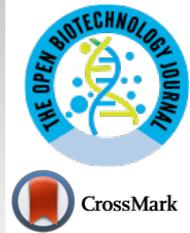




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## RESEARCH ARTICLE

### A Comparative Proteomic Study of *Thermobifida Cellulosilytica* TB100<sup>T</sup> Secretome Grown on Carboxymethylcellulose and Rice Straw

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#### Abstract:

#### Background:

Cellulose, the major component of the plant cell wall, is the most abundant and cheap polymer on earth. It can be used by varieties of cellulolytic enzymes. Cellulases can hydrolyze cellulose to its glucose monomers, which can be fermented to many biotechnological products, such as biochemicals, bioplastics, and biofuels. Actinomycetes are potential sources of cellulases.

#### Objective:

The current study sheds light on the cellulolytic activity of *Thermobifida cellulosilytica*, a previously isolated thermophilic actinomycete, and the analysis of the lignocellulases produced in the secretome as a result of induction by different carbon sources.

#### Methods:

The cellulolytic activity was qualitatively confirmed by Congo red method showing a large halo zone around the colonies. The activity was also assayed using the 3,5-dinitrosalicylic acid (DNS) method. The secretome analysis was conducted by liquid chromatography-tandem mass spectroscopy (LC-MS/MS) based proteomic approach.

#### Results:

The cellulolytic activity increased by two folds upon the growth of *T. cellulosilytica* on rice straw (RS) as a complex substrate comparatively to Carboxymethylcellulose (CMC) as a simple one. These results were highly assured by LC-MS/MS. Where more proteins (n=31) were produced in the RS secretome, CMC produced only six proteins, including only one cellulase. Different classes of proteins produced in the RS secretome were cellulases (26%), hemicellulases (16%), proteases (10%), and others (48%).

#### Conclusion:

Lignocellulases are inducible enzymes. RS as a complex substrate induced *T. cellulosilytica* for the expression of more lignocellulolytic enzymes than CMC.

**Keywords:** Carboxymethylcellulose , Carboxymethylcellulase , *Thermobifida cellulosilytica* , Rice straw , Secretome analysis , LC-MS/MS .

#### Article History

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## 1. INTRODUCTION

The lignocellulosic materials, left behind agricultural and forestry industries, are mainly composed of cellulose 40-50%,

hemicellulose 20-40%, and lignin 20-30% of plant dry weight [1]. Their production was estimated by  $1.5 \times 10^{12}$  tons per year [2]. Cellulose is a linear polymer ranging from 10,000-15,000 glucose units linked by  $\beta$ -1,4 glycosidic bonds [3] and may reach up to 25,000 glucose units [4]. Due to its highly ordered crystalline structure, water insolubility as well as the presence of a tough lignin layer around it, cellulose is slowly/hardly degraded and unusable, leading to its disposition and

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accumulation as waste in nature [5]. This may lead to environmental pollution [6]. So, intervention to accelerate cellulose degradation and conversion to its fermentable sugars that can be used for biorefinery and industrial purposes is a must.

The efficient hydrolysis of cellulosic waste materials, has been employed either chemically or enzymatically [4, 7]. Chemical hydrolysis methods of cellulose degradation include incineration (the routine method), pyrolysis, steam explosion, and acidic or alkaline treatment [8]. Nowadays, application of these chemical methods has been dramatically limited and restricted only to be a preliminary/pre-treatment step before enzymatic hydrolysis [9] due to their many disadvantages including environmental pollution, health problems, requirement of large equipment, and production of toxic chemicals or inhibitors as furfural or even undesirable non-sugar by-product [4, 5, 7].

On the other hand, the enzymatic degradation of cellulose can overcome all these drawbacks being clean, highly specific, and eco-friendly [7, 10]. The bioconversion of cellulosic wastes into its simple sugars has been employed by different hydrolytic and oxidative enzymes such as cellulases, hemicellulases (xylanases), lytic polysaccharide monoxygenases (LPMO) and peroxidases [1, 11]. Cellulases are a group of three enzymes; endoglucanases or CMCase (carboxymethyl-cellulases) (E.C. 3.2.1.4), exoglucanases or CBHs (cellobiohydrolases) (E.C. 3.2.1.91) and  $\beta$ -glucosidases (BGLs) or cellobiases (E.C. 3.2.1.21), acting synergistically for complete hydrolysis of cellulose [4]. Microbial lignocellulolytic enzymes are mainly produced from bacteria, fungi, and actinomycetes, the most effective secretors of lignocelluloses [12].

*Thermobifida cellulositytica* TB100<sup>T</sup> strain is an actinomycete that has been isolated from manure compost region [13]. This strain showed 97.4% similarity to *Thermobifida fusca*, one of the well-characterized cellulolytic actinomycetes [13]. *T. cellulositytica* becomes the subject of many recent researches due to its production of many important industrial enzymes such as cutinases and lignocellulases. Cutinases are efficiently used in plastic biodegradation, especially hydrolysis of PET (polyethylene terephthalate) [14]. Lignocellulases composed of cellulases, hemicellulases, and ligases are responsible for effective hydrolysis of lignocellulosic substrates [15].

Previous studies are focused on the identification of culture conditions, phenotypic, genotypic and taxonomic data of *T. cellulositytica* TB100<sup>T</sup> [13]. Little studies assessed the cellulolytic activity of this thermophilic strain [16]. Our research aimed to evaluate the cellulolytic potential of *T. cellulositytica* grown on different carbon sources. This is the first work concerned with studying the effect of substrate type on the expression of different cellulases in the secretome collected from *T. cellulositytica* using LC-MS/MS based proteomic analysis. This study can help the researchers interested in the production of thermophilic cellulases for industrial use.

## 2. MATERIALS AND METHODS

### 2.1. Bacteria and Culture Conditions

*Thermobifida cellulositytica* TB100<sup>T</sup> (DSM 44535) used in

the present study was ordered from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen), Germany. The lyophilized strain was aseptically resuspended in 1 ml of Czapek peptone broth containing: sucrose 30 g/L, NaNO<sub>3</sub> 3 g/L, K<sub>2</sub>HPO<sub>4</sub> 1 g/L, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.5 g/L, KCl 0.5 g/L, FeSO<sub>4</sub>·7H<sub>2</sub>O 0.01 g/L, yeast extract 2 g/L, peptone 5 g/L and water to 1L. PH was adjusted to 7.3. A volume of 100  $\mu$ L was added to 5 mL Czapek peptone broth for refreshment and was incubated at 45°C for 3-5 days. A glycerol stock was prepared and preserved at -80°C.

### 2.2. Qualitative Detection of The Cellulolytic Activity of *T. cellulositytica*

The selected strain was cultivated on CMC agar media containing: CMC 0.5 g/L, NaNO<sub>3</sub> 0.1 g/L, K<sub>2</sub>HPO<sub>4</sub> 0.1 g/L, MgSO<sub>4</sub> 0.05 g/L, yeast extract 0.05 g/L, agar 20 g/L and water to 1L by spot inoculation method. The plates were incubated at 50°C for 72 h then flooded with 0.1% Congo red dye, gently shaken for 20 min, and excess dye was poured off. Finally, the plates were washed with 1M NaCl, gently shaken for 15 min and excess NaCl was poured off. The formation of a clear halo zone around the colony, that can be visualized as a purple color when flooded with 1 M HCl is considered as a positive result [17].

### 2.3. Effect of Different Substrates on Cellulase Production

Cellulase production was determined using CMC and RS, individually, as simple and complex substrate in the production medium containing the following constituents: CMC 5 g/L or pre-treated RS 1% (w/v), MgSO<sub>4</sub>·7H<sub>2</sub>O 0.2 g/L, KNO<sub>3</sub> 0.75 g/L, K<sub>2</sub>HPO<sub>4</sub> 0.5 g/L, FeSO<sub>4</sub>·7H<sub>2</sub>O 0.02 g/L, CaCl<sub>2</sub> 0.04 g/L, yeast extract 2 g/L and water to 1 L, pH was adjusted to 7.5. The RS was thermally and chemically pre-treated by NaOH. RS treatment was done as described by Taniguchi [18] as following: RS was cut into small pieces, washed with tap water for removal of surface dust, and soaked in 2.5% NaOH for 1h with a proportion of 1:10 (w/v). The mixture was autoclaved at 121°C for 30 min and then filtered. The residue was washed with distilled water several times till the supernatant pH became neutral. Finally, the residue was filtered again and dried in the oven at 80°C for 10 min. The microorganism was added to the flasks containing the previous production media with the corresponding substrates. The flasks were incubated at 50°C, shaken at 150 rpm for 11 days. A sample of one mL was periodically withdrawn every 24 h interval for assay of cellulase activity of *T. cellulositytica*.

### 2.4. Cellulase Assay

After the specified incubation time, one milliliter of the production medium was withdrawn and centrifuged at 7000 xg for 10 min. Two hundred and fifty microliters of CFE (Cell Free Extract) was added to a reaction mixture composed of: 500  $\mu$ L of CMC and 250  $\mu$ L of 50 mM phosphate buffer, pH was adjusted to 7. The reaction mixture was incubated at 50°C for 2 h, and the resulted reducing sugars were quantified by the DNS method [19] using a microtiter plate reader (Tecan Sunrise, Switzerland). A standard curve was constructed based on at least six different glucose concentrations to estimate the

reducing sugars released. One international unit of enzymatic activity was defined as the amount of enzyme that released 1  $\mu\text{mol}$  of reducing sugar per min.

## 2.5. Partial Characterization of Crude Extract Produced from *T. cellulositytica*

The CFE resulted from *T. cellulositytica* growing on CMC as sole carbon source was used for characterization as following:

### 2.5.1. Optimum PH

Different buffer systems with different pH were prepared. One hundred mM Citrate buffer with pH 4.5, 5 and 5.5 and 100 mM phosphate buffer with pH 6, 6.5, 7, 7.5, 8 and 8.5 were used. The reaction mix was made with each prepared buffer and incubated at 50°C for 2 h. The enzyme activity was assayed by the DNS method.

### 2.5.2. Optimum Temperature

Five hundred microliters of CMC 0.5%, 250  $\mu\text{L}$  buffer pH 8 and 250  $\mu\text{L}$  supernatant were mixed in Eppendorf tubes. The tubes were incubated at different temperatures (4, 25, 30, 37, 50, and 70°C) for 2 h. The enzyme activity was assayed by the DNS method.

### 2.5.3. Optimum Substrate Concentration

A typical reaction was prepared as following: in Eppendorf tubes, 500  $\mu\text{L}$  of different concentrations of CMC (0.1%, 0.25%, 0.5%, 0.75%, and 1%), 250  $\mu\text{L}$  buffer pH 8 and 250  $\mu\text{L}$  supernatant were mixed. The tubes were then incubated at 50°C for 2 h. A negative control tube containing distilled water instead of supernatant was incubated under the same conditions. The reducing sugar content was measured using the DNS method.

## 2.6. Secretome Analysis of *T. cellulositytica* Grown on Different Cellulose Substrates

### 2.6.1. Strain Cultivation and Secretomic Protein Extraction

*T. cellulositytica* TB100<sup>T</sup> strain was cultivated on the production medium containing the corresponding substrates (0.5% CMC and 1% RS, separately). The flasks were incubated at 50°C with shaking at 150 rpm for 7 days. The secretome from each substrate was harvested by centrifugation at 7000  $\times g$  at 4°C for 15 min twice to remove the bacterial pellets completely. The supernatant was concentrated and diafiltered through Vivaspin dialysis membrane with 5 kDa MWCO (GE Healthcare Life Sciences, Uppsala, Sweden) with 10 mM  $\text{PO}_4^{3-}$  buffer pH 7, to one-tenth its starting volume. The protein concentration of each sample was determined using the bicinchoninic acid assay method (BCA). The resultant volume aliquots were preserved at -80°C.

### 2.6.2. Protein Digestion and LC-MS/MS Analysis

The secretory proteins from each sample were chemically denatured by 8 M urea, reduced with 200 mM DTT (dithiothreitol) for 30 min at room temperature, alkylated with

1 M IAA (iodoacetamide) for 45 min to 1 h at room temperature in the dark and then enzymatically digested by trypsin containing procaine. The tryptic digestion was performed at 37 °C for 18 h (overnight) and then terminated by acidification with 100% FA (formic acid) to pH range 2-3 [20]. The resulting peptides were analyzed by Eksper NanoLC425 HPLC system (Eksigent, USA) for chromatographic separation. Peptides (with 2  $\mu\text{L}$  injection volume) from each sample were loaded onto trapping cartridge (10 x 0.5 mm) packed with CHROMXP C18CL 5 $\mu\text{m}$  particles, washed using mobile phase A (0.1% FA in DI-Water) at a flow rate of 10  $\mu\text{L}/\text{min}$  for 3 min. Subsequently, peptides were fractionated on the analytical column (150 x 0.3 mm) packed with CHROMXP C18CL, 120 Å, 3  $\mu\text{m}$  particles. The mobile phase A and B (0.1% FA in acetonitrile) were run at a flow rate of 2  $\mu\text{L}/\text{min}$  over 55 min to establish gradient elution that was 38 min 3-30% B; followed by 5 min 30-40% B and 2 min 40-80% B; maintained at 80% B for 3 min and finally equilibrated at 3% B.

Peptide fractions eluted from the HPLC system were directly injected into the mass spectrometer with nanospray<sup>®</sup> Ion Source. Data acquisition was set with a Triple TOF<sup>™</sup> 5600<sup>+</sup> system (AB SCIEX software), and the MS data were acquired in the positive ion mode, with a selected mass range of 400-1250  $m/z$ . Smart IDA (Information Dependent Acquisition) was obtained through high-resolution TOF/MS survey scan followed by a product ion scan resulted from MS/MS analysis for the most abundant 40 ions whose threshold was over 150 counts, and charge ranged from +2 to +5 with cycle speed of 1.5 s.

### 2.6.3. Mass Spectrometric Data Analysis

Data acquisition was performed with Analyst<sup>®</sup> TF 1.7.1 (AB SCIEX software). MS raw data files from the Triple TOF<sup>™</sup> 5600<sup>+</sup> system were converted into MGF (Mascot Generic Format) files, which were then analyzed by Peptide Shaker (v1.16.27) for protein identification. The databases used for searching *T. cellulositytica* TB100 were Swiss-prot and TrEMBL database containing 3,587 proteins [21]. The X!Tandem Algorithm was used for peptide identification. The data search parameters were set up as follows: Trypsin digestion with a maximum of two missed cleavage sites was considered along with fixed modification of cysteines by carbamidomethylation. Acetylation of lysine residues, peptide N termini, deamidation of asparagine and glutamine residues; and methionine oxidation were set as variable modifications. The presence of signal peptide sequences was determined using the ProtParam database [22].

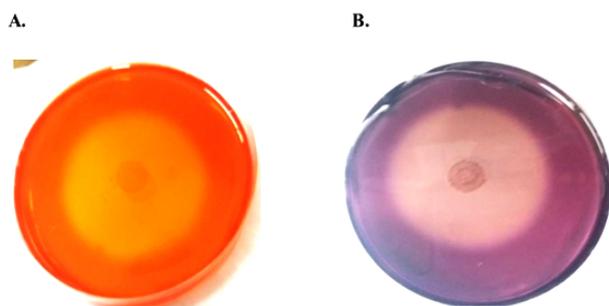
### 2.6.4. Statistical Analysis

All experiments were done in at least two independent replicates, and the presented data are the mean values of replicates  $\pm$  standard deviation using Microsoft Excel 2010 (Microsoft, USA). Differences between means were examined by one-way ANOVA (analysis of variance) followed by the Student's *t* test using SPSS 22.0 software (SPSS Inc., Chicago, USA). The significance of differences was considered at a probability value less than 0.05.

### 3. RESULTS

#### 3.1. Microorganism and its Cellulolytic Activity

The cellulase activity of *T. cellulosilytica* was confirmed by Congo red method. It produced a wide clear halo zone around the colonies due to the degradation of CMC (Fig. 1).



**Fig. (1).** Screening the cellulolytic activity of *T. cellulosilytica* using Congo red method. The microorganism was spotted in the center of the plate containing CMC agar media. A wide clearance zone formed around the colonies in the left picture after addition of Congo red. For good contrast, 1M HCl was added after destaining step leading to the darkness of the color of Congo red in the right picture.

#### 3.2. Cellulolytic Activity Assay Using Different Carbon Sources

The cellulolytic activity of *T. cellulosilytica* TB100<sup>T</sup> was estimated upon its growth on either CMC or RS. The cellulase production was gradually increased till reaching its maximum activity that was estimated at  $0.132 \pm 0.080$  IU/mL and  $0.298 \pm 0.161$  IU/mL on the 8<sup>th</sup> day and 7<sup>th</sup> day of incubation for both CMC and RS substrates, respectively. Subsequently, the cellulolytic activity decreased with longer incubation (Fig. 2).

#### 3.3. Partial Characterization of Crude Extract

The cellulolytic activity of the CFE was optimal at pH 4.5 and 7.5 evaluated by  $0.137$  and  $0.132$  IU/mL, respectively, (Fig. 3A). The optimum temperature of cellulase was 50°C with a maximum activity of  $0.136$  IU/mL. Cellulase enzyme was able to tolerate temperature up to 70°C (Fig. 3B). The concentration of 0.75% of CMC was found to induce the highest amount of cellulase estimated by  $0.132$  IU/mL (Fig. 3C).

#### 3.4. Secretome Analysis

The extracellular proteins of *T. cellulosilytica* TB100<sup>T</sup>, secreted in the presence of different substrates, were confidently identified by LC-MS/MS based proteomic analysis. After the enzymatic digestion of the secretome proteins by trypsin, a total of 9 peptides and 127 peptides were detected in the MS/MS spectra of CMC and RS, (Supplementary files 2 and 3). The resulted peptides were then searched against different databases giving 6 proteins and 31 proteins were produced in the CMC and RS, respectively, (Supplementary files 4 and 5).

The molecular weight and isoelectric point (PI) of all identified proteins were ranged from 15-106.3 kDa and 3.52-6.43 as shown in Tables 1 and 2. Most produced proteins had an acidic pH and extracellularly secreted by the action of signal peptides.

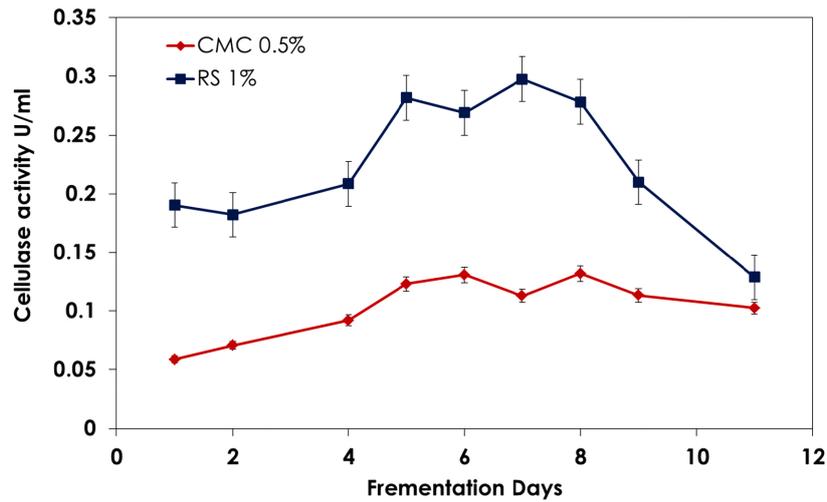
The secretomic produced proteins were functionally categorized and pie charted according to their roles in the deconstruction of lignocellulosic biomass (Fig. 4). Regarding CMC, the only secreted cellulolytic enzyme was glucanase (7%). On the other hand, the proteins detected in the RS secretome were grouped as cellulases (26%), hemicellulases (16%), proteases (10%), and other proteins (48%) as shown in (Fig. 4). Out of 31 proteins, 9 proteins (8.43) belonged to glycoside hydrolases (GHs) which were classified into different families according to (Carbohydrate Active enZymes) CAZY database (<http://www.cazy.org>) [23]. The GH families included were GH6 (2), GH9 (2), GH10 (2), GH43 (1), GH48 (1) and GH81 (1). In addition, ten enzymes were annotated as containing carbohydrate binding modules (CBMs).

### 4. DISCUSSION

Actinomycetes are considered as potential sources of bioactive secondary metabolites like antibiotics, enzymes and growth factors [12]. Cellulose active enzymes, “cellulases” are considered as important enzymes certainly produced from actinobacteria, including *T. cellulosilytica* TB100<sup>T</sup>. An additional thermophilic property of *T. cellulosilytica* TB100<sup>T</sup> strain makes it a potential source for the production of thermostable cellulases, the cause of choice of the provided actinomycete strain in this study.

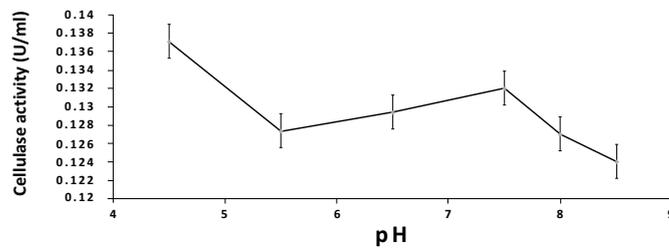
We confirmed the cellulolytic activity of *T. cellulosilytica* TB100<sup>T</sup> by Congo red which is the most widely used method for preliminary screening of cellulolytic actinomycetes, bacteria and fungi [5]. Congo red binds strongly with  $\beta$ -1,4-D glycoside linkage in polysaccharides, forming red complex [24]. This strong complex does not form except with polymers [25]. So, a large clear halo zone formed around colonies due to degradation of CMC polymer as a role of cellulase produced from *T. cellulosilytica*. Although the formation of a large clear zone on CMC agar plates, it doesn't imply good productivity in liquid media [17].

It is also worth noting that the maximum CMCase activity of a thermophilic actinomycete, *Thermomonospora fusca* BD25 which is the closest one to the strain of our study, was also weak and estimated at  $0.05$  IU/mL, nearly half the CMCase activity from our strain of interest [26]. Similarly, lower weak cellulolytic activity was produced maximally from *Geobacillus* sp. DUSELR7 on day 7 and from *Brevibacillus* sp. DUSELG12 on day 10 as  $0.058$  IU/mL and  $0.02$  IU/mL respectively [27]. In another study, the cellulase activity of *Bacillus subtilis* AS3 was  $0.07$  IU/mL in basal control medium and increased 6 folds to be  $0.43$  IU/mL after cultivation in the optimized medium using Plackett-Burman design [28].

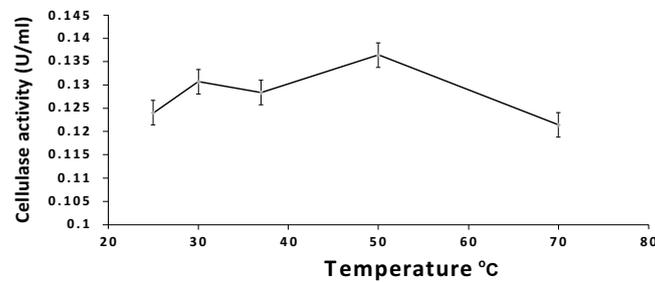


**Fig. (2).** Effect of incubation period/fermentation time on the cellulolytic activity of *T. cellulossilytica* using different carbon sources, CMC and RS. The symbols (♦) with a red line representing 0.5% CMC and (■) with blue line representing 1% RS.

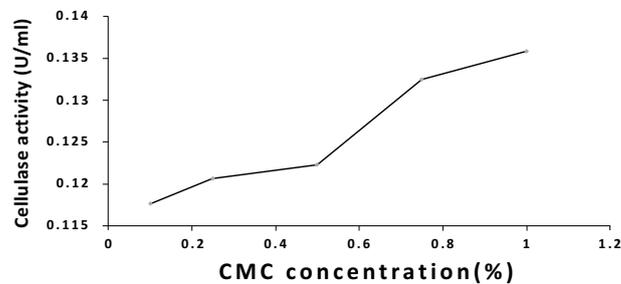
A.



B.



C.



**Fig. (3).** Partial characterization of cellulase including:  
 A. Effect of different pH (▲) on cellulase production.  
 B. Effect of different incubation temperatures (♦) on cellulase production.  
 C. Effect of different CMC concentration (■) on cellulase production.

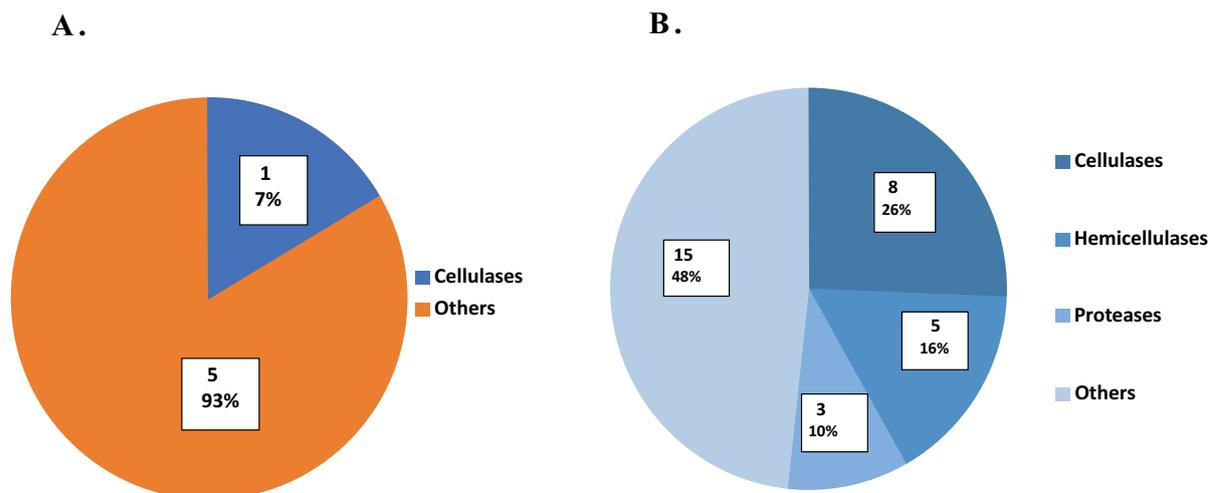


Fig. (4). Different classes of proteins identified in the *T. cellulositytica* TB100 secretome using (A). CMC as a simple substrate and (B). rice straw as a complex substrate after analysis by LC-MS/MS data.

Table 1. Functional classification of proteins detected in the secretome of *T. cellulositytica* TB100 using CMC as simple substrate.

| Description/ Protein Name                    | Uniprot Accession NO. | MW (kDa) | Coverage% | Peptides Matched | Amino Acid No. | Protein Family                | PI   | CBM  | Signal Peptide |
|--|-----------------------|----------|-----------|------------------|----------------|-------------------------------|------|------|----------------|
| <b>Cellulases</b><br>Glucanase               | A0A147KIV9            | 61.9     | 2.38      | 2                | 588            | GH6                           | 4.30 | CBM2 | N              |
| <b>Others</b><br>Dihydrolipoyl dehydrogenase | A0A147KD21            | 48.3     | 14.16     | 7                | 459            | Pyr_redox_2                   | 5.44 | -    | N              |
| NADH dehydrogenase                           | A0A147KF40            | 50.0     | 0         | 1                | 459            | Pyr_redox_2                   | 9    | -    | N              |
| Glutamine synthetase                         | A0A147KFJ5            | 53.2     | 0         | 1                | 474            | Gln-synt_C                    | 5.01 | -    | N              |
| Phospho-2-dehydro-3 deoxyheptonate aldolase  | A0A147KJ47            | 50.8     | 0         | 1                | 468            | DHAP_synth_2                  | 6.11 | -    | N              |
| Restriction endonuclease subunit R           | A0A147KL42            | 117.4    | 0         | 1                | 1054           | Helicase ATP-binding [HSDR_N] | 5.60 | -    | N              |

Table 2. Functional classification of proteins detected in the secretome of *T. cellulositytica* TB100 using RS as complex substrate.

| Description/ Protein Name              | Uniprot Accession NO. | MW (kDa) | Coverage% | Peptides Matched | Amino Acid No. | Protein Family | PI   | CBM    | Signal Peptide |
|--|-----------------------|----------|-----------|------------------|----------------|----------------|------|--------|----------------|
| <b>Cellulases</b><br>Endoglucanase     | A0A147KLC2            | 68.3     | 5.02      | 2                | 618            | Cellulase      | 4.51 | CBM3   | Y              |
| Cellulose 1,4-beta-cellobiosidase      | A0A147KIK5            | 106.3    | 15.9      | 16               | 981            | GH48           | 4.35 | CBM2   | Y              |
| Glucanase                              | A0A147KI07            | 46.6     | 17.4      | 11               | 454            | GH6            | 5.77 | CBM2   | N              |
| Endoglucanase                          | A0A0A7A855            | 46.2     | 4.78      | 3                | 439            | Cellulase      | 4.71 | CBM2   | Y              |
| Glycoside Glycoside hydrolase          | A0A147KHU4            | 100.8    | 1.59      | 1                | 941            | GH81           | 5.03 | -      | Y              |
| Endoglucanase                          | A0A147KHF1            | 103.8    | 16.17     | 14               | 965            | GH9            | 4.67 | CBM2,3 | N              |
| Endoglucanase                          | A0A147KMV8            | 102.4    | 17.45     | 15               | 957            | GH9            | 4.14 | CBM2   | N              |
| Glucanase                              | A0A147KIV9            | 61.9     | 34.69     | 17               | 588            | GH6            | 4.30 | CBM2   | N              |
| <b>Hemicellulases</b><br>Beta-xylanase | A0A147KDP3            | 53.4     | 11.02     | 8                | 490            | GH10           | 5.41 | CBM2   | Y              |
| Beta-xylanase                          | A0A147KJ27            | 50.5     | 21.1      | 8                | 474            | GH10           | 4.49 | CBM2   | Y              |
| Xyloglucanase                          | A0A147KMS2            | 97.8     | 2.29      | 5                | 919            | -              | 4.29 | CBM2   | Y              |
| Xylose isomerase                       | A0A147KE49            | 43.1     | 3.12      | 1                | 385            | AP_endonuc_2   | 5.09 | -      | N              |
| Beta-xylosidase                        | A0A147KMT4            | 61.8     | 2.18      | 3                | 550            | GH43           | 5.88 | -      | N              |

(Table 2) cont.....

| Description/ Protein Name   | Uniprot Accession NO. | MW (kDa) | Coverage% | Peptides Matched | Amino Acid No. | Protein Family | PI    | CBM | Signal Peptide |
|---|-----------------------|----------|-----------|------------------|----------------|----------------|-------|-----|----------------|
| <b>Proteases</b>  |                       |          |           |                  |                |                |       |     |                |
| Aminopeptidase Y  | A0A147KFZ9            | 53.8     | 8.74      | 6                | 515            | Peptidase_M28  | 5     | -   | Y              |
| Peptidase S1  | A0A147KH40            | 38.2     | 2.95      | 1                | 373            | Peptidase S1   | 4.48  | -   | Y              |
| ATP-dependent Clp protease ATP-binding subunit ClpX                 | A0A147KHB4            | 46.9     | 3.3       | 1                | 424            | AAA            | 5.03  | -   | N              |
| <b>Others</b>   |                       |          |           |                  |                |                |       |     |                |
| Glutamyl-tRNA amidotransferase                                      | A0A147KH75            | 16.3     | 6.67      | 1                | 150            | -              | 6.43  | -   | N              |
| Uncharacterized protein   | A0A147KDP0            | 22.5     | 9.05      | 1                | 210            | -              | 11.15 | -   | N              |
| Uncharacterized protein   | A0A147KM68            | 27.2     | 4.38      | 1                | 251            | HTH GntR       | 6.02  | -   | N              |
| Peptide ABC transporter substrate-binding protein                   | A0A147KIK0            | 63.5     | 8.86      | 6                | 587            | SBP_bac_5      | 4.08  | -   | Y              |
| Growth inhibitor PemK   | A0A147KJW7            | 17.3     | 5.33      | 1                | 150            | PemK_toxin     | 5.37  | -   | N              |
| Glycine/betaine ABC transporter substrate-binding protein           | A0A147KDQ0            | 33.9     | 10.19     | 2                | 314            | OpuAC          | 4.02  | -   | Y              |
| Dihydrolipoyl dehydrogenase   | A0A147KD21            | 48.3     | 10.24     | 5                | 459            | Pyr_redox_2    | 5.44  | -   | N              |
| Surface protein   | A0A147KIW2            | 39.2     | 2.34      | 1                | 384            | TED            | 4.34  | -   | Y              |
| Glutamate-binding protein   | A0A147KMF2            | 29.7     | 11.58     | 3                | 285            | SBP_bac_3      | 4.06  | -   | Y              |
| Branched-chain amino acid ABC transporter substrate-binding protein | A0A147KMP9            | 42.2     | 1.93      | 1                | 414            | PBP6           | 3.52  | -   | N              |
| Cell division protein DivIVA  | A0A147KL19            | 31.6     | 6.71      | 2                | 283            | DivIVA         | 4.96  | -   | N              |
| Cellulose-binding protein   | A0A147KKN7            | 25.0     | 4.05      | 1                | 222            | LPMO10         | 8.40  | -   | Y              |
| Protein GrpE  | A0A147KM11            | 27.7     | 3.49      | 1                | 258            | GrpE           | 4.32  | -   | N              |
| 50S ribosomal protein L11   | A0A147KL98            | 15.0     | 9.86      | 1                | 142            | Ribosomal_L11  | 9.10  | -   | N              |
| RNA helicase [Fragment]   | A0A147KIV8            | 89.5     | 1.24      | 1                | 804            | rRNA_proc_arch | 9.02  | -   | N              |

On the other hand, there are some actinobacterial strains in other studies that produced CM Case with moderate and high maximum activity including actinomycete BAY 21 with  $1.381 \pm 0.024$  IU/mL [7] and *Streptomyces* DSK59 with 20 IU/mL [12]. Another alkalothermophilic actinomycete produced CM Case with the activity of 23 IU/mL, 8.5 IU/mL and 12.5 IU/mL when cellulose paper powder, wheat bran and corncob were used as the substrate, respectively [29].

Regarding the cellulase, it is produced gradually till reaching maximum activity followed by a decline with longer incubation. Longer incubation period enhances the production of proteases. Proteases degrade subsequent enzymes resulting in diminished cellulolytic activity [30]. Prasad reported (2013) other reasons related to feedback inhibition occurred by the accumulation of end products [31].

Unlike most researches interested in the isolation of cellulolytic strains, the first study directed toward secretomic analysis of *T. cellulosilytica* TB100<sup>T</sup> was conducted [32]. The secretory protein profiles of *T. cellulosilytica* grown on different carbon sources, including CMC and RS, were compared. The proteomic study revealed a significant up-regulation in the lignocellulolytic enzymes expression of in the case of RS than CMC.

Lignocellulases are inducible enzymes [20]. Their production is dependent on the type or nature of the substrate used the culture media, which is known as substrate-induced phenomena [33, 34]. Do Vale and his colleagues (2012) showed that the complexity of the substrate affected the variety of the produced enzymes [35]. The CMC secretome contained six secretory proteins involving only one cellulase (glucanase). This low expression level of cellulases produced in the CMC secretome has an advantage of better isolation, purification, and studying the kinetic parameters of this cellulase, leading to

decrease production cost on the large industrial scale.

On the contrary, more lignocelluloses (31 proteins) were identified in the RS secretome. The different classes of proteins produced were cellulases, hemicellulases, proteases, and other proteins. These varieties of enzymes were produced in the RS secretome due to its complex composition of cellulose (41%), hemicellulose (20%) tightly packed with lignin (12%) [36].

The lignin content of RS hinders the accessibility of the hydrolytic enzymes to their substrates [20]. Therefore, thermal and chemical pretreatment of RS is an essential process to make their cellulosic content more accessible to cellulases [37]. However, RS pretreatment is two-sided coin having drawbacks, including the formation of inhibitory products such as phenolics, aliphatic acids, and furan derivatives making feedback inhibition on the hydrolytic enzymes and further the fermentation process [38].

Most of the produced proteins contain carbohydrate-binding modules (CBMs) previously known as cellulose then carbohydrate binding domains (CBDs). CBMs are non-catalytic domains covalently bonded to the hydrolytic enzymes of different GH families. Like GHs, CBMs were classified into families ranged from family1 to 49 according to amino acid sequence similarities [39]. CBMs are responsible for protein-polysaccharide recognition [40]. They can target (hemi) cellulases to their carbohydrate polymers by increasing their concentrations at the substrates sites. Then, they change the carbohydrate structure, promoting their degradation by the effect of the hydrolytic enzymes [39, 41]. So, CBMs have a positive effect on increasing the rate and yield of the lignocellulolytic enzymes.

The absence of BGLs was noticed in the secretome of *T. cellulosilytica* grown on the corresponding substrates. This result was assured by quantitative assay for BGLs activity

using pNPG (para nitro phenyl beta- D-glucopyranoside) as a substrate showing its negligible activity (data not shown). Our similar result was reported by other researches referring to the cause of the intracellular formation of this enzyme [42]. So, commercial cellulase preparations should be amended with BGLs from other sources, especially fungal ones, which are the highest BGL producers for efficient lignocellulolytic biomass hydrolysis [43].

The production, activity, and stability of actinomycetes' cellulases are greatly affected by pH and temperature [44]. Thermophilic cellulase system showed stability and activity at a wide pH range from 4.5-8.5 with a slight non-significant increase at pH 4.5 and 7.5 ( $p < 0.05$ ). Other researches showed similar results in which the optimum pH range of cellulases was generally reported from 4.2-5.2 [45]. However, Prasad and co-workers (2013) assured that maximum cellulase activity from *Streptomyces griseorubens* (Accession No. AB184139) was at pH 7 [31]. The activity of CM Case from *Bacillus* sp. SMIA-2 was found to be optimum at pH 8 [46]. The main reason may be attributed to the complex cellulase system required for complete cellulose degradation. This complex system is composed of 3 different enzymes acting synergistically [47]. Each enzyme has its optimum pH that ensures proper working.

Cellulase isolated from *T. cellulosilytica* showed the maximum activity at 50°C with 94% of retained activity at 70°C. Other researchers showed similar results from *Streptomyces lividans*66, *Bacillus* sp., MSL2 strain, *Bacillus* sp DUSELR13, and *Geobacillus* sp WSUCF1 [48, 49]. This higher temperature reaction leads to faster reaction rates and better degradation of cellulosic materials [46]. The maximum cellulolytic activity achieved at 0.75% concentration of CMC. Higher concentrations were too viscous that hindered the cellulolytic assay.

## CONCLUSION

*T. cellulosilytica* is a promising source of thermostable cellulases that are active over a wide range of pH and temperature. Also, LC-MS/MS based proteomic analysis revealed that *T. cellulosilytica* has variable cellulases that are efficient for the degradation of simple/complex lignocellulosic materials. *T. cellulosilytica* is an interesting actinomycete for more studies in the production of thermostable cellulases for successful applications, such as biofuel production.

## ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

## HUMAN AND ANIMAL RIGHTS

No animals/humans were used for studies that are the basis of this research.

## CONSENT FOR PUBLICATION

Not applicable.

## AVAILABILITY OF DATA AND MATERIALS

Not applicable.

## FUNDING

None.

## CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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## SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's web site along with the published article.

Supplementary file 1: Pellets of *T. cellulosilytica* grown on 0.5% CMC and 1% RS after 2 days and 7 days of incubation.

Supplementary file 2: Peptides identified in the CMC secretome by LC-MS/MS technique.

Supplementary file 3: Proteins identified in the CMC secretome by LC-MS/MS technique.

Supplementary file 4: Peptides identified in the RS secretome by LC-MS/MS technique.

Supplementary file 5: Proteins identified in the RS secretome by LC-MS/MS technique.

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