-1,4-β-D-glucases which cleave internal glucosidic bonds and 1,4-β-D-glucosidase, which cleaves glucose units from cellooligosaccharides [15, 18]. The enzymatic hydrolysis of cellulose is considered to require the action of both *endo*-β- and *exo*- β –glucanases [29]. Endoglucanases (1,4- β-D-glucan glucanohydrolase, EC 3.2.1.4) are characterized by their activity toward substituted cellulose derivatives such as carboxymethylcellulose (CMC). In contrast, exocellulases (1,4- β-D-glucanohydrolase, EC 3.2.1.91) have been operationally defined by their ability to degrade microcrystalline Avicel, but not CMC. The term "Avicelase" is therefore commonly regarded as synonymous with exoglucanase or cellobiohydrolase (CBH). However, some inconsistencies with cellobiohydrolase behavior have been reported [6, 16, 27, 7]. Cellulase protein has also been produced from sugarcane bagasse by *Aspergillus niger* and *T. reesei* [2, 17].

Biosynthesis of cellulase was also made on lignocellulosic materials such as sugar beet pulp and alkaline extracted sugar beet pulp cellulose [21]. Some microorganisms are reported to produce enzyme system containing multiactivity [3, 11, 28]. For example, *C. thermocellum* and *C. cellulolyticum,* gram-positive, thermophilic and anaerobic bacteria, produce a multienzyme complex (cellulosome) when grown on cellulose as the substrate.

Species of the genus *Bacillus* are industrially important as they have a high growth rate, are able to secrete proteins extracellularly and are considered relatively safe to use with regard to health and environmental aspects [26]. An interesting feature of the genus is its well characterized ability to degrade amorphous substrates, such as CMC, despite the inability to degrade Avicel [20, 22, 1].

## II. Materials and Methods

### A. Materials

Thermophilic bacteria *Geobacillus stearothermophilus* was identified by Central Laboratories, Faculty of Agriculture, Alexandria University, Egypt. All media components and chemicals used were of highest purity grade available

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commercially. Sugarcane Bagasse (SCB) was obtained locally. All the experiments were performed independently in triplicate and the results given here are the mean of three values.

*B. Pretreatment*

100 g of the washed and ground SCB was treated separately with 2000ml of 4% solution of NaOH, autoclaved at 121ºC for 30 min. The material recovered by filtration was washed with distilled water until pH 7.0 and dried at 65ºC to constant weight [18].

*C. Isolation and Preservation of Thermophilic Bacteria*

Only four thermophilic bacterial isolates were isolated from different soil samples of Aswan City (Egypt). All isolates assayed for Avicelase enzyme activity to select the most potent one (Avicelase higher producer) from others to achieve the comparative study of Avicelase production by the most potent using UntSCB and treated one. Isolation minimal medium (IMM) of the following compositions, $gL^{-1}$ (NaCl, 5.0; $KH_2PO_4$, 1.0; $MgSO_4.7H_2O$, 0.2; $K_2HPO_4$, 1.0; $(NH_4)_2SO_4$, 1.0; and $FeSO_4$, 0.05) [30] and 50 g/L powder of untreated SCB (UntSCB) was added as the sole carbon source (particle size < 5mm). The pH at 7.0 was controlled using 2N HCl and 2N NaOH. Medium was autoclaved for 20 min, and inoculated by soil suspension (1% v/v) and incubated at 50ºC ±2 for 72h and then the filtrate was assayed for cellulase production. The cultures were further purified by sub-culturing on the same medium.

To preserve *G. stearothermophilus*, and other new isolates refrigeration can be used for short-term storage of bacterial cultures, but for long period "the deep-freezing" process was used in which a pure bacterial culture placed in a suspending liquid and quick-frozen at temperature from -50ºC to -95ºC. The culture can usually be thawed and cultured even several years later.

*D. Cellulase Production*

As mentioned above, the IMM was prepared for cellulase production; 50 g/L of both treated SCB (TSCB) and UntSCB were added separately. The medium was autoclaved and incubated at 50ºC for 72h. The pH and inoculum size of the medium were adjusted to 7.0 and 1.0 ml bacterial suspension ($16.76 \times 10^3$ cells/ml), respectively. The protein content was determined as described by [5], using bovine serum albumin as standard.

*E. Enzyme Assay*

Liquid samples obtained (crude enzyme) from production culture filtrate. The filtrates were stored at 4ºC for enzyme assay. Avicelase (EC 3.2.1.91) activity was assayed by incubation for 24h at 80ºC in a shaking incubator. Reaction mixture (1ml) contained a 1% (w/v) suspension of microcrystalline cellulose (Avicel) in (0.1M) Na-succinate pH 6.0. After removal of solids by centrifugation, aliquots of the supernatants were assayed for the release of reducing sugars. One unit of enzyme corresponds to the release of 1µmol of glucose equivalent per minute [6].

The optimum pH was determined by assaying the enzyme activity over a pH range "3.0 – 9.0". The pH stability was determined by preincubating the enzyme in universal buffer (pH 3.0 – 9.0) for 4h in the absence of SCB followed by Avicelase assay at pH 6.0 (0.1M Na-succinate). The optimum temperature was determined by assays between 30 and 80ºC. Temperature stability was determined by preincubating the enzyme for 10 min at various temperatures in the absence of SCB followed by Avicelase assay at 50ºC.

The optimum (substrate concentration) SCB concentration was determined by assaying the enzyme activity over a SCB range (10 – 70 g/L) (w/v). The optimum inoculum size was determined by assaying the enzyme activity over a volume range (10 – 80 ml/L).

*F. Purification of Avicelase Enzyme*

*G. stearothermophilus* was allowed to grow under the optimal static conditions on both TSCB and UntSCB for Avicelase production. At the end of incubation period, the cell-free supernatant was obtained by centrifugation at 15,000 rpm for 10 min; the supernatant was filtered using 0.45µm GHP cellulose acetate filter (Hydrophilic). All the purification steps for TSCB and UntSCB were carried out at 10ºC unless otherwise specified. The cell-free supernatant was saturated with ammonium sulphate, then centrifugation at 15,000 rpm for 15 min, and the pellet was resuspended in enzyme buffer at pH 6.0 in order to determine both the enzyme activity and protein content according to the method of [5]. The calculation of the solid ammonium sulphate to be added at any concentration was obtained by the chart of [13] as mentioned by [12]. The obtained enzyme preparations for TSCB and UntSCB were dialyzed against sucrose 30 % (w/v). The dialyzed enzymes (1ml) was subsequently loaded on a gel-filtration using a 12.5×1.7 cm column, flow rate 1ml $min^{-1}$ of Sephadex G-200 (mesh, 200µ), which was previously equilibrated with the same buffer of enzyme activity, then eluted with the same buffer.

## III. Results and Discussion

One of the important criteria taken into account for the choice of thermophilic industrially important producer strain is its ability to secrete enzymes on cheap and local substrates. In this study, comparison of Avicelase enzyme production on TSCB and UntSCB at 50ºC ±2 by bacterial thermophilic *G. stearothermophilus* strain isolated from different soil samples.

Only four thermophilic bacterial isolates (i.e. ASC-1, ASC-2, ASC-3 and ASC-4) were found and assayed for Avicelase enzyme activity using UntSCB and TSCB under investigation (Table I). It was found that the maximum production of Avicelase (0.27 and 1.04 IU/ml) was obtained from ASC-3 isolate after 72h at 50ºC for both UntSCB and TSCB, respectively. Other isolates (ASC-1, ASC-2 and ASC-4) were not good Avicelase enzyme producers from both UntSCB and TSCB (Table I). Only ASC-3 isolate was selected as the most

potent producer and identified as *Geobacillus stearothermophilus* used in this study.

Screening of some physical and chemical properties which affecting on enzyme production were investigated for comparison study of Avicelase production from both UntSCB and TSCB by the higher Avicelase producer *G. stearothermophilus* strain under study. Results shown in (Fig. 1) indicated that pH 7.0 was more suitable for Avicelase (0.30 and 1.57 IU/ml) for both UntSCB and TSCB, respectively. Either increase or decrease in pH beyond the optimum value showed decline in enzyme activities. Avicelase enzyme showed good pH stability between "5-8" and "4-9" for UntSCB and TSCB, respectively.

The results in (Fig. 2) showed that the flasks containing minimal medium with the sole carbon source (UntSCB & TSCB) supported maximal Avicelase (0.36 and 1.63 IU/ml) activities at 50ºC in both UntSCB and TSCB, respectively. However, Avicelase showed good temperature stability between (30-80ºC) for both treated and untreated SCB.

Of the various substrate concentrations used in the medium (Fig. 3), the presence of 60 and 50 g/L substrate supported maximal Avicelase (0.81 and 1.94 IU/ml) activities for both UntSCB and TSCB, respectively.

Production of Avicelase was evaluated up to 80 ml/L inoculum size (Fig. 4). A gradual increase in the production of Avicelase over an inoculum size up to 70 ml/L (0.52 IU/ml) was observed in UntSCB and up to 80 ml/L (1.59 IU/ml) in TSCB.

The effect of different incubation periods on the production of Avicelase in both UntSCB and TSCB as shown in (Fig. 5). Both were evaluated up to 96h, and supported maximal Avicelase over a period up to 48h (1.06 IU/ml) was observed in UntSCB and up to 24h (1.99 IU/ml) was observed in treated substrate. The results of all parameters controlling enzyme production were summarized in (Table II).

Purification of Avicelase enzyme by loading on a gel-filtration column of Sephadex G-200 (mesh, 200µ) yielded an elution profile that revealed the presence of only one peak obtained from each substrate (UntSCB & TSCB) after collection of fractions up to 50 each containing 5ml eluted purified enzyme. The fractions that constituted peak from UntSCB (13-19) and (25-31) were obtained from TSCB (Fig. 6). These fractions were assayed for Avicelase activity correlated with the protein elution profile i.e. absorbance at 280nm. The purification procedure steps were summarized in (Table III).

According to [18], the comparative study was made on various carbon sources on the production of cellulase using strains of *T. reesei* QM 9414, 97, 177 and Tm3. Pretreatment of sugarcane bagasse and rice straw offers very digestible cellulose and potentially less inhibition. According to the results obtained by [8], indicate that *B. subtilis* AU-1 resembles other *Bacillus* species in that it lacks a complete cellulase system [31, 14, 24], producing only exo-CMCase. The observation that poor growth occurs in cultures containing either filter paper or Sigmacell (Avicel) as the major carbon source leads to the conclusion that *B. subtilis* AU-1 produces only very weak exo-1,4-β-glucanase activity, which is also supported by the fact that a very low level of this enzyme is detected in the culture when compared with other cellulolytic microorganisms [19, 23].

In conclusion, *G. stearothermophilus* strain produced Avicelase enzyme under thermophilic conditions from sugarcane bagasse. This strain shown as better Avicelase producer strain maybe reported in this study. The comparative study displayed that the treated SCB found to be the most effective inducer for Avicelase enzyme activities. However, UntSCB showed lower Avicelase inducing power when serve individually as inducer for Avicelase in the medium, the TSCB was able to induce Avicelase enzyme to maximal activities in all experiment results.

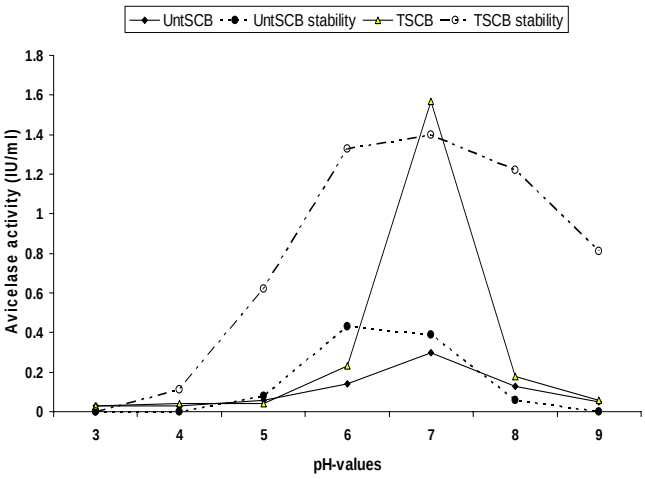


Fig. 1 Effect of different pH-values and stability on Avicelase production from UntSCB and TSCB

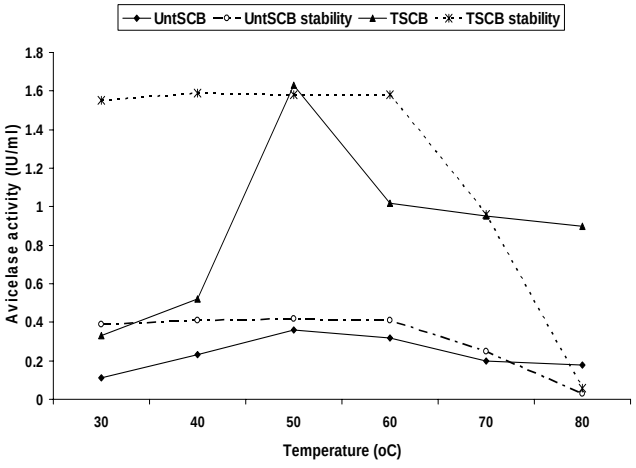


Fig. 2 Effect of different temperatures and stability on Avicelase production from UntSCB and TSCB

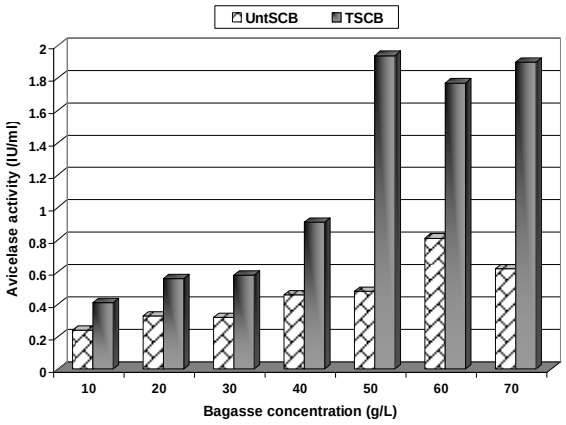


Fig. 3 Effect of different SCB concentrations (UntSCB and TSCB) on Avicelase production

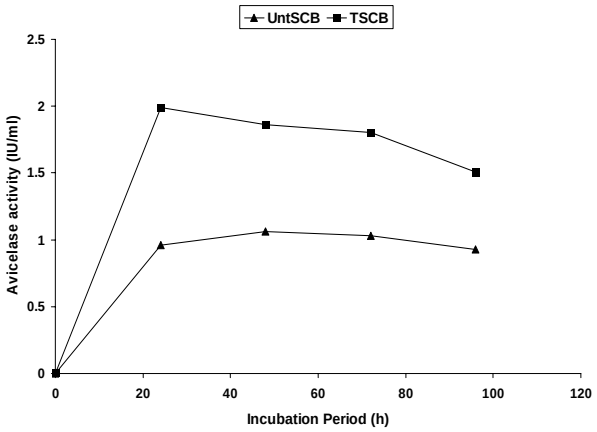


Fig. 5 Effect of different incubation periods on Avicelase production from UntSCB and TSCB

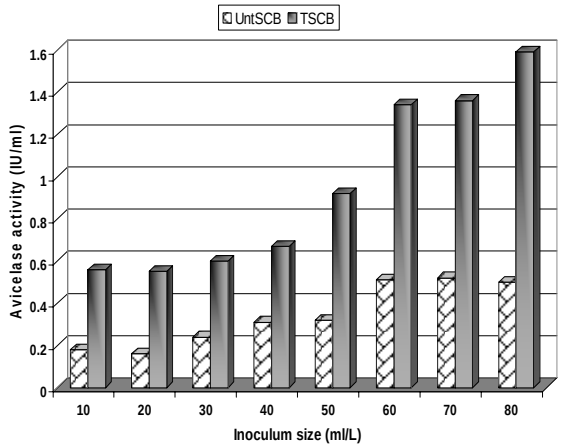


Fig. 4 Effect of different inoculum sizes on Avicelase production from UntSCB and TSCB

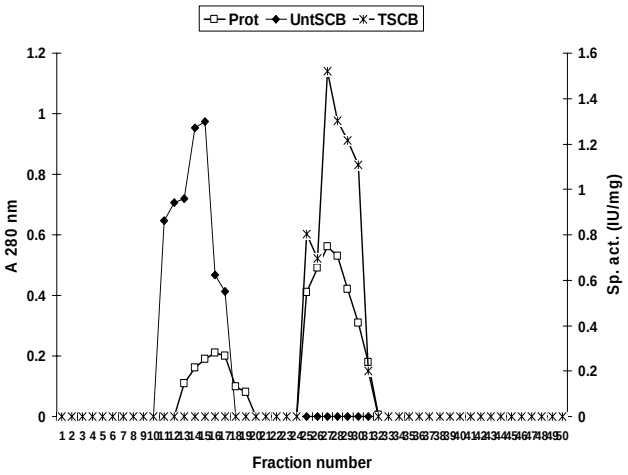


Fig. 6 Sephadex gel-column chromatography fractions of purified Avicelase specific activity

TABLE I
SCREENING OF AVICELASE ACTIVITY, EXTRACELLULAR PROTEIN AND SPECIFIC ACTIVITY PRODUCED BY FOUR THERMOPHILIC BACTERIAL ISOLATES USING UNTSCB AND TSCB

| Isolate Symbol | UntSCB | | | TSCB | | |
|---|---|---|---|---|---|---|
| | Avi. act. (IU/ml) | Extracellular Protein (mg/ml) | Sp. act. (IU $mg^{-1}$) | Avi. act. (IU/ml) | Extracellular Protein (mg/ml) | Sp. act. (IU $mg^{-1}$) |
| **ASC-1** | 0.09 | 0.81 | 0.111 | 0.29 | 1.70 | 0.170 |
| **ASC-2** | 0.06 | 0.93 | 0.064 | 0.23 | 1.66 | 0.138 |
| **ASC-3*** | 0.27 | 2.11 | 0.127 | 1.04 | 3.15 | 0.330 |
| **ASC-4** | 0.10 | 1.22 | 0.082 | 0.31 | 1.42 | 0.218 |

*Selected as most potent isolate

TABLE II
SUMMARY OF PARAMETERS CONTROLLING THE PRODUCTION OF AVICELASE ENZYME FROM UNTSCB AND TSCB

| Parameter | Result | UntSCB | | | Result | TSCB | | |
|---|---|---|---|---|---|---|---|---|
| | | Avi. act. (IU/ml) | Prot. cont. (mg/ml) | Sp. act. (IU $mg^{-1}$) | | Avi. act. (IU/ml) | Prot. cont. (mg/ml) | Sp. act. (IU $mg^{-1}$) |
| **pH** | 7 | 0.30 | 0.49 | 0.612 | 7 | 1.57 | 2.31 | 0.680 |
| **Temp (ºC)** | 50 | 0.36 | 1.95 | 0.185 | 50 | 1.63 | 1.44 | 1.312 |
| **SCB conc. (g/L)** | 60 | 0.81 | 0.92 | 0.880 | 50 | 1.94 | 0.88 | 2.204 |
| **Inocul. size (ml/L)** | 70 | 0.52 | 0.62 | 0.839 | 80 | 1.59 | 0.96 | 1.656 |
| **Incu. per. (h)** | 48 | 1.06 | 1.61 | 0.658 | 24 | 1.99 | 0.81 | 2.457 |

TABLE III
SUMMARY OF PURIFICATION PROCEDURES OF AVICELASE ENZYME

| Purification step | UntSCB | | | | | TSCB | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Avi. act. (IU/ml) | Prot. cont. (mg/ml) | Sp. act. (IU $mg^{-1}$) | Purif. fold | Yield (%) | Avi. act. (IU/ml) | Prot. cont. (mg/ml) | Sp. act. (IU $mg^{-1}$) | Purif. fold | Yield (%) |
| Culture broth | 0.33 | 2.44 | 0.135 | 1 | 100 | 1.17 | 3.06 | 0.382 | 1 | 100 |
| $(NH_4)_2SO_4$ (60%) | 0.29 | 1.03 | 0.281 | 2.08 | 87.9 | 0.96 | 2.30 | 0.417 | 1.09 | 82 |
| Dialysis | 0.16 | 0.54 | 0.296 | 2.19 | 48.5 | 0.79 | 0.91 | 0.868 | 2.27 | 67.5 |
| CGF* (G-200) | 0.11 | 0.35 | 0.314 | 2.32 | 33.3 | 0.56 | 0.60 | 0.933 | 2.44 | 47.9 |

*Column gel-filtration



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