Enhanced Cellulase Production by a Novel Thermophilic *Bacillus licheniformis* 2D55: Characterization and Application in Lignocellulosic Saccharification

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Effects of nutritional and physicochemical factors were investigated for cellulase production by the newly isolated thermophilic strain *Bacillus licheniformis* 2D55 (Accession No. KT799651). The optimum cellulase production in shake flask fermentation was attained at 60 °C, pH 3.5, 180 rpm, and in a medium containing untreated sugarcane bagasse and pre-treated rice husk at 7% (w/v), urea, 1 g/L, peptone, 11.0 g/L, $Mg(SO4)_2$, 0.40 g/L, $CaCl_2$, 0.03 g/L, Tween 80, 0.2% (w/v), and 3% inoculum. The highest carboxymethyl cellulase (CMCase), filtre paperase (FPase), and β-glucosidase produced under the optimized conditions were 29.4 U/mL, 12.9 U/mL, and 0.06 U/mL, respectively, after 18 h of fermentation. Optimization of the parameters increased the CMCase, FPase, and β-glucosidase activities by 77.4-fold, 44.5-fold, and 10-fold, respectively. The crude enzyme was highly active and stable over broad temperature (50 to 80 °C) and pH (3.5 to 10.0) ranges with optimum temperature at 65 °C and 80 ºC for CMCase and FPase, respectively. The optimum pH for CMCase and FPase was 7.5 and 6.0, respectively. Saccharification of sugar cane bagasse and rice husk by crude cellulase resulted in perspective yields of 0.348 and 0.301 g g^{-1} dry substrate of reducing sugars. These results suggest prospects of thermostable cellulase from *B. licheniformis* 2D55 in application for bio-sugar production and other industrial bioprocess applications involving high temperatures.

Keywords: Bacillus licheniformis 2D55; Thermostable cellulase; Lignocellulosic saccharification; Optimization

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INTRODUCTION

Lignocellulosic materials are one of the most abundant renewable sources of biomass, and they are composed mainly of cellulose, hemicellulose, and lignin (Hossain and Aldous 2012). Global production of agro-waste and its by-products amounts to about 180 billion tons per year (Kim *et al*. 2006). Rice husk (RH) is an agricultural waste product of the rice-milling industries. It is produced in great quantities in rice-producing countries such as Malaysia and Thailand. The annual world output of rice husk amounts to approximately 80 million tons (Li and Wang 2008). A large amount of RH is disposed of by burning, which causes environmental pollution.

Sugarcane is a crop cultivated in many countries around the world. Large amounts of sugarcane bagasse (BAG) are generated by the sugarcane industry during sugar and ethanol production. Sugarcane bagasse has been utilized for heat and electricity generation in countries such as Brazil (Dias *et al*. 2012); however, the majority of bagasse still constitutes environmental pollution in many parts of the world. As abundant and renewable resources, much attention has been focused on the conversion of agro-waste biomass into value-added products such as bioethanol (Madhavan *et al*. 2012), hydrogen (Alonso *et al*. 2010), and cellulase enzymes (Acharya and Chaudhary 2012; Harun *et al*. 2012). Therefore, RH and BAG could present cheaper carbon substrates for cellulase production compared with carboxymethylcellulose (CMC) and Avicel®.

Enzymatic hydrolysis of lignocellulosic biomass complicates the biofuel production process. Pre-treatment is usually required to deconstruct the tough complex structures in lignocellulosic biomass prior to hydrolysis. Acid and alkali pre-treatment technologies have been used on lignocellulosic biomass (Krishnan *et al*. 2010; Sindhu *et al*. 2011); most thermal pre-treatment requires high temperatures above 100 °C (Nitsos *et al*. 2013; Zheng and Rehmann 2014). However, fungal cellulases available for saccharification have optimum activities at 50 °C. Therefore, incompatibility in both temperatures requires cooling after pre-treatment, which in turn adds cost and lengthens production times (Liang *et al*. 2010).

The bioconversion of lignocellulosic biomass to ethanol under thermophilic conditions has advantages including higher mass transfer rate, lower viscosity, improved hydrolysis, low risk of contamination, and increased flexibility of the process design. However, the lack of robust cellulases that function at high temperatures and over a wide pH range remains a challenge (Rastogi *et al*. 2010). A number of thermophilic cellulolytic bacteria, including *Brevibacillus*, *Anoxybacillus*, *Geobacillus*, *Bacillus*, *Thermobifida*, and *Clostridium* species, have been identified in various environments.

The costs of cellulases hinder biomass hydrolysis for biofuel production. Therefore, cost-effectiveness in cellulase production remains an important phase of the economic process of bioethanol production. The extent of enzyme production by a bacterial culture is a function of the medium components and fermentation conditions (Singh *et al*. 2014). Therefore, cellulase production can be greatly improved by the optimization of media composition, especially the carbon and nitrogen sources, and physical conditions such as pH, temperature, and agitation (Lynd *et al*. 2002).

The industrial usefulness of cellulase enzyme can be improved by exploiting its functional efficiency under extreme conditions (Ladeira *et al.* 2015). Compared to mesophilic environment, microbes in thermophilic environment such as during composting could be a potential source of cellulase enzyme with desirable functional properties for industrial application.

We have isolated a novel thermophilic bacterium identified as *Bacillus licheniformis* 2D55. This bacterium utilizes microcrystalline cellulose and different agro-wastes for cellulase production. The present study aimed at optimizing the growth medium and physiological conditions for cellulase production. The crude enzyme was characterized and further investigated for enzymatic saccharification of cellulosic biomass.

EXPERIMENTAL

Materials

Bacterium and inoculum preparation

Bacillus licheniformis 2D55 (KT799651), a thermophilic bacteria, was isolated from the co-composting process of oil palm empty fruit bunch (OPEFB) with chicken manure. The isolate was stored in 20% glycerol at -80 °C. The bacterial stock was cultured aerobically for 24 h at 50 °C and 180 rpm in a shaking incubator. The cultures were grown to log phase ($OD_{600\ nm}$ = 1.0) prior to inoculation.

Substrate preparation and pre-treatment

Rice husk was obtained from Bernass Bhd, a rice processing factory in Sekinchan, Selangor, Malaysia, while sugarcane bagasse was obtained from a local sugarcane drink extractor in Serdang, Malaysia. Both substrates were washed thoroughly with tap water and dried at 60 °C for 24 h. Both substrates were then milled into 0.25-mm particles using a grinder (Retsch SM 200, Rostfrei, Hann, Germany) and stored at 4 °C until use. Rice husk was pre-treated by soaking in 2% NaOH for 2 h at a solid liquid ratio of 1:10, followed by autoclaving at 121 °C for 10 min. It was then washed several times with tap water to a neutral pH, oven-dried at 60 °C, and stored at 4 °C for future use.

Methods

Medium formulation

The basal medium used in this study contained the following: 1.0 g/L KH_2PO_4, 1.145 g/L K_2HPO_4, 0.5 g/L $MgSO_4.7H_2O$, 5.0 g/L NH_4SO_4, 0.05 g/L $CaCl_2.2H_2O$, and 1 mL Nitsch's trace element solution (2.2 g $MnSO_4$, 0.5 g $ZnSO_4$, 0.5 g H_3BO_3, 0.016 g $CuSO_4$, 0.025 g Na_2MoO_4, and 0.046 g $CoCl_2$) (Rastogi *et al*. 2009). The pH of the medium was corrected to pH 7. A mixture of untreated bagasse and NaOH pre-treated rice husk (ratio 1:1) at 10 g/L was added as a carbon source for fermentation.

Fermentation

All fermentations were carried out in a 100-mL flask containing 50 mL of basal medium sterilized at 121 °C for 15 min. A 5% inoculum of bacterial cells was added into the medium, which was incubated at 50 °C and 180 rpm for 18 h. The fermentation broth was centrifuged at 10,000 × *g* for 10 min. The supernatant was withdrawn and used as a crude enzyme for determining cellulase activities.

Optimization of physicochemical and nutritional parameters for cellulase production

The cellulase production by *B. licheniformis* 2D55 was optimized using a one factor at a time (OFAT) approach. The effects of various factors such as incubation temperature (35 to 65 °C), pH (2.0 to 5.0) using glycine-HCl and citrate buffers, and carbon source concentration (1 to 8%) were tested. The effects of inorganic nitrogen (5 g/L) and organic nitrogen sources at concentrations of 5.86 g/L peptone, 17.8 g/L chicken manure, and 5 g/L of yeast extract, meat extract, soytone, and tryptone were also examined. The concentration of nitrogen sources used in this study was equivalent to 1 g/L elemental nitrogen, which was determined using the Folin-Ciocalteu reagent method (Bailey 1967). The effects of Mg $(SO4)_2$, $CaCl_2$, surfactants, and inoculum concentration were also determined. The kinetics of cell growth on cellulase production were studied at the

optimized medium. Aliquots were withdrawn at 6 h interval and cell growth were estimated by spread plate method (log_{10}CFU/ml).

Analytical methods

Cellulase activity was determined using the method of Wood and Bhat (1988). The activity of carboxymethyl cellulase (CMCase) was determined by measuring the release of reducing sugars from CMC. The crude enzyme (0.5 mL) was reacted with 0.5 mL of 1% CMC in 0.05 M phosphate buffer (pH 7) and incubated at 50 °C for 30 min. Filter paperase (FPase) was determined by measuring the reducing sugar released from the filter paper. In this reaction, 0.5 mL crude enzyme was mixed with a 1 × 6 cm (Whatman No. 1) filter paper immersed in 1.5 mL of phosphate buffer pH 7 and incubated at 50 °C for 1 h. The reducing sugars liberated were measured using the dinitrosalicylic acid (DNS) method (Miller 1959). DNS was supplied by R & M chemicals (Essex, UK). The reactions were stopped by the addition of 3 mL of DNS. One unit of enzyme activity was determined as the enzyme content required to liberate 1 µmol of reducing sugar/min under assay conditions. For the beta-glucosidase assay, p-nitrophenyl liberated from p-nitrophenyl-beta-D-glucopyranoside was determined at 400 nm. The reaction mixture was incubated at 50 °C for 30 min. One unit of beta-glucosidase activity was defined as the enzyme content that liberated 1 µmol p-nitrophenol per min under assay conditions.

Characterization of crude cellulases of Bacillus licheniformis 2D55

The optimum pH for CMCase and FPase activity was studied over a wide pH range 3.0 to 11.0 using different assay buffers. The assay was conducted in a 2.0 mL reaction volume containing 0.5 mL of appropriately diluted enzyme and 1.5 mL of 1% CMC in 50 mM sodium citrate (pH 3.0 to 6.0), sodium phosphate (pH 6.0 to 8.0), tris-HCl (pH 8.0 to 9.0), glycine-NaOH (pH 9.0 to 10.5). The relative enzyme activity at other pHs was expressed as a percentage of activity at pH optimum, while the optimum pH was used to determine the optimum temperature of assay carried out at temperature range 40 to 100 °C under the above assay conditions. Thermal stability of CMCase and FPase of crude enzyme was carried out by determining the enzyme activity remaining after inoculating the enzyme at temperature range 40 to 100 °C over a period of 240 h. The residual activity was determined at optimum pH and temperature using DNS method described above and was expressed as a percentage of the initial activity at 100%.

Enzymatic saccharification

Rice husk and sugar cane bagasse were pretreated with 2% NaOH as earlier described above. Enzymatic saccharification was carried out according to (Remli *et al.* 2013) with some modification. Briefly, crude cellulase 10 FPU in 50 mM phosphate buffer (pH 7.5) was inoculated with 5% w/v pretreated rice husk. Sodium azide 0.02% w/v was added to inhibit microbial contamination. Incubation was carried out at 60 °C under shaking at 180 rpm. Samples (1.0 mL) were withdrawn after 24 h, then centrifuged at 10,000 rpm for 10 min. The total reducing sugar was assayed from the supernatant using DNS method as stated above.

Statistical analysis

The values shown are the means of triplicates ± standard deviation. Data were analyzed by one way analysis of variance (ANOVA). Duncan's multiple range was used

to compare the means among treatment groups. Differences of $P < 0.05$ were considered significant using SAS software package version 9.4 (SAS Institute Inc., Cary, NC, USA).

RESULTS AND DISCUSSION

Effect of Temperature on Cellulase Production

Temperature is a critical factor affecting enzyme production. *B. licheniformis* 2D55 produced cellulases at temperatures ranging from 35 °C to 65 °C (Fig. 1). Generally, cellulase activity increases with increasing temperature. The activity of CMCase was higher than that of FPase and β-glucosidase at all temperatures. Cellulase production peaked at 60 °C with CMCase at 0.63 U/mL, FPase at 0.42 U/mL, and β-glucosidase at 0.006 U/mL. However, cellulase production was negatively affected at 65 °C. This result could be due to alterations in the physical properties of the cell membrane or the repression of some proteins in the metabolic pathways (Sethi *et al*. 2013). Sadhu *et al.* (2014) reported that *Bacillus* sp. produced high amounts of cellulase at an optimum temperature of 50 °C. Enzyme production at higher temperatures was better for thermophilic *B. licheniformis* MVS1 (Acharya and Chaudhary 2012). Rastogi *et al.* (2010) also pointed out that producing cellulase at high temperatures leads to better substrate solubility and low viscosity. In sum, *B. licheniformis* 2D55 was able to tolerate a wide range of temperatures (45 to 65 °C) for better cellulase production**.**

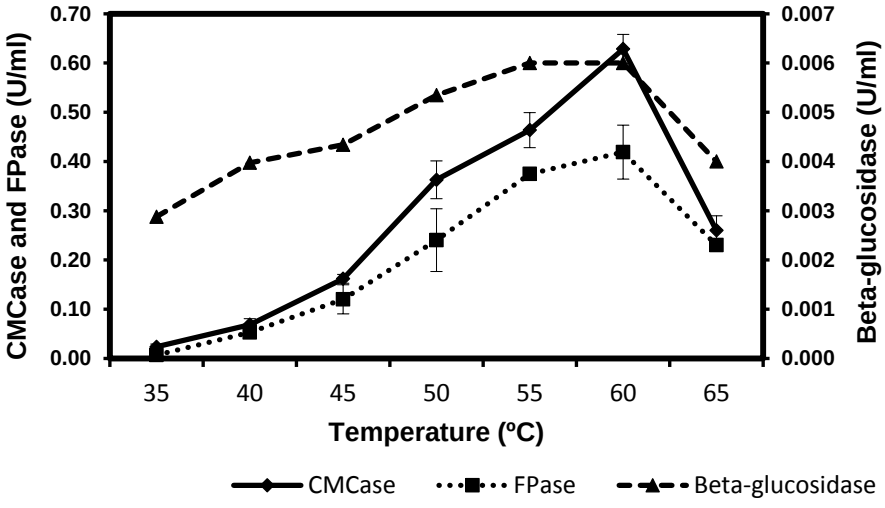

Fig. 1. Effect of temperature on cellulase production by *B. licheniformis* 2D55

Effect of pH on Cellulase Production

The influence of pH on cellulase production by *B. licheniformis* 2D55 was studied by varying the pH between 2.0 and 5.0. Results showed that cellulase production was favoured in acidic pH, with the most cellulase produced at pH 3.5 (Fig. 2), where the CMCase, FPase, and beta-glucosidase yields were 3.25 U/mL, 1.07 U/mL, and 0.011 U/mL, respectively. Cellulase production was drastically reduced at pH 4 and above. It has been reported that acidic pH induces the production of cellulases in thermophilic cellulolytic *Bacillus* sp. (Patel *et al.* 2005). The high tolerance of this strain to acidic pH suggests that it has the potential for use in the harsh environments encountered during industrial fermentation.

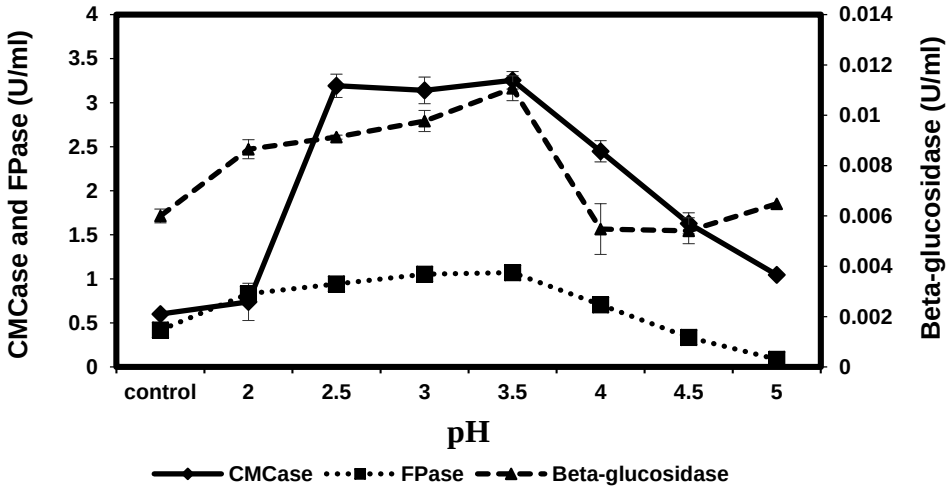

Fig. 2. Effect of pH on the cellulase production of *B. licheniformis* 2D55

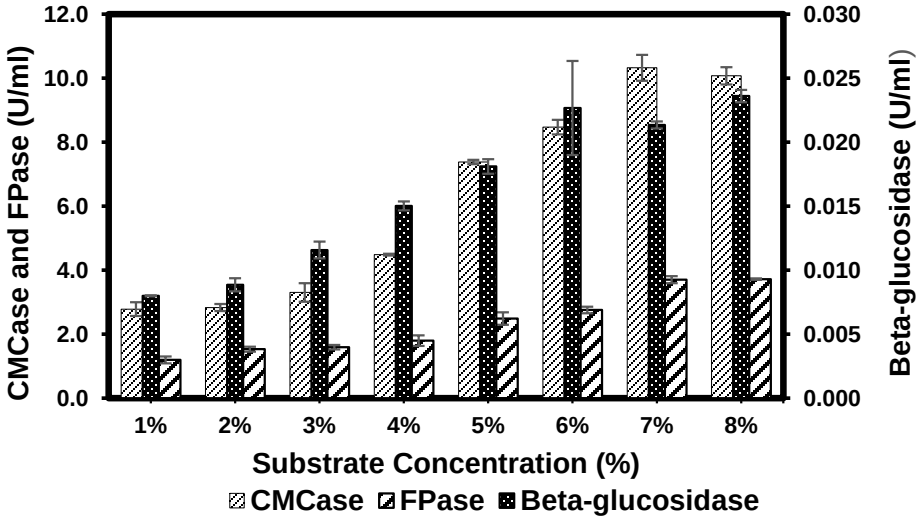

Fig. 3. Effect of substrate concentration on cellulase production by *B. licheniformis* 2D55. The control was 1 % substrate concentration. The values shown are the means of triplicates ± standard deviation.

Effect of Substrate Concentration on Cellulase Production

Figure 3 shows the effects of different substrate concentrations on cellulase production. The maximum CMCase (10.3 U/mL) was obtained at 7% concentration, while FPase and β-glucosidase increased to 3.7 U/mL and 0.024 U/mL, respectively. While 42 g/L Avicel® concentration is utilized by *Geobacillus stearothermophilus* for cellulase production, 5% substrate concentration is optimal for *Pseudomanas aeruginosa* (Abdel-Fattah *et al.* 2007; Agarwal *et al*. 2014). A substrate concentration of 8% is optimal for cellulase production by *Bacillus* sp. at 50 °C (Sadhu *et al*. 2014). A low substrate concentration increased the yield and reaction rate of hydrolysis; however, high substrate concentrations caused substrate inhibition and resulted in low enzyme formation (Liu and Yang 2007). In contrast, cellulase production by *B. licheniformis* 2D55 was favoured at high substrate concentrations. High substrate concentration might stimulate promoters controlling metabolic genes in *B. licheniformis* 2D55.

Effect of Inorganic Nitrogen Sources on Cellulase Production

Cellulase production by *B. licheniformis* 2D55 was evaluated with different inorganic nitrogen sources (Fig. 4). CMCase was enhanced at 12.7 U/mL in the presence of urea, while the rest of the substrates did not noticeably differ from the control. FPase activity was optimal (7.1 U/mL) when ammonium nitrate was a nitrogen source. Ammonium chloride, urea, and potassium nitrate improved FPase activity compared with ammonium sulphate. This result differed from a report showing that *B. licheniformis* KIBGE-IB2 cellulase activity was not enhanced by inorganic nitrogen (Karim *et al.* 2015). In *Bacillus* sp., cellulase activity is higher with ammonium chloride (Sadhu *et al*. 2013). Hence, there are species-specific preferences in utilizing inorganic compounds for cellulase production.

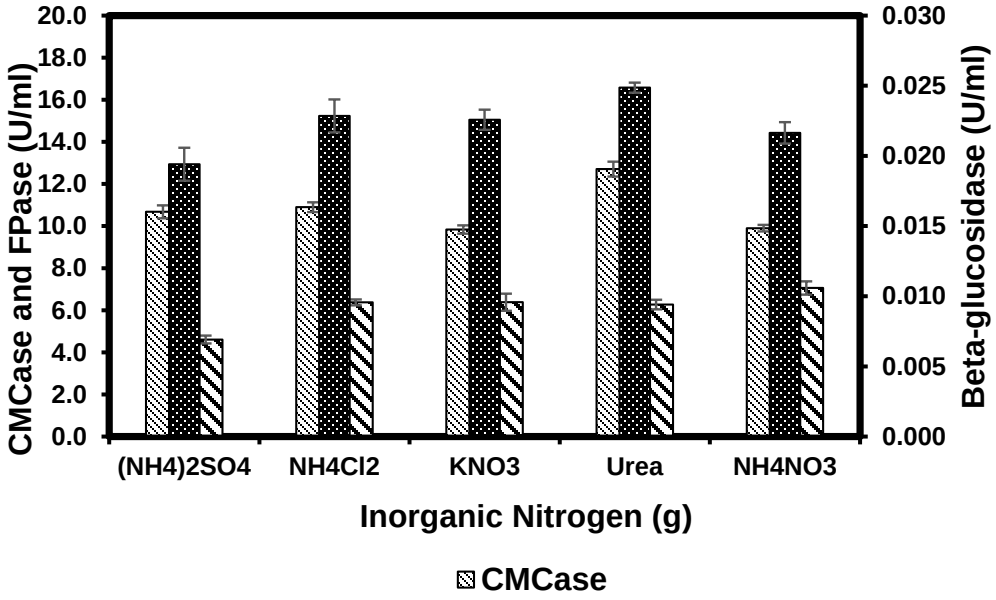

Fig. 4. Effect of inorganic nitrogen sources on cellulase production by *B. licheniformis* 2D55. $(NH4)_2SO4$ was used as the control.

Effect of Organic Nitrogen Sources on Cellulase Production

The addition of different organic nitrogen sources had varying effects on cellulase production (Fig. 5). The optimum levels of CMCase at 18.6 U/mL and FPase at 8.7 U/mL were obtained when peptone was used. Other organic nitrogen sources did not differ greatly from the control. This result indicated that peptone was the most important factor affecting cellulase expression by *B. licheniformis* 2D55.

Cellulase production significantly increased with increasing peptone (Table 1); 11.0 g/L peptone yielded 22.77 U/mL, 8.74 U/mL, and 0.048 U/mL of CMCase, FPase, and β-glucosidase, respectively. However, there was no significant difference in FPase activities at all concentrations. β-glucosidase showed low activity in all of the experiments. Karim *et al.* (2015) also reported that cellulase production by *B. licheniformis* KIBGE-IB12 increased with increasing peptone and yeast extract concentration. Cellulase activity increased when peptone (20 g/L) was used as a nitrogen source by *Bacillus sp*. (Das *et al.* 2010).

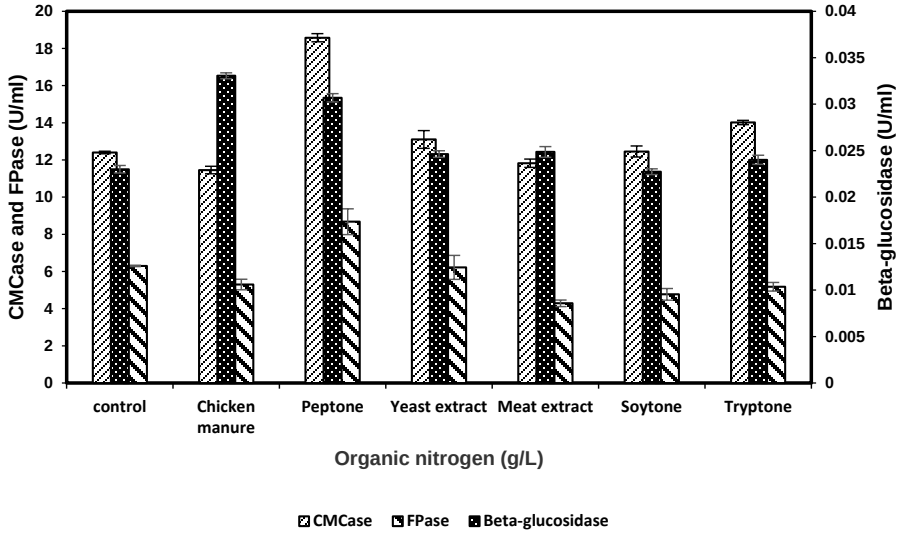

Fig. 5. Effect of organic nitrogen sources on cellulase production by *B. licheniformis* 2D55. A flask containing only urea was used as the control.

Table 1. Effect of Peptone Concentration on Cellulase Production

Peptone Concentration (g/L)	CMCase (U/mL)	FPase (U/mL)	β-glucosidase (U/mL)
5.9	17.63 ± 0.93^{c}	8.26 ± 0.20^{a}	0.031 ± 0.001^{c}
8.5	20.57 ± 0.23^{b}	8.26 ± 0.05^{a}	0.037 ± 0.001^{b}
11.0	22.77 ± 0.33^{a}	8.28 ± 0.12^{a}	0.048 ± 0.002^{a}
13.6	20.11 ± 0.42^{b}	8.74 ± 0.34^{a}	0.048 ± 0.002^{a}

The values shown are the means of triplicates ± standard deviation. Mean values in the same column with different superscripts are significantly different ($P < 0.05$).

Effect of $Mg(SO_4)_2$ and $CaCl_2$ Concentrations on Cellulase Production

The influence of different mineral salts on cellulase production was studied by adding different concentrations of magnesium sulphate and calcium chloride to the basal medium. Magnesium sulphate at 0.4 g/L increased the CMCase, FPase, and β-glucosidase activity to 24.46, 10.09, and 0.05 U/mL, respectively (Table 2).

Table 2. Effect of $Mg(SO_4)_2$ and $CaCl_2$ Concentrations on Cellulase Production

Mineral Source	Concentration (g/L)	CMCase (U/mL)	FPase (U/mL)	β-glucosidase (U/mL)
$Mg(SO4)_2$	0.2	21.43 ± 0.25^{b}	10.59 ± 0.02^{a}	0.04 ± 0.004^{b}
	0.4	24.46 ± 0.22^{a}	11.04 ± 0.23^{ab}	0.05 ± 0.002^{a}
	0.6	23.43 ± 0.22^{a}	10.38 ± 0.49^{ab}	0.05 ± 0.009^{a}
	0.8	20.10 ± 0.75^{c}	10.18 ± 0.45^{b}	0.04 ± 0.004^{b}
	1.0	20.51 ± 0.3b^{c}	10.12 ± 0.04^{b}	0.05 ± 0.004^{b}
$CaCl_2$	0.03	25.1± 0.63^{a}	9.5 ± 0.08^{a}	0.05^{a}
	0.05	22.7 ± 0.14^{b}	10.0 ± 0.09^{a}	0.05^{a}
	0.07	22.3 ± 0.61^{bc}	9.0 ± 0.65^{b}	0.05^{a}
	0.09	21.5 ± 0.24^{bc}	9.0 ± 0.36^{b}	0.05^{a}
	0.11	21.3 ± 0.46^{c}	9.0 ± 0.19^{b}	0.04^{a}

The values shown are the means of triplicates ± standard deviation. Mean values in the same column with different superscripts are significantly different ($P < 0.05$).

Increased calcium chloride did not improve enzyme activity. Therefore, the addition of magnesium sulphate and calcium chloride at lower concentrations of 0.4 and 0.03 g/L, respectively, were sufficient. The incorporation of optimal salts into the culture medium increases the utilization of substrate by bacteria (Gao *et al*. 2010). The dissociation of salts into ions in an aqueous solution affects the circulation of water molecules through the cell membrane as well as the catalytic stability of enzymes (Karim *et al*. 2015).

Effect of Additives on Cellulase Production

The addition of different surfactants at 0.2% concentration (Fig. 6) had varying effects on cellulase production. Of the various additives used to enhance cellulase production, tween 80 showed a better stimulatory effect on CMCase (27.1 U/mL). Tween 20, SDS, palm olein, and olive oil were similar to the control and showed no significant effect on FPase activities. However, EDTA had a negative effect on CMCase and β-glucosidase activities. The stimulatory effect of tween 80 on CMCase activities may be due to its positive influence on cell membrane permeability.

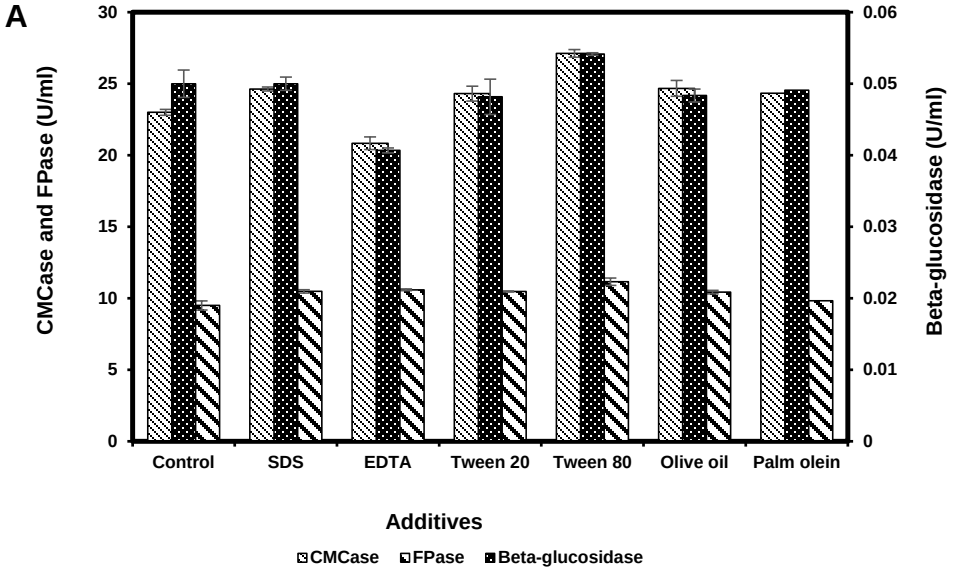

Fig. 6. Effect of additives on cellulase production by *Bacillus licheniformis* 2D55

Effect of Inoculum Size and Growth on Cellulase Production

Inoculum optimization is necessary to uphold the equilibrium between the increasing biomass and the available nutrients for maximal enzyme yield (Kumar *et al.* 2014). With respect to inoculum size (1.0 to 10.0% v/v), the maximum CMCase (29.4 U/mL), FPase (12.9 U/mL), and beta-glucosidase (0.06 U/mL) values were found at 3% v/v (Fig. 7a). This result was similar to another report including a maximum inoculum size of 3% *B. amyloliquefaciens* C23 (Abou-Taleb *et al.* 2009). Das *et al.* (2010) reported that the best inoculum concentration for *Bacillus* sp. grown on CMC was 7%. The results for cell growth (Fig. 7b) revealed that both cell growth and cellulase production increased in the log phase and reached maximum at 18 h and started to decrease gradually after 30 h. The maximum CMCase and FPase extended between 18 to 24 h when the culture reached a stationary phase. Ladiera *et al.* (2015) reported maximum production of CMCase in thermophilic *Bacillus* sp. SMIA-2 at 168th h when the culture reached the death phase.

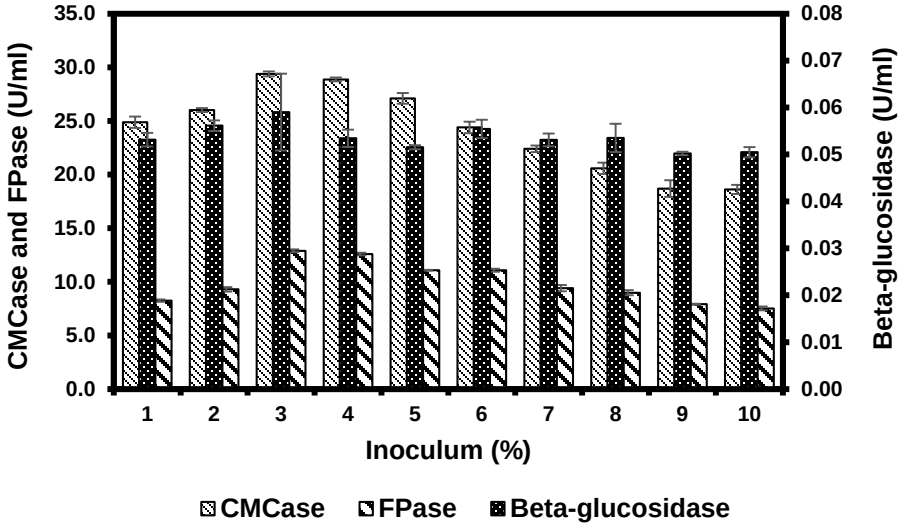

Fig. 7a. Effect of inoculum size on cellulase production by *Bacillus licheniformis* 2D55

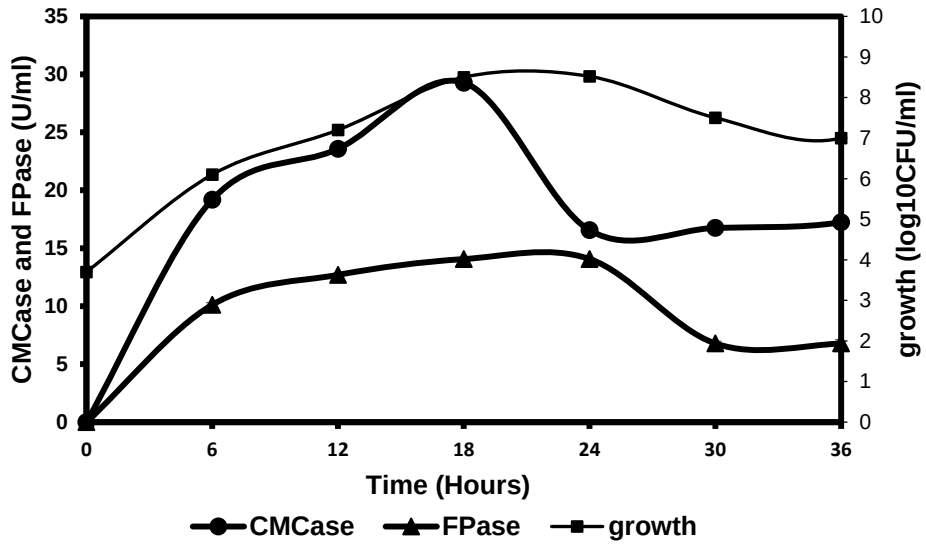

Fig. 7b. Growth profile and cellulase production by *B. licheniformis* 2D55 after 36 h of fermentation

Comparison of Cellulase Production at Optimum Conditions

The optimization of cellulase production has been studied in bacterial and fungal strains isolated from different environments (Table 3). Assareh *et al.* (2012) produced more CMCase (143.50 U/mL) from *Geobacillus sp.* than that reported in this study. Notably, different carbon substrates were utilized for fermentation, which influenced cellulase activities. *B. amyloliquefaciens* UNPVD-22 hydrolyzed wheat bran with CMCase activity at 13.0 U/mL, while wheat straw hydrolysis by *B. licheniformis* MVS1 produced CMCase and FPase activities of 0.120 U/mL and 0.505 U/mL, respectively.

Table 3. Cellulase Production by Bacterial and Fungal sp. Grown on Different Carbon Sources at Optimum Conditions

Organism	Temperature (°C)	Carbon Source	CMCase (U/mL)	FPase (U/mL)	β-glucosidase (U/mL)	References
Aspergillus terreus	40	*Groundnut shell	2.183	0.171	n.d	(Vyas *et al*. 2005)
B. alkalophilus S39	30	*CMC	2.40	1.2	3.61	(Abou-Taleb *et al*. 2009)
B. amyloliquefaciens C2	45	*CMC	2.39	1.18	3.53	(Abou-Taleb *et al*. 2009)
B. subtilis AS3	45	**Wild grass	0.57	nd	nd	(Deka *et al*. 2013)
B. licheniformis MVS1	50	*Wheat straw	0.120	0.505	nd	(Acharya and Chaudhary 2012)
B. subtilis BY-2	37	*Corn flour	3.56	nd	nd	(Yang *et al*. 2014)
Cellulomonas	40	*Coir powder	0.0336	nd	nd	(Immanuel *et al*. 2006)
B. subtilis	35	**CMC	22.9	nd	nd	(Asha and Sakthivel 2014)
B. amyloliquefaciens SS35	40	*CMC	0.693	nd	nd	(Singh *et al.* 2014)
B. subtilis	40	*Coconut cake	0.9	nd	nd	(Sethi *et al*. 2013)
B. amyloliquefaciens UNPDV-22	42.2	**Wheat bran	13	nd	nd	(Vasudeo and Lew 2011)
Geobacillus sp T1	50	*Barley straw	143.50	nd	nd	(Assareh *et al*. 2012)
B. licheniformis 2D55	60	*Sugarcane bagasse and rice husk	29.4	12.9	0.06	This study

*, One variable at a time; **, Plackett-Burman and CCD; nd, not determined

The optimum conditions for cellulase production by *B. licheniformis* 2D55 (29.4 U/mL CMCase, 12.9 U/mL FPase, and 0.06 U/mL β-glucosidase) were 60 °C, pH 3.5, with 7% mixtures of pretreated rice husk and untreated sugarcane bagasse, 5 g/L urea, 11 g/L peptone, 0.4 g/L $MgSO_4$, 0.03 g/L $CaCl_2$, 0.2% tween 80, and 3% inoculum.

Characterization of Cellulase Enzyme

Effect of pH and temperature on cellulase activity

The effect of pH on CMCase and FPase activity of the enzyme was examined from pH 3.0 to 10.5 (Fig 8a). CMCase and FPase exhibited optimum activity in sodium phosphate buffer pH 7.5 and 6.0, respectively. It is interesting to note that high activity was recorded on the either extreme side of the optimum pH, which shows that the enzyme has a characteristically broad range of pH activity. It was also evident that CMCase and FPase still retained more than 70% of their optimum activity over the pH range of 3.5 to 10.

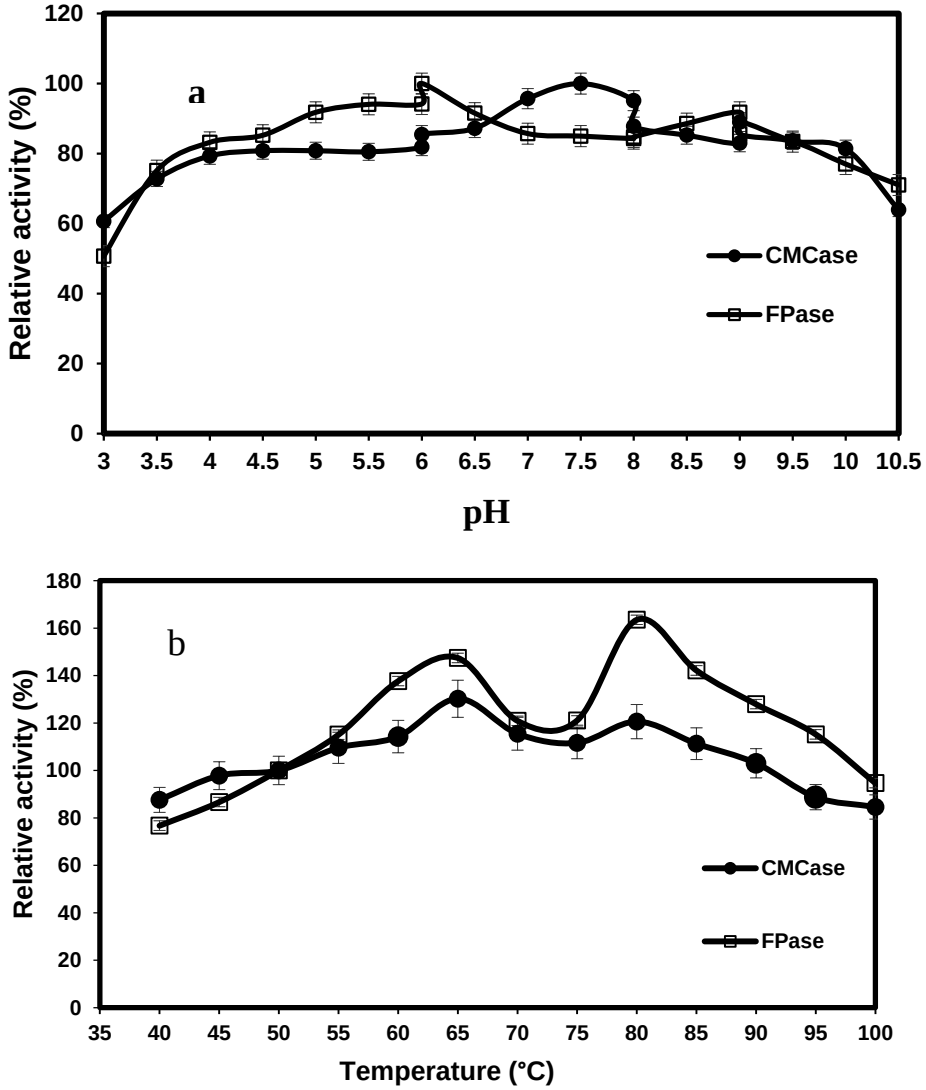

Fig. 8. Effect of pH (a) and temperature (b) on CMCase and FPase activity produced from *B. licheniformis 2D55.* Error bars smaller than the symbols are not shown.

The key characteristic of cellulase secreted by most *Bacillus sp* is its activity at broad pH range (Mawadza *et al.* 2000). Slightly similar optimum CMCase activity was found at pH 7 for *B. vallismortis* RG-07 (Gaur and Tiwari 2015), *Bacillus sp*. (Sadhu *et al.* 2013). In the case of *B. licheniformis* JK7 pH 5.0 has been reported for optimum activity (Seo *et al.* 2013) as well as *Bacillus* and *Geobacillus sp*. (Rastogi *et al.* 2010). Not much studies have been reported on the influence of pH on FPase activity, so detailed comparison with previous studies was not possible. However, avicellase activity from *Bacillus sp*. SMIA-2 exhibited optimum pH at 7.5 (Ladeira *et al.* 2015).

The crude enzyme was recorded to show activity over a broad range of temperature (40 to 100 °C) with optimum activity of CMCase and FPase first at 65 °C and then 80 °C (Fig 8b), thus suggesting that the enzyme has two optimum temperatures. Since the crude enzyme was produced from mixture of rice husk and sugar cane bagasse, a possible explanation for this occurrence could be that both substrates induced different set of enzymes. Different sets of enzymes are induced by different substrates (Liang *et al.* 2010). Similar results was observed for a thermophilic *Brevibacillus* JXL as different optimum temperatures were observed in crude cellulase induced by glucose and by cellobiose (Liang *et al.* 2009, 2010a). Recently, cellulase from a thermophilic *B. vallismortis* RG-07 recorded optimum activity at 65 °C, and 105 °C (Gaur and Tiwari 2015). The complete genome sequence of *B. licheniformis* ATCC 14580 also revealed that the strain has two endoglucanases belonging to two different hydrolase families, hence optimum temperatures for their activity may differ (Rey *et al.* 2004).

Effect of temperature on cellulase stability

The thermostability studies of crude enzyme revealed that CMCase activity was completely stable in a broad temperature range of 50 to 70 °C incubation over 24 h. This was followed by a linear decrease of about 18% residual activity up to 120 h at temperature 50 and 60 °C (Fig. 9a,b). However, at temperature 70 and 80 °C, about 60 and 50% residual activity up to 144 h was observed. It should be noted that the CMCase activity loss was only about 58% of its original activity over 240 h at 60 °C incubation temperature. With further increase in incubation temperature, to 90 and 100 °C, CMCase activity retained about 74% and 64% residual activity respectively, at 1 h incubation. Previous report showed that thermostable cellulase from *B. licheniformis* JK7 (Seo *et al.* 2013), retained only about 25% of its original activity when incubated at 80 °C for 1 h. Another *Bacillus* sp. isolated from deep surface of homestake gold mine exhibited a thermostable cellulase (Rastogi *et al.* 2009); however, the enzyme completely lost its activity over 1 h incubation at 90 and 100 °C.

Contrary to CMCase, FPase activity demonstrated optimum stability (100 %) only at 50 and 60 °C for about 24 h (Fig. 9c,d) which later decreased, retaining about 50% of its optimum activity after 240 h of incubation. The CMCase activity decrease to about 80% residual activity at incubation temperatures 70 and 80 °C after 24 h and retained less than 20% of its maximum activity at 192 h of incubation. The FPase activity was fairly unstable at temperatures 90 and 100 °C and lost more than 45% of its optimum activity over 6 h of incubation. *B. licheniformis* 2D55 demonstrated a highly thermostable FPase activity; however our results could not be compared with previous studies due to lack of data on thermostability studies on FPase activity from *Bacillus* sp. Hence, we report for the first time to the best of our knowledge a thermostable FPase in this study.

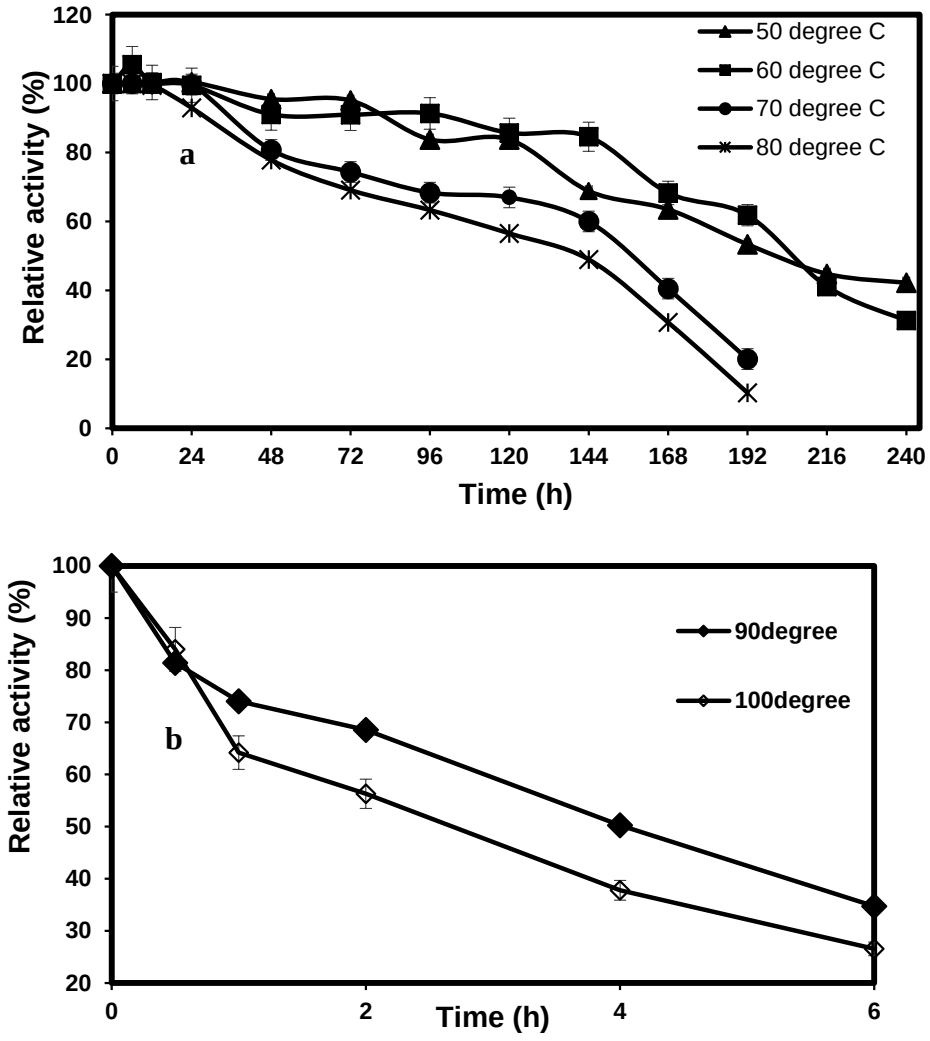

Fig. 9 a, b. Thermal stability of CMCase produced by *B. licheniformis* 2D55. Error bars smaller than the symbols are not shown.

Enzymatic saccharification for sugar production

In order to examine the effect of crude cellulase on lignocellulose degradation, sugarcane bagasse and rice husk were used as feed stocks for production of reducing reducing sugars. As shown on Table 4, the optimum reducing sugar of 17.4 g/L and 19.2 g/L was liberated from pretreated sugarcane bagasse and rice husk, respectively. Alkaline pretreatment may increase surface area and reduce crystallinity in sugarcane bagasse than rice husk. This results concord with those of Akhtar *et al.* (2001), who reported that 2% NaOH was more effective for enzymatic saccharification of sugarcane bagasse than for rice straw by cellulases from *B. subtilis.*

The enzyme was also able to hydrolyse the untreated rice husk and sugarcane bagasse, but to a lower extent. Low saccharification rate in untreated lignocellulosic waste is attributed to the high lignin content. Hence, restricting the penetration of more enzymes into the constricted network of plant cell wall (Alrumman, 2016).

A yield of reducing sugars liberated from 1 g of dry feed stock at 0.348 and 0.301 g g^{-1} (dry substrate) were observed for pretreated sugarcane bagasse and rice husk, respectively. There are few reports on reducing sugar production using cellulase from bacteria. A yield of reducing sugar of 0.214 and 0.157 g g^{-1} (dry substrate) was obtained from saccharification of wheat straw and rice straw respectively, using crude cellulase from *Fomitopsis* sp. RCK2010 (Deswal *et al.* 2011). Yu and Li (2015) recorded a yield of reducing sugar at 0.678 and 0.502 g g^{-1} (dry substrate) from alkaline pretreated corn stover and rice straw using crude cellulase (100 U mL^{-1}) from *Gracibacillus* sp. SK1. Optimization studies on enzymatic saccharification could improve the hydrolysis yield of sugars produced by the enzyme.

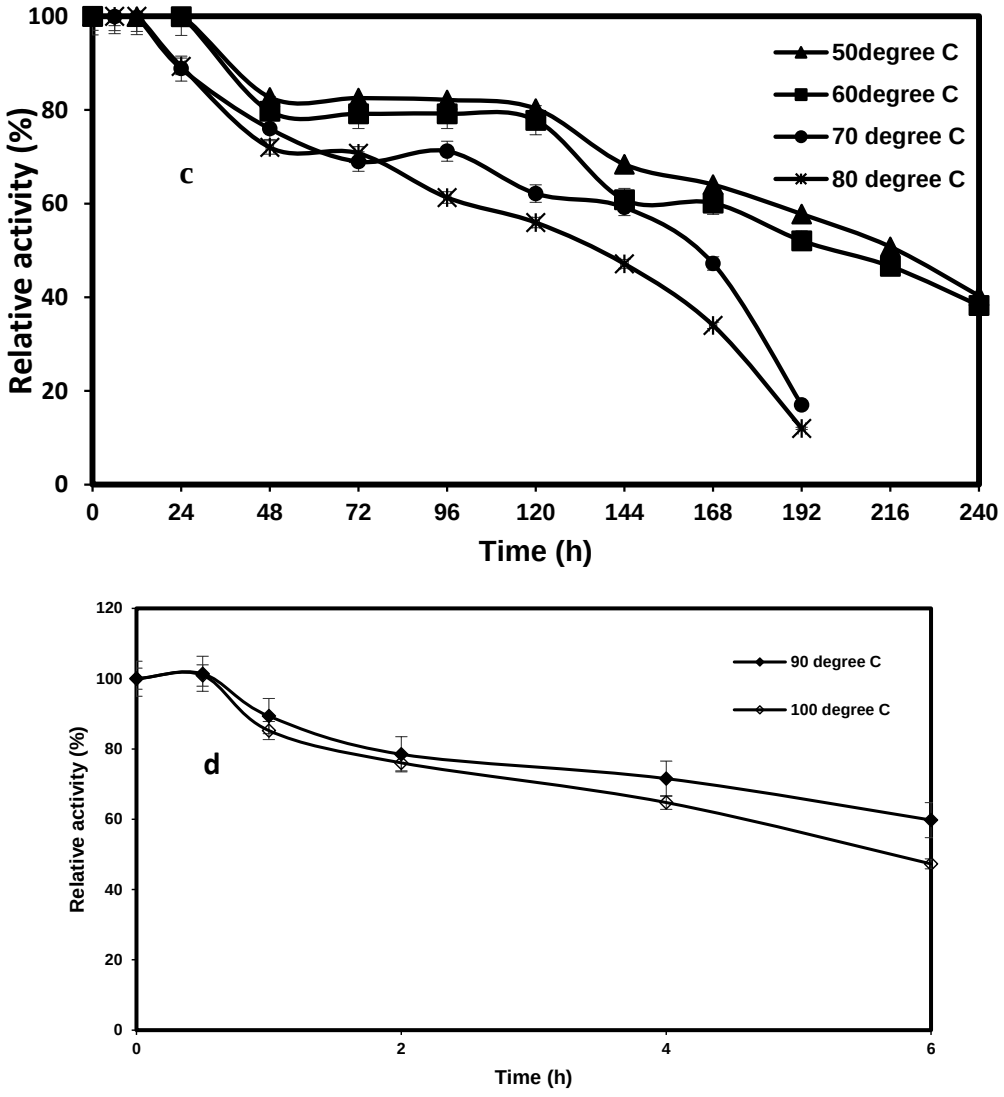

Fig. 9 c, d. Thermal stability of FPase produced by *B. licheniformis* 2D55. Error bars smaller than the symbols are not shown.

Table 4. Reducing Sugar Production from Biomass Hydrolysis using Crude Cellulase from *Bacillus licheniformis* 2D55

Biomass (g)	Reducing sugar (g/L)	Yield (g/g dry substrate)
Untreated sugarcane bagasse	4.1 ± 0.19	0.078
Untreated rice husk	1.5 ± 0.18	0.03
Pretreated sugarcane bagasse	17.4 ± 0.04	0.348
Pretreated rice husk	19.2 ± 0.1	0.301

CONCLUSIONS

1. Cellulase production by thermocellulolytic *B. licheniformis* 2D55 was enhanced by the optimization of nutritional and cultural conditions with CMCase production at 46.6-fold, FPase 30.7-fold, and β-glucosidase production at 10-fold
2. The cellulase production at high temperature of 60 °C makes *B. licheniformis* 2D55 potentially effective for the production of thermostable enzymes for sugar production from agro-waste saccharification.
3. CMCase and FPase activity exhibited broad pH and temperature range of (3 to 10.5) and (40-100 °C). With excellent stability at high temperatures, other possible industrial applications in processes that run at high temperature and uncontrolled pH could be exploited
4. Cellulases from *B. licheniformis* 2D55 were effective in hydrolysis of both pretreated sugarcane bagasse and rice husk. Hence, the enzyme can be applied for bioconversion of agricultural residues to bio-sugars.

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