

Studying the Stability of Cellulases and Xylanase Produced by Thermophilic and Alkaliphilic Bacterial Strains Isolated from Agricultural Wastes

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Abstract: Two *Bacillus* strains (MAM-29 and MAM-38) were isolated from agriculture wastes and were identified by 16SrRNA as *Bacillus* sp. with Accession number KF542653 for MAM-29 which represent a new isolate and as *Bacillus thuringensis* for MAM-38, as mentioned in a previous study. These isolates produced different cellulases and xylanase. The pH and temperature stability of cellulases and xylanase crude enzymes were studied for the two isolates MAM-29 and MAM-38. Studying the pH stability for these crude enzymes at different pH values (3.0, 4.0, 4.8, 5.0, 5.6, 6.0, 7.0 and 7.6) indicated that the cellulases and xylanase enzymes were with high activity and remains stable at higher pH values for both isolates MAM-29 and MAM-38. When studying the crude enzymes stability at different temperatures (60°C, 70°C and 80°C) for different periods of time, in case of isolate MAM-29, the CMCase, FPase, Avicelase, Xylanase and extracellular protein retain about (94%, 96%, 99%, 95% and 95% respectively) of their activity at 80°C for 60 min. In case of isolate MAM-38, the CMCase, FPase, Avicelase, Xylanase and extracellular protein retain about (98%, 97%, 91%, 91% and 97% respectively) of their activity at 80°C for 60 min.

Key words: Avicelase • CMCase • FPase • Stability • Xylanase

INTRODUCTION

The bioconversion of cellulose to fermentable sugars requires the synergistic action of complete cellulase system comprising of endoglucanase (EC 3.2.1.4) which act randomly on soluble and insoluble cellulose chains, exoglucanase (cellobiohydrolases; EC 3.2.1.91) which liberate cellobiose from the reducing and non-reducing ends of cellulose chains and β -glucosidases (EC 3.2.1.21) which liberate glucose from cellobiose [1,2]. The hemicellulase system involves among other endo-1, 4- β -D-xylanase (EC 3.2.1.8), which cleaves internal bonds in the xylan chain; β -xylosidases (EC 3.2.1.37), which cleaves xylooligosaccharides to produce xylose [3]. Xylanase (endo-1, 4- β -D-xylan xylanohydrolase; EC 3.2.1.8) catalyzes the hydrolysis of xylan to produce a mixture of shorter xylo-oligosaccharides, xylose and xylobiose [4]. There is an increasing demand for cellulases

in the market for various applications, among which the bioconversion of lignocellulosic biomass for ethanol production is the major one [5]. Besides this, cellulases have many other potential applications as well, for example, formulation of washing powder, animal feed production [6], textile industry, pulp and paper industry, starch processing, grain alcohol fermentation, malting and brewing, extraction of fruits and vegetable juices [7]. The thermostability characteristics of cellulases enzyme system is a key to industrial interest of cellulose hydrolysis. Thermostable cellulolytic enzymes have wide applications in food and sugar industries where high temperature process such as pasteurization is used [8]. Cellulase production from bacteria can be an advantage as the enzyme production rate is normally higher due to bacterial high growth rate [9]. Reports on bacterial hydrolytic enzymes by SSF, however, are primarily confined to only *Bacillus* spp. [10] which could be

attributed to their ability to the substrate particles to produce filamentous cells for penetration and to their specific need for water activity. Among these, *Bacillus licheniformis* enzymes are reported to have a marginally higher thermostability [11]. The majority of commercial cellulases are extracellular enzymes produced by mesophilic microorganisms. Since the use of cellulose degrading enzymes is related to industrial processes operating at high temperatures, application of thermostable enzymes produced by thermophilic microorganisms appears to be advantageous [12]. Colombatto [13, 14] examined benefits from adding extracellular enzymes derived from thermophilic microorganisms to ruminant diets in view of the advantages of thermostable enzymes, called thermozyms, compared to their mesophilic counterparts.

Recently, much attention has been paid to the thermophilic bio-processing of cellulosic biomass to biofuels which, due to the use of elevated temperatures, offers several potential advantages such as improved hydrolysis of cellulosic substrates, higher mass transfer rates leading to better substrate solubility, lowered risk of potential contamination and increased flexibility with respect to process design thus improving the overall economics of the process. However, the cellulose recalcitrance to biodegradation poses several major bottle necks in the thermophilic digestion of biomass with one being the lack of robust cellulases that can function efficiently at high temperatures and at a broad range of pH. Thus, thermophilic cellulose degrading bacteria and their enzymes have great potential in the development of viable technologies for the production of alternative fuels from agricultural, forestry and municipal cellulosic wastes (MCWs) [15]. Enzymes produced by mesophilic fungi such as *Trichoderma* and *Aspergillus* spp. normally have a temperature optimum between 45 and 60°C, which is lower than temperatures found during the more advanced stages of silage processing. Ideally, enzymes must act rapidly during the crucial first stage of ensilage before they become thermally inactivated [16]. Nevertheless, silages with high temperatures are not desired because high temperatures have been associated with growth of undesired microorganisms such as *Clostridium* sp. [17].

Enzymes require an optimal activity and stability for a prolonged period of time to be effective. Colombatto *et al.* [14] demonstrated that mesophilic enzymes preserve at least 70% of their activity for 48 h at 39°C at a pH ranging from 4.0 to 6.8; enzymes from thermophilic microorganisms often have greater stability than enzymes from mesophilic

microbes. By increasing enzyme stability, the yield of products to be used in ruminant diets can be increased. In addition, direct use of enzymes as additives in rations or food supplements to act inside the rumen is may prove practical. Such enzymes must be stable under the physicochemical conditions of the rumen, such as a pH of about 6.0 and temperatures of up to 40°C as well as resistance to salts and proteases. Such characteristics have been described by Vieille and Zeikus [18] for thermozyms. Compared to mesozymes, these enzymes have a broad tolerance to pH variation, greater resistance to denaturing agents and increased stability and activity at elevated temperatures. Bacteria belonging to the genus *Bacillus* have been used for isolation of hemicellulases [19]. Among these, some *Bacillus* species are reported to produce enzymes that have a marginally higher thermostability [20]. Owing to the increasing biotechnological importance of thermostable xylanases, many thermophilic fungi have been examined for xylanases production [21 and 22]. These strains include *Thermoascus auranticus*, *Talaromyces emersonii*, *Thermomyces lanuginosus*, *Melanocarpus albomyces* and *Sporotrichum thermophile* [23].

This study aimed to study the stability of cellulases and xylanase produced by bacterial strains which tolerate harsh conditions (high temperature and alkalinity).

MATERIALS AND METHODS

Preparation of Agricultural Wastes: The lignocellulosic material (Rice straw) was dried and milled into small pieces (3-5 mm). The milled agriculture waste used for studying the stability of cellulases and xylanase and for SSF as substrates.

Bacterial Strains: Two bacterial *Bacillus* isolates (MAM-29 and MAM-38) were isolated from agriculture wastes and were identified by 16S-rRNA. The two *Bacillus* strains were facultative thermophilic and alkaliphilic as mentioned in previous study.

Enzyme Extraction: According to Abo-State [24], the enzymes were extracted from the fermented flasks with 100 ml of distilled water. The whole content was filtered and squeezed through muslin cloth. The filtered extract was centrifuged at 8000 rpm for 15 min. by cooling centrifuge (4°C). The clear supernatant was used as crude enzymes for enzyme assay and extracellular protein determination.

Enzyme Assay:

CMCase Assay: Endoglucanase, Carboxymethyl cellulase (CMCase) activity was determined according to Wang *et al.* [25]. One ml of the crude enzyme supernatant was incubated with 1 ml of 1% CMC in 0.1 M sodium acetate buffer solution pH 5.0 for 30 min at 63°C. The resulted reducing sugars were determined according to Miller [26] by dinitrosalicylic acid (DNS) reagent (product of Sigma/Aldrich, USA). The resulted reducing sugars were determined at 540 nm by spectrophotometer (LW-V-200RS UV/VIS, Germany). The concentration of resulted reducing sugars was determined using glucose standard curve. One unit of CMCase, is the micro mole of glucose liberated per ml of culture filtrate (crude enzyme) per minute.

FPase Assay: Total cellulase (FPase) activity was the crude enzyme supernatant was determined as described by Gadgil *et al.* [27]. One ml of the crude enzyme supernatant was incubated with 2 ml of 0.1 M citrate buffer (pH 4.8) containing 50 mg Whatman No. 1 filter paper. After incubation for 1 h at 50°C, the resulted reducing sugars were determined by DNS reagent as previously mentioned. One unit of FPase is the micromole of glucose librated per ml of culture filtrate per minute.

Avicelase Assay: Avicelase activity was determined according to Li and Gao [28]. One ml of the crude enzyme supernatant was incubated with 1 ml of 2% (w/v) Avicel (product of Sigma, St. Louis, USA) in 0.1 M phosphate-citrate buffer (pH 6.6) at 40°C for 2 h. The resulted reducing sugars were determined by DNS reagent as previously mentioned. One unit of Avicelase is the micromole of glucose librated per ml of culture filtrate per minute.

Xylanase Assay: Xylanase assay was determined according to Gawande and Kamat [29]. One ml of the crude enzyme supernatant was mixed with 1 ml of 2% xylan from brichwood (product of Sigma/Aldrich, St. Louis, USA) in sodium acetate buffer (pH 5.5) and incubated at 50°C for 30 min. The released reducing sugar was determined by DNS reagent as previously mentioned. Standard curve was determined by xylose. One unit of xylanase is the micromole of xylose librated per ml of culture filtrate per minute under the assay conditions.

Extracellular Protein Determination: Protein was determined according to Lowry *et al.* [30]. One ml of the

crude enzyme supernatant was used and 5.0 ml reaction mixture was added in a clean dry test tube. The tubes were kept at room temperature for 10 min. Then 0.5 ml of Folin reagent (product of Fluka, Switzerland) was added to the previous mixture. The tubes were leaved for 20 min. at room temperature and the absorbance was measured at 720 nm by spectrophotometer.

Studying Enzymes Stability:

pH Stability: Ten grams of milled Rice straw in 250 ml Erlenmeyer flasks were moistened with 20 ml distilled water and autoclaved at 121°C for 20 min. Then, they were inoculated with 5.0 ml of the selected bacterial isolate and incubated at 37°C for 48 h. After that the crude enzymes were extracted as previously described. The pH stability of the enzymes was studied by using the crude enzyme. During the assay of CMCase, FPase, avicelase and xylanase, all the substrates were dissolved in mellvaine buffer with pH (3.0, 4.0, 4.8, 5.0, 5.6, 7.0 and 7.6) and this to study the stability of these enzymes at different pH values.

Temperature Stability: Five ml of distilled water added to ten grams of milled rice straw in 250 ml Erlenmeyer flasks and autoclaved for 20 min at 121°C. Then they inoculated with 5.0 ml of the selected bacterial isolate and incubated at 37°C for 48 hr. The crude enzymes extracted as previously described. These crude enzymes were incubated in water bath at different temperatures 60°C for (10 min, 30 min and 60 min), 70°C for (10 min, 30 min, 60 min and 90 min) and 80°C for (10 min, 30 min, 60 min). Then the substrates were added to complete the enzymes assay as previously described.

RESULTS AND DISCUSSION

The pH and temperature stability of the enzyme are very important factors when we intend to study the industrial importance of the enzyme [31]. Studying the crude enzyme stability at different pH values (3.0, 4.0, 4.8, 5.0, 5.6, 6.0, 6.6, 7.0 and 7.6) revealed that all the enzymes activities were best at high pH values but with low activities at low acidic pH for both the two isolates MAM-29 and MAM-38 as presented in Fig. 1 and 2, respectively. This can be explained on the basis that all the enzymes (CMCase, FPase, avicelase and xylanase) beared the alkaliphilic conditions so they are stable and retained their activities at higher pH values. Extracellular endo-1, 4-β-D-glucanase showed its activity in both acidic

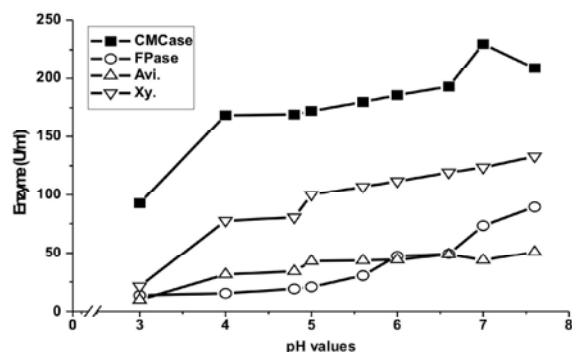


Fig. 1: Effect of different pH values on cellulases and xylanases stability produced by isolate MAM-29.

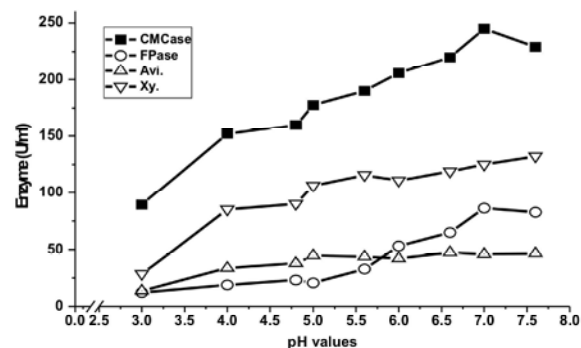


Fig. 2: Effect of different pH values on cellulases and xylanases stability produced by isolate MAM-38.

and alkaline pH but maximum enzyme activity was detected in succinate buffer pH 5.0. It retained 73% and 66% of its maximal activity at pH 3.0 and 9.0, respectively. At pH 10.0, endo-1, 4- β -D-glucanase lost its activity rapidly and only 34% activity was retained [32]. The pH range from 5 to 11 was used to study the effect of pH on xylanase activity. The optimum activity was at 6.5, but a significant level of activity was observed at a range of pH from 5 to 7.5 (above 80% of activity) and there was another peak at pH 8.5 (about 60% of activity) and at pH 11 (about 10% of activity) [33]. A thermostable, alkaline active xylanase was purified to homogeneity from the culture supplement of an alkaliphilic *Bacillus halodurans* S7, which was isolated from a soda lake in the Ethiopian Rift Valley. When assayed at 70°C, it was optimally active pH 9.0-9.5. The optimum temperature for the activity was 75°C and pH 9 and 70°C at pH 10. The enzyme was stable over a broad range and showed good thermal stability when incubated at 65°C in pH 9 buffer [34].

By studying the crude enzyme stability at different temperatures (60°C, 70°C and 80°C), results in the present study revealed that in case of isolate MAM-29 at 60°C (for 120 min), the CMCase, FPase, avicelase, xylanase and extracellular protein retained about 97%, 97%, 99%, 99% and 95% of their activity respectively. At 70°C (for 90 min), CMCase, FPase, avicelase, xylanase and extracellular protein retained about 98%, 98%, 100%, 97% and 95% of their activity respectively. Finally, at 80°C (for 60 min.), CMCase, FPase, avicelase, xylanase and extracellular protein retained about 94%, 96%, 99%, 95% and 95% of their activity respectively as shown in Fig. 3-7. In case of isolate MAM-38, at 60°C (for 120 min), the CMCase and FPase retained 100% but avicelase, xylanase and extracellular protein retained 91%, 98% and 99% of their activity, respectively. At 70°C (for 90 min), CMCase

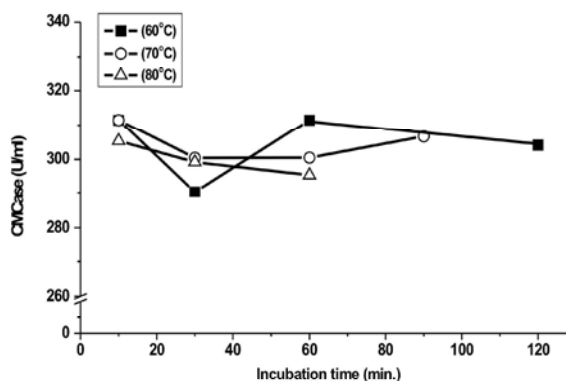


Fig. 3: Effect of temperature on CMCase stability produced by isolate MAM-29.

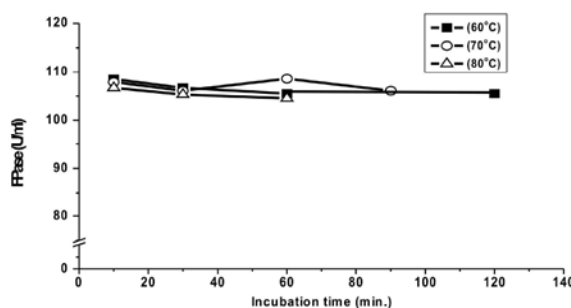


Fig. 4: Effect of temperature on FPase stability produced by isolate MAM-29.

retained 100% but FPase, avicelase & xylanase retained about 95% of their activity. The extracellular protein retained 99% of its activity. At 80°C (for 60 min), CMCase, FPase, avicelase, xylanase and extracellular protein retained about 98%, 97%, 91%, 91% and 97% of their activity respectively as shown in Fig. 8-12. Thermal stability is a very important aspect when considering the industrial application of enzymes. The xylanase from *B. coagulans* BL69 is relatively stable at 50°C, with

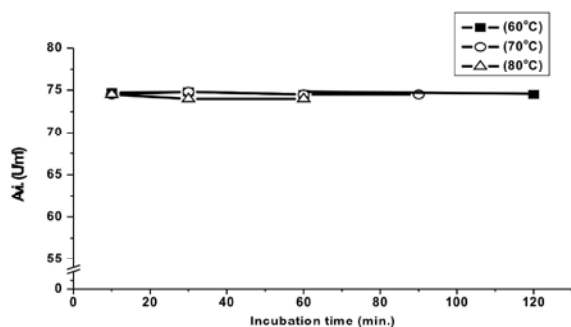


Fig. 5: Effect of temperature on Avicelase stability produced by isolate MAM-29.

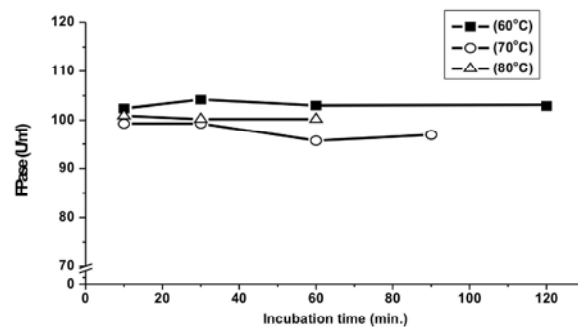


Fig. 9: Effect of temperature on FPase stability produced by isolate MAM-38.

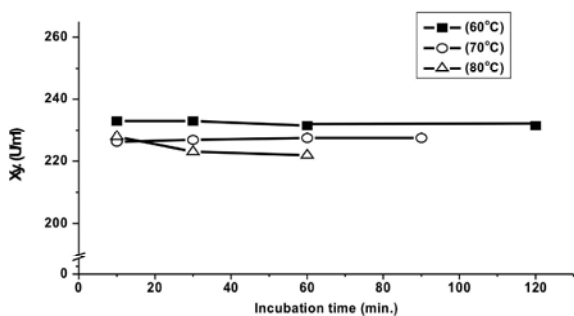


Fig. 6: Effect of temperature on xylanase stability produced by isolate MAM-29.

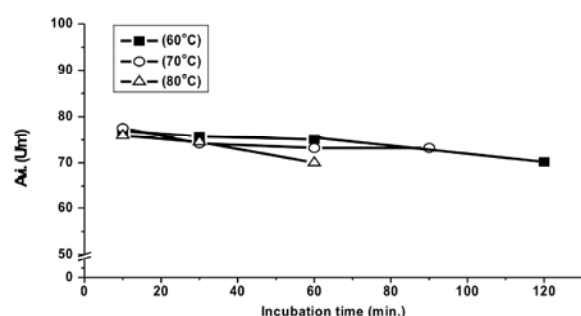


Fig. 10: Effect of temperature on Avicelase stability produced by isolate MAM-38.

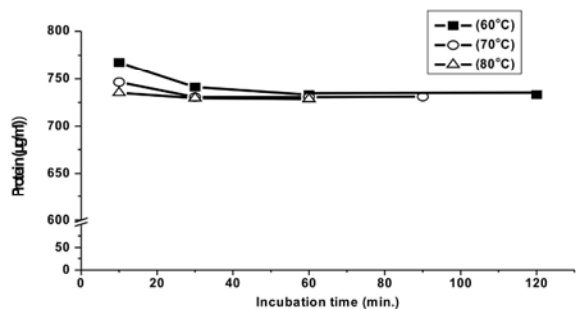


Fig. 7: Effect of temperature on protein stability produced by isolate MAM-29.

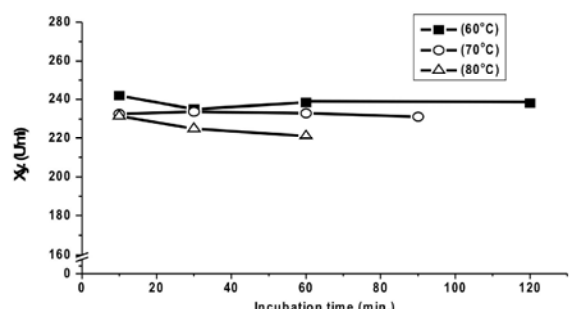


Fig. 11: Effect of temperature on xylanase stability produced by isolate MAM-38.

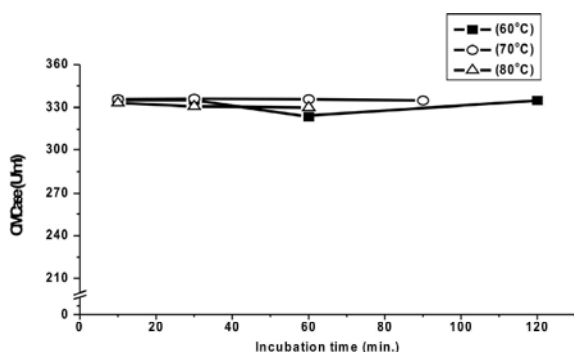


Fig. 8: Effect of temperature on CMCase stability produced by isolate MAM-38.

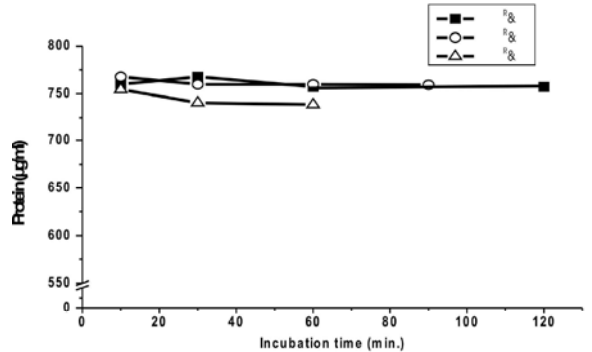


Fig. 12: Effect of temperature on protein stability produced by isolate MAM-38.

activity decreasing sharply above 60°C. Thirty-four percentage activities was maintained when incubated at 70°C, 5 min and 20% at 80°C. Under storage conditions of 7°C for 7 days and -20°C for 30 days, no detectable loss of activity was observed [35].

Extracellular endo-1, 4-β-D-glucanase activity was performed at different temperatures ranging from 25°C to 70°C. The enzyme showed its activity in wide temperature range with optimum of 60°C. It has been described that increasing temperature has the general effect of increasing enzyme activity and optimum temperature for endo-1, 4-β-D-glucanase from *B. pumilus* was 60°C [36]. The enzyme retained 82% of its original activity at 70°C. In addition, at 80°C the enzyme activity decreased up to 42%, whereas endo-1, 4-β-D-glucanase from *B. pumilus* EB3 retained only 66% of its maximum activity at 70°C [37]. The xylanase is very stable at temperatures up to 60°C, decreasing its stability above 65°C. At 90°C, the activity decreases progressively, occurring complete thermal inactivation after 1 hr at this temperature [38]. Enzyme extracted from *B. pumilus* showed stability over a broad range of pH from 6 to 10 and temperature from 55 to 70°C. A xylanase comprising two components active over a pH range of 6-10 and temperature range of 50-60°C has been characterized from an alkalothermophilic (AT) *Bacillus* strain by Dey *et al.* [39]. Similar results were observed for *Bacillus* sp. [40, 41]. The optimum temperature for bacterial xylanase was found in the range of 50-80°C [31, 42]. Several reports have revealed that the optimum pH for xylanase produced from bacteria does not usually exceed pH 7, as in *Bacillus* sp. cases [43, 44] with a range of 6-6.5. However, optimum pH from some *Bacillus* sp. has also been reported in alkaline range of 7-9 [45].

As explained by Sα-Pereira *et al.* [38] and Park *et al.* [46] the difference in pH and temperature tolerance for xylanase excreted may be due to the effect of different enzymes mixtures excreted and or the post-translational modifications in xylanase excretion process, such as glycosylation, that improve stability in more extreme pH and temperature conditions. Few xylanases are reported to be active and stable at alkaline pH and high temperature [47, 48]. Optimal temperature and pH for the CMCase activity were determined to be 50°C and 6.5, respectively for *B. subtilis* subsp. *Subtilis* A-53. More than 70% of original CMCase activity was maintained at relative low temperature ranging from 20 to 40°C after 24h incubation at 50°C [49]. Also, the optimum temperature

and pH for the CMCase activity of the purified cellulase were found to be 50°C and pH 7.0, respectively for *B. amyloliquefaciens* DL-3 [50].

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