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CHARACTERIZATION OF CELLULASES FROM THERMOPHILIC BACILLI AND THEIR APPLICATION FOR THE SACCHARIFICATION OF SUGARCANE BAGASSE

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Abstract

Cellulases from thermophilic bacteria are likely to be more stable and are required in countless biotechnological applications. *Bacillus* being a house of thermostable proteins has frequently been studied for the production of cellulases. During the process of composting, distinct temperature changes are observed including significant rise that favors the growth of thermophiles with the ability to degrade plant based waste materials. Therefore, isolation of bacterial strains was carried out from a composting site; screening of the isolates revealed capability of three of the isolates, MAH14, MAH66 and MAH80, to produce endoglucanase (EG) and β-glucosidase (BGL) at 50°C. On the basis of biochemical characters MAH66 and MAH80 were identified as the strains of *Bacillus licheniformis* whereas, MAH14 was identified as *Bacillus subtilis*. The cellulase preparations from these bacteria were studied for their thermostability by investigating the half-life ($t_{1/2}$) and melting temperature (T_m). The MAH80 cellulase exhibited more stability with a T_m of 60°C and a $t_{1/2}$ of 70 min at 50°C.The prospected application of cellulase crude preparation from the strains was estimated by carrying out experiments on the saccharification of untreated and alkali-treated sugarcane bagasse (SB) and the data was compared with the saccharificaiton of CMC. Hydrolysis rate from MAH14 of untreated SB was higher as compared to alkali pretreated SB and CMC. The amount of reducing sugars liberated by the action of cellulase preparation from MAH 66 on untreated SB (1.7 mg mL^{-1}) was higher than on alkali treated SB (0.29 mg mL^{-1}). While the enzyme from MAH14 yielded almost equal amount of sugars when applied either on alkali treated SB (0.56 mg mL-1) or on CMC (0.6 mg mL^{-1}). Results from this study revealed the capability of indigenous thermophilic bacterial strains for cellulase production and biomass saccharification.

Key words: *Bacillu*s, β-Glucosidase, Endoglucanase, Thermostability

Introduction

Cellulases assumed to include exo-acting and endo-acting hydrolases that perform an active role in the biodegrdation and bioconversion of lignocellulosic (LC) biomass, mainly cellulose into numerous value-added chemicals (Merino & Cherry, 2007). The action of exo-enzymes reduces the chain ends of polymers while endo-acting enzymes place random cut in the chain of polysaccharide (Horn *et al*., 2012). Indeed, complete disintegration of cellulose requires the synergistic operation of (i) Endoglucanase (EG) that indefinitely cuts inner bonds in the cellulose chain, mostly amorphous regions, (ii) Exoglucanase (EXG) acts on terminal and releases monomers and (iii) β-glucosidase (BGL) that hydrolyzes oligosaccharides and disaccharides (Singhania *et al*., 2013).

Genes encoding cellulases are widely disseminated amongst all living forms, though they are mostly studied in fungi and bacteria (Lo *et al*., 2009). Fungal cellulases predominate over bacterial cellulases in commercial processes; the most comprehensively studied fungal cellulases are produced by *Aspergillus* (Sohail *et al*., 2009) and *Trichoderma* (Hasunuma *et al*., 2013). *T. reesei* is demonstrated as a model organism for its high yields and competent cellulase induction mechanism. Despite of their widespread industrial application, most of the fungal cellulases do not exhibit thermal stability and hence research has been diverted to find out new and novel thermostable counterparts (Miller *et al*., 2010). In contrast, bacterial cellulases provide an advantage of greater stability and can easily be expressed in the presence of LC substrates; however, they are yet to replace fungal cellulases for their employment on large scale (Seiboth *et al*., 2011).

Thermophilic/ thermotolerant bacteria have many biotechnological applications mainly attributed to their property to survive, grow and produce industrially important enzymes under extreme conditions (Attri & Garg, 2014). Under aerobic conditions many cellulose degrading bacilli consume cellulose through the production of sufficient quantities of cellulase which are rarely complexed at the cell surface (Wachinger *et al*., 1989) therefore, the enzyme can easily be retrieved from culture supernatants.

Thermophilic cellulolytic strains of bacteria have been isolated from different sources for instance, soil, rumen, brewery sludge, manure, domestic solid wastes (Gilkes *et al.*, 1991), compost sites (Kinet *et al.*, 2015), gold mines (Rastogi *et al*., 2010) and deep sea (Dipasquale *et al.*, 2014). Such bacteria exhibit growth at temperatures ranging from 50-80°C and produce thermo-active enzymes (Zambare *et al*., 2011). In eubacteria, several members of Actinomycetales (aerobic bacteria) and Clostridiales (anaerobic bacteria) have been described for their cellulolytic potential including *Fervidobacterium nodosum* (Wang *et al*., 2010), *Acidothermus cellulolyticus*, *Acetobacterxylinum*, *Rhodothermus* and *Thermotoga neopolitana* (Lynd *et al*., 2002). Additionally, if a thermostable enzyme can sustain its activity at a broader range of pH, such enzymes contend well for bioconversion of LC biomass (Bhalla *et al*., 2013). Indeed thermostable cellulases with stability over broad range of pH have been obtained from strains of *Paenibacillus* and *Bacillus* (Maki *et al*., 2009). Similarly, alkali-stable and halo-tolerant cellulases have been reported from the strains of *B. subtilis* (Asha & Sakthivel, 2014) and *B. vallismortis* (Gaur & Tiwari, 2015) isolated from soil. Whereas, cellulase from *B. licheniformis* was found to require microaerophilic conditions in addition to high temperature to degrade cellulosic substrates (Fujimoto *et al*., 2011).

Cellulases in general, and thermostable cellulases, in particular, have tremendous applications in waste-biorefinery processes where they incur 40% reduction in the cost by minimizing the requirement of pretreatment (Lynd *et al*., 2008). Sugarcane bagasse (SB), a residue generated after extracting juice from sugarcane, is an abundantly available waste with a prospect to be utilized as biorefinery substrate. Sugarcane is a major crop in South American and Asian countries (Kim & Dale, 2004) hence SB can be used as a raw material for many fermentation industries (Xue *et al*., 2016) in such countries. The bioconversion of SB into simple sugars involves a concerted and synergistic action among cellulases for subsequent conversion into lactic acid, biofuel, methane, hydrogen and other valuable chemicals. In perspective to significance of thermophilic cellulose degrading bacteria in LC biomass conversion, few of the indigenous thermotolerant *Bacillus* strains were evaluated for cellulase production; thermostability of the enzymes was assessed and the enzymatic saccharification of SB was studied.

Materials and Methods

Isolation of aerobic thermotolerant/thermophilic cellulolytic bacteria: The bacterial strains were isolated from compost samples and from soil near an area where plant materials were routinely burnt. The samples were transferred to different sets of flasks containing mineral salt medium (MSM) described by Mandels & Weber (1969) supplemented separately with 0.5% carboxymethyl cellulose (CMC) and SB and kept in an orbital incubator at 150 rpm and 50°C for 72 h. An aliquot from each flask (5 ml) was sub-cultured to another flask containing MSM with CMC and incubated under the same conditions for 72 h. After incubation, the contents present in each flask were serially diluted for the isolation of bacteria using MSM-CMC agar plates at 50°C for 72 h. Cellulase producing bacterial isolates were screened by iodine staining method (Kasana *et al*., 2008) and preserved on Nutrient agar and MSM-CMC agar slants for the routine work while stock cultures were saved in 70% glycerol.

Screening for cell associated and cell-free cellulase production from *Bacillus* strains: The isolates capable to grow and produce extracellular cellulase were initially screened by cultivating on MSM-CMC agar plates at 50°C and visualizing the clear zones around the cellulolytic colonies using iodine method (Kasana *et al.*, 2008). The isolates exhibiting clear zones around their colonies were further screened by adopting the same method and cultivated at 60 and 70ºC. In other set of experiments, the extracellular cellulase activity was screened in cell-free culture supernatant (CFCS). The strains were separately grown at 37°C and 50°C in MSM-CMC broth and the CFCS was added into the wells of CMC-agar plates. The plates were kept at 37°C and 50°C for 2 h after staining by iodine method.

Cellulase production: The inoculum was prepared by transferring a single colony of the isolate from MSM-CMC agar plate to MSM-CMC broth and incubated at 50°C until an OD of 0.3 at 600 nm was attained. An aliquot of 0.5 mL was transferred to a 5 mL of fresh MSM-CMC and incubated in an orbital incubator at 50°C for 24 h at 150 rpm. The CFCS obtained after centrifugation at 4000 rpm for 20 min was assayed as described by Sohail *et al*., (2016) for endoglucanase (EG), β-glucosidase (BGL) and Filter paperase (FPase) activities.

Identification of the isolates: The promising bacterial isolates were identified following standard microbiological and biochemical protocols. Cultural characteristics were noted by cultivating the isolates on Nutrient agar and morphology was observed by Gram staining. Different tests for identification were performed such as catalase, Indole, MR-VP, oxidase, nitrate reduction, casein, citrate, starch and gelatin utilization tests and sugar fermentation tests.

Estimation of melting temperature (T_m) and Half-life ($t_{½}$) of crude Cellulase preparation: Thermostability of the isolates was evaluated by investigating melting temperatures (T_m) and half-life ($t_{1/2}$) of the enzyme. CFCS were incubated at different temperatures (from 50-80°C) for 30 min and were assayed for any residual activity (RA). T_m was calculated by plotting RA against temperature. The half-lives of the enzymes were determined at 50ºC by placing the CFCS for variable duration and plotting RA against time. To determine T_m or $t_{½}$, activity in the unheated enzyme was taken as 100%.

Pretreatment of SB by using alkali: The substrate (SB) was obtained from a local supplier, dried, powdered to maintain 100µm mesh size and pretreated as mention by Shariq and Sohail (2020). Briefly, a sample of 1 g SB was pretreated with alkali at room temperature for overnight using 50 mL of 1% (w/v) NaOH solution. After decanting the alkali, SB was excessively washed with tap water until pH of the wash-through became neutral and then SB was dried at 60°C in an oven.

Saccharification of commercially purified substrates and SB: The potential of the CFCS from the three strains MAH14, MAH66 and MAH80 to saccharify untreated and pretreated SB was determined and compared with the results obtained using a soluble substrate, CMC. The enzyme preparations were standardized to 10 IFPU (International Filter Paperase Unit) g^{-1} of substrate. Saccharification was commenced by loading CFCS to 0.25g substrate buffered with sodium citrate buffer of pH 4.8 containing 0.2% (w/v) sodium-azide in a final volume of 25 mL. Flasks were incubated at 50°C for 48 to 52 h by shaking at 100 rpm. Aliquots were drawn at different time intervals and assayed for reducing sugars by DNS method (Miller, 1959).The rate of hydrolysis of the substrate and yield of reducing sugars were calculated by plotting reducing sugars (mg h^{-1}) against time.

Results and Discussion

Aerobic thermophiles have the ability of producing extracellular thermostable enzymes which have many industrial and commercial applications. Although, thermophiles can easily be obtained from habitat facing naturally higher temperatures, however, they can also be isolated from mesophilic environments. In this study, 117 isolates were obtained from different compost and garden soil samples; 95 strains were found to be cellulolytic by plate screening method (data not shown). The cellulase positive (95) isolates were screened for the production of the enzyme at temperatures ranging from 37-70°C by spot inoculating the cultures on MSM-CMC agar plates followed by iodine method. The outcome showed that 89 isolates produced cellulase at 55°C. Further deduction showed that 47 strains were good producers (with zone ≥30 mm), 34 isolates moderate producers (with zone 15-29 mm) and remaining 14 strains were classified as low producers (data not shown). Consequently, the good producers were cultivated in broth and assessed for cellulase activity.

The extracellular cellulase activity in CFCS was determined by agar-well diffusion method at different temperatures from 37°C to 70°C. The strains, MAH37, MAH41, MAH44, MAH49, MAH58 and MAH70 produced higher levels of cellulase at 50°C (data not shown). Nonetheless, the zones of clearance exhibited by these five strains were smaller than observed for the strains MAH 14, MAH 66 and MAH 80. These three strains, not only produced more cellulase at 37ºC, rather the enzyme produced at this temperature displayed more activity at 50ºC (Table 1). However, the enzyme activity was decreased at 60°C and 70°C. At higher temperature, the loss of activity of cellulase could be attributed to the thermal denaturation (Rajoka *et al*., 2004) or towards restriction of microbial growth (Sohail *et al*., 2013).

On the basis of the zones of clearance by spot culture and CFCS, 47 isolates were selected for quantification of extracellular cellulase activity using shake flask method (data not shown). Isolates were cultured at 50, 60 and 70°C and CFCS were assayed for EG, BGL and FPase activities at temperatures 50 to 70°C. At 50°C, MAH66 produced the highest titers of 11.45 IU mL^{-1}, 10.43 IU mL^{-1} and 3.8 IU mL^{-1} of EG, BGL and FPase, respectively (Table 2). MAH14 yielded the maximum EG (11.5 IU/ml) at 60°C while for MAH80 50ºC was found to be the most suitable temperature for the production of EG, BGL and FPase. Thus these 3 isolates were selected for further studies.

These isolates were identified by following Bergey's Manual of Systematic Bacteriology and all the biochemical tests (Table 3) were found in accordance to the characteristics of *B. subtilis* (MAH14) and *B. licheniformis* (MAH66 and MAH80. Previously, cellulase production from thermophilic strains of *B. subtilis* CY5 (Ray *et al*., 2007), *B. licheniformis* GXN151 (Liu *et al*., 2004) and *B. licheniformis* RT17 (Tariq *et al*., 2018) have also been reported.

Considering the vital importance of thermostability of the enzymes for their application at industrial or commercial scale, thermal stability of cellulase from MAH14, MAH66, and MAH80 was evaluated by determining two of the important parameters i.e. T_m and $t_{1/2}$. T_m of EG, BGL and FPase from MAH14 was determined as 62, 60 and 52°C, respectively (Fig. 1a). While the EG from MAH66 (Fig. 1b) had a lower T_m of 58°C. The EG (T_m 65ºC) and BGL (T_m 60ºC) produced by MAH80 were found as the most heat stable amongst the cellulase tested in this study (Fig. 1c). Sohail *et al*., (2013) determined a T_m of 68 and 69ºC for EG and BGL, respectively, from a mutant strain of *A. niger*, which were similar to the values obtained in the present study.

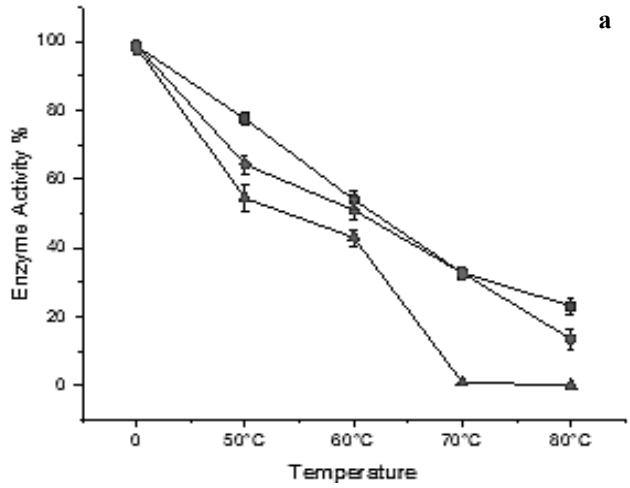
Table 1. Screening of bacterial isolates for extracellular cellulase production at 37 and 50ºC. The isolates were cultivated at two different temperatures and cell-free activity was evaluated by agar-well diffusion method at 37-70ºC.

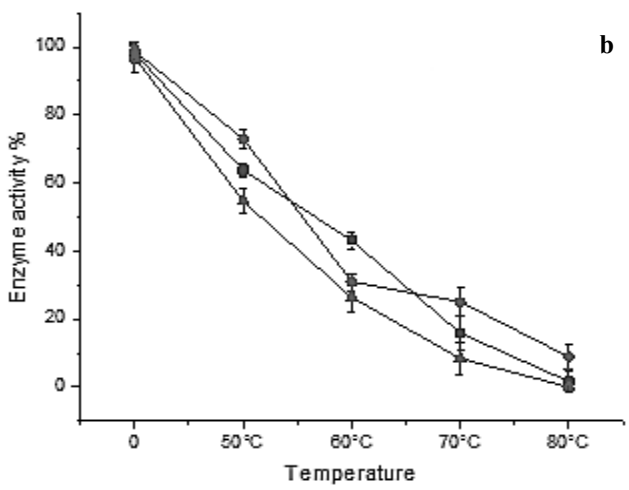
Bacterial isolates	Production temperature (°C)	Zone of clearance (mm)			
		37°C	50°C	60°C	70°C
MAH14	37	12	28	30	10
	50	19	32	39	25
MAH66	37	9	11	37	0
	50	8	20	22	10
MAH80	37	9	36	23	14
	50	4	40	19	21

Table 2. Production of Endoglucanase (EG), β-glucosidase (BGL) and Filter paperase (FPase) activity at different temperatures.

Isolates	EG			BGL			FPase		
	50°C	60°C	70°C	50°C	60°C	70°C	50°C	60°C	70°C
MAH 14	10.43 (1.634)	11.5 (0.97)	7.34 (1.551)	9.47 (0.127)	8.56 (0.78)	7.98 (0.111)	3.76 (1.452)	3.12 (0.145)	3 (0.362)
MAH 66	11.45 (1.543)	9.98 (0.662)	10.56 (0.343)	10.43 (1.812)	9.87 (0.333)	9.76 (0.131)	3.8 (1.453)	3.34 (0.323)	3.61 (0.553)
MAH 80	9.34 (0.445)	7.56 (1.397)	7.11 (1.985)	8.9 (1.667)	8.34 (0.774)	6.64 (0.455)	3.12 (1.554)	3.19 (0.323)	1.99 (0.232)

*Values in parentheses represent standard deviation





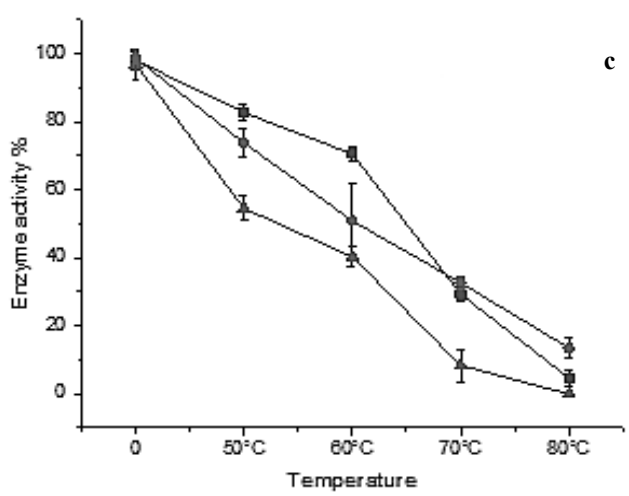
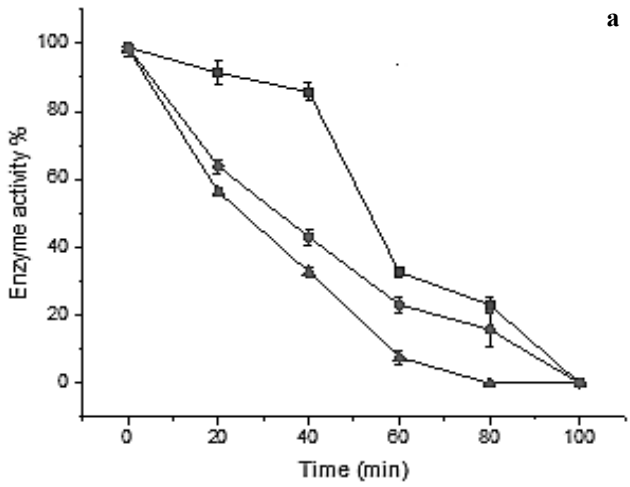
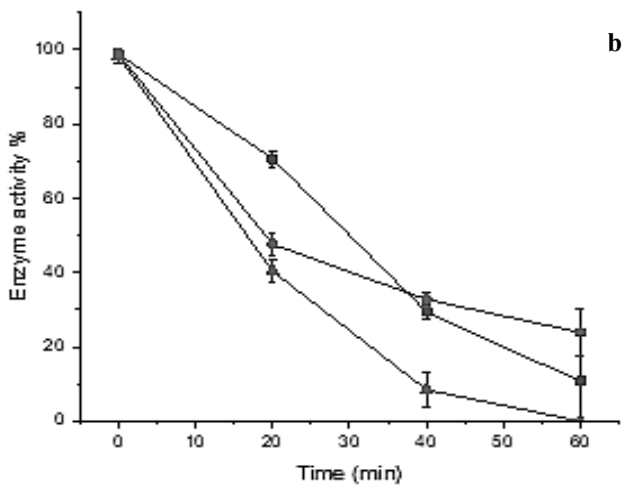


Fig. 1. Melting temperatures of endoglucanase (-■-) β-glucosidase (-●-) and filter paperase (-▲-) from the strains (a) *B. subtilis* (MAH14), *B. licheniformis* (MAH66) and and (c) *B. licheniformis* (MAH80).





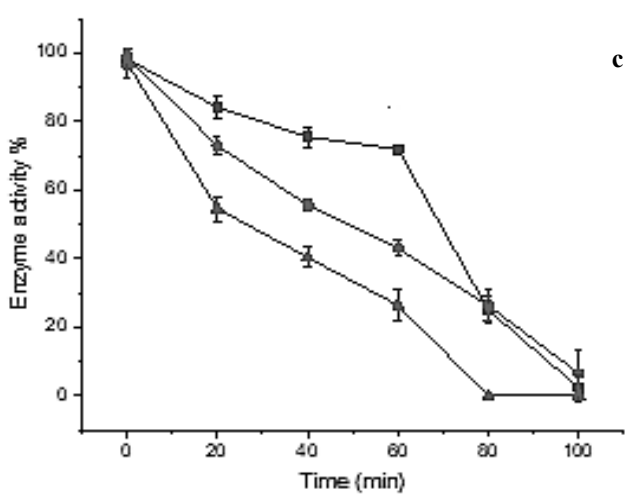


Fig. 2. Determination of half-life of endoglucanase (-■-) β-glucosidase (-●-) and filter paperase (-▲-) from the strains (a) *B. subtilis* (MAH14), *B. licheniformis* (MAH66) and and (c) *B. licheniformis* (MAH80).

The application of thermostable enzyme acquired from thermophilic bacteria with an extended half-life renders the rate of reaction higher and hence decreases the requirement of the amount of the enzyme (Haki & Rakish, 2003), therefore, such enzymes remain in demand. The elucidation of thermostability of the enzymes studied in terms of half-lives at 50°C showed that the EG ($t_{1/2}$ 70 min) from MAH80 (Fig. 2) was more stable than that of the enzymes from the other strains. The BGL and FPase from MAH14 were least stable with half-lives of less than 45 min. BGL was reported previously for having shorter half-life than EG in the cellulase preparation by *A. niger* MS82 (Sohail *et al*., 2013).

Table 3. Biochemical tests performed to identify the strains MAH14, MAH66 and MAH80.

Biochemical tests*	MAH14	MAH66	MAH80
Gram Stain	+	+	+
Glucose	+	+	+
Fructose	+	+	+
Mannose	+	+	+
Mannitol	+	+	+
Sucrose	+	+	+
Arabinose	+	+	+
Xylose	±	+	+
Galactose	+	+	+
Rhamnose	+	+	+
Lactose	+	+	+
Starch hydrolysis	+	+	+
Indole	±	-	-
Methyl red (MR)	±	-	-
Voges Proskauer (VP)	±	+	+
Citrate	+	-	-
Catalase	+	+	+
7% NaCl	+	+	+
Oxidase	-	-	-
Motility	+	+	+
Nitrate	-	+	+
Casein hydrolysis	+	+	+
Gelatin hydrolysis	+	+	+

*+ Indicate growth of bacterial strain or positive test while – sign shows no growth or negative test

The capacity of cellulase from *B. subtilis* MAH14, *B. licheniformis* MAH66 and MAH80 was estimated for the hydrolysis of untreated sugarcane bagasse (SB), alkali-pretreated SB and CMC at 50ºC as most of the enzymes retained significant activities at this temperature (Table 4). The results were recorded in terms of productivity (mg mL^{-1} h^{-1}) and total yield of reducing sugars. The cellulase preparation from MAH66 yielded the highest amount of reducing sugars from CMC (0.87 mg mL^{-1}). In comparison with CMC and alkali-pretreated SB, saccharification of untreated SB by the enzyme from MAH66, released more sugars (1.72 mg mL^{-1}) and hence yielded the highest productivity of 69 mg L^{-1} h^{-1} (Fig. 3). Tariq *et al.*, (2018) also reported about the efficient hydrolysis of SB than CMC by the cellulase preparation from *B. licheniformis* R17 and attributed it to synergistic action among components of cellulase to saccharify a crude LC substrate. The hydrolysis of untreated SB by the enzyme from MAH80 produced more amount of reducing sugar (1 mg mL^{-1}) as compared to the hydrolysis of alkali-pretreated SB (0.4 mg mL^{-1}). In contrast to this finding, Yoon *et al.*, (2011) obtained more reducing sugar by the saccharification of alkali-pretreated SB than untreated SB. On the other hand, alkaline pretreatment reportedly releases irrecoverable salts (Zheng *et al.*, 2009) that inhibited cellulase activity as was observed in this study. Nonetheless, the cost of pretreatment can be avoided and the environment can be safeguarded from discharge of toxic acid or alkali. However, further studies are required to explore ultimate candidature of these strains for future biotechnological applications.

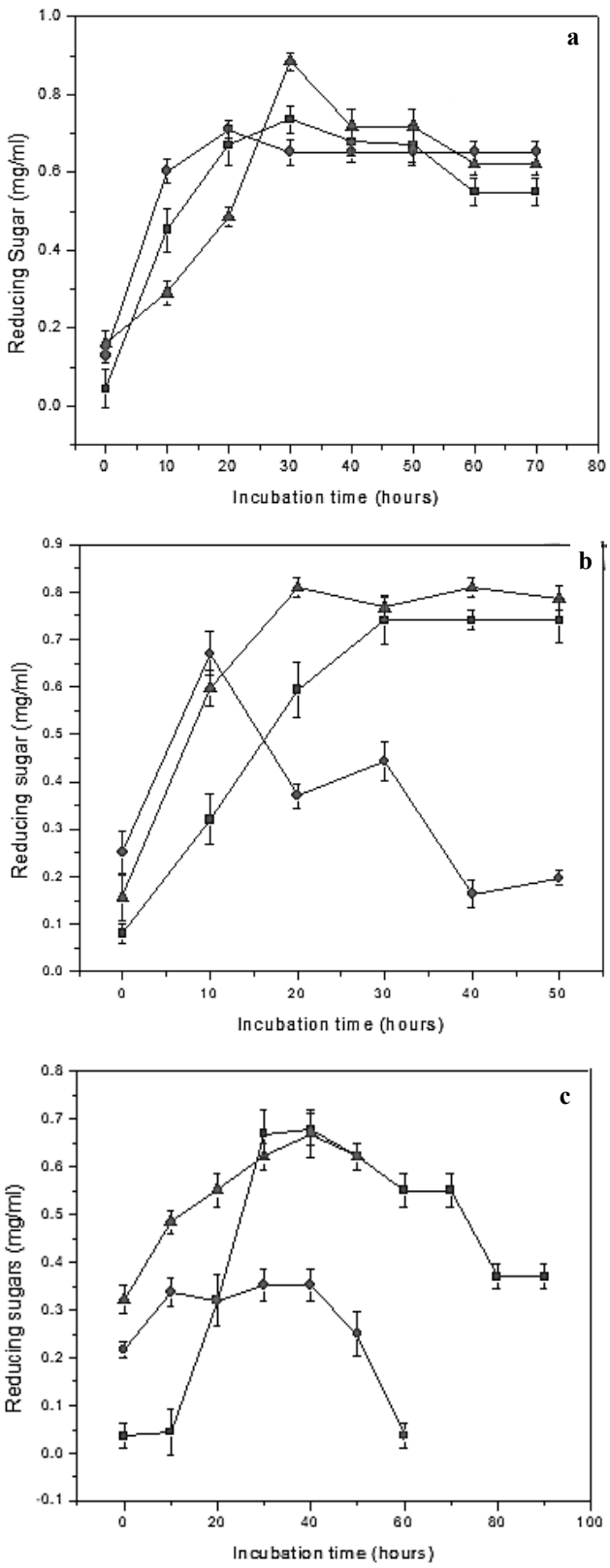


Fig. 3. Saccharification of (a) CMC (b) Untreated and (c) Alkali treated SB by cellulase preparation from the strains (a) *B. subtilis* (MAH14), *B. licheniformis* (MAH66) and and (c) *B. licheniformis* (MAH80).

Table 4. Saccharification of carboxymethyl cellulose (CMC) and sugarcane bagasse (SB).

Substrates	Release of reducing sugar (R; mg $mL^{-1}h^{-1}$) and Productivity (P; mg L^{-1} h^{-1}) of the enzyme solutions from isolates on different substrates					
	MAH14		MAH66		MAH80	
	(R)	(P)	(R)	(P)	(R)	(P)
CMC	0.6	24	0.87	35	0.74	29.6
Untreated SB	0.62	25	1.72	69	1	40
Alkali-pretreated SB	0.56	22.6	0.29	11.6	0.43	17.25

Conclusion

Aerobic thermophilic cellulolytic bacteria can be isolated from soil and compost resource rich in organic plant waste material. The cellulase preparation from the promising strains of *B. subtilis* and *B. licheniformis* were characterized for their thermal stability. The enzyme preparation was found efficient for the saccharification of sugarcane bagasse.

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