Biotechnological Potential of Bacterial Endoglucanase from Marine and Hypersaline Environments in Saccharification of Lignocellulosic Biomass Pre-Treated with Alkali or Ionic Liquid

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Research Article

Keywords: extracellular enzymes, endoglucanase, halophiles, Pretreatment, ionic liquids, lignocellulosic biofuel

Posted Date: June 2nd, 2021

DOI: https://doi.org/10.21203/rs.3.rs-552634/v1

Abstract

In second-generation biofuel production, the recalcitrant plant biomass requires pretreatment prior to enzymatic hydrolysis. Pretreatment with alkali or ionic liquids (IL) such as 1-butyl 3-methyl immidazolium chloride ([Bmim][Cl]), are efficient but the residual salt in former, or IL in later process inhibits downstream enzymatic saccharification and thus require extensive washing. Recent studies have established IL tolerance by moderate halophilic bacteria being contributed by their general salt adaptation strategies. Objective of the present study is to examine whether the same holds true for their extracellular enzymes, and eventually select a few for future exploitation. In this direction, ten distinct endoglucanase positive (≥3 mm halo zone in congo-red cellulolytic assay) colonies were picked each from decomposed wood material of the hypersaline Sambhar Lake (SLW), in Rajasthan; and Bichitrapur (BPW) mangrove in Orissa. SLW and BPW samples had total salinities of 21.54% and 2.18%; and their isolates had optimum NaCl requirement of 10-15% and 1-5% respectively. The extracellular endoglucanase of SLW isolates were active in 5-25% NaCl but those from BPW remain active in only up to 5% NaCl. Interestingly, SLW endoglucanases also performed better in 10% and some even in 30% (v/v)[Bmim][Cl]. Endoglucanase secreted by two SLW isolates, identified by their 16S rRNA gene sequence as *Salipaludibacillus* sp. were effectively used for in situ enzymatic hydrolysis of both alkali and [Bmim][Cl] pretreated rice straw. However, endoglucanase from BPW isolate *Salinicola* sp. could hydrolyze seawater washed alkali-pretreated biomass thereby expanding their industrial applicability in coastal areas.

Introduction

Increasing worldwide demand of fossil fuels and its escalating price has led to environmental and economic concerns. Consequently, biofuel, particularly from lignocellulosic biomass has received wide attention, since lignocellulose is a renewable, abundant and inexpensive resource (Brandt et al. 2013). In lignocellulosic biofuel production process, one key upstream step is enzymatic hydrolysis of substrate that produce the reducing sugars, which in subsequent downstream steps are converted to ethanol by well-established microbial action (Souza 2013). For complete enzymatic hydrolysis, synergistic activity of endoglucanase (EC 3.2.1.4.), exoglucanase (EC 3.2.1.91.) and β-glucosidase (E.C. 3.2.1.21) is required (Ilmberger et al. 2011). The bottleneck in the entire enzymatic hydrolysis process is recalcitrant nature of plant biomass (Kumar et al. 2009). Lignocellulose consist of crystalline cellulose entangled with lignin and hemicellulose, forms complex matrix, which makes it mostly inaccessible for enzymes in native state (Sun et al. 2016).

Complex structure of plant biomass must be disrupted either mechanically and/or using temperature, or corrosive chemicals to make the cellulose accessible for enzymatic hydrolysis (Agbor et al. 2011; Akkharasinphonrat et al. 2017; Amnuaycheewa et al. 2017; Kumar et al. 2009; Rodiahwati and Sriariyanun 2016). An ideal pretreatment method should render lignocellulosic biomass completely susceptible to enzymatic hydrolysis by avoiding formation of inhibitory by-product. For many years, pretreatment and enzymatic hydrolysis processes have been rationalized to make biofuel production economically viable. However, both these processes have always been dealt independently, that might

have resulted in limited success in delivering feasible enzymes that could be used efficiently with pretreated biomass.

Ionic liquid (IL) pretreatment is widely considered to be a promising process because of its high efficiency to solubilize cellulose under moderate temperature (Shill et al. 2011; Sriariyanun et al. 2015; Wang et al. 2014). A remarkable feature of ILs is that it can be finely tuned by selecting appropriate combinations of cations and anions (Yang 2009). But, inhibitory effect of the residual ILs on the enzymatic activity limits bioconversion (Engel et al. 2010; Sriariyanun et al. 2015). Even most of the commercial cellulases are inhibited or have limited activity in residual ILs and thus require repeated washing (Zhang et al. 2011). Many strategies have been developed to mitigate inhibitory effect of IL and maintain its efficiency, which includes modifications of enzyme properties to improve tolerance to ILs and searching for a novel IL tolerant cellulase (Park et al. 2012; Sriariyanun et al. 2016; Xu et al. 2014).

It has been reported that denaturing effect of ILs and salts on enzymes are similar (Raddadi et al. 2013). Thus, due to the adaptation to high saline environments, extracellular enzymes from halophilic bacteria are hypothesized to be stable in IL (Ilmberger et al. 2011). Many studies have reported the correlation of salt and IL tolerance in cellulase producing bacteria. Recently, there is a report about salt stable cellulase, which retained its activity up 30% (v/v) ILs (Ilmberger and Streit 2010; Shivanand et al. 2013). Cellulase of *Paenibacillus tarimensis*, isolated from Tunisian salt lake, exhibited IL tolerance potency (Raddadi et al. 2013).

On the other hand, the unique swampy, partially anaerobic and saline condition makes mangroves promising source for exploring microbial resources (Gao et al. 2010; Jiang et al. 2006). Cellulolytic microbes are important members in such environment because of the presence of fallen leaves of mangrove trees (Gao et al. 2014). Thus, mangrove forests provide an opportunity to identify microorganisms with novel cellulase enzyme. Despite this interest, no one to the best of our knowledge has studied the behavior of cellulase in presence of IL from mangrove environment. Therefore, investigation and understanding on enzymes from this environment in presence of IL is needed.

Considering the importance of IL tolerant cellulase in biofuel industry, we targeted both moderate and extreme saline environments for exploration of IL tolerant endoglucanase and characterize those with efficient capability to hydrolyze alkali or IL pretreated lignocellulosic biomass.

Materials And Methods

Chemicals and reagents

Carboxymethyl cellulose sodium salt (CMC), esculin hydrate and 1-butyl 3-methyl immidazolium chloride ([Bmim][Cl]) were purchased from Sigma-Aldrich (St. Louis, USA). 3, 5-Dinitrosalicylic acid (DNSA) was obtained from Merck (Mumbai, India) and ferric ammonium citrate was from MP Biomedicals (Illkirch, France). All other chemicals used in this study were purchased from Himedia Laboratories Pvt. Ltd. (Mumbai, India).

Sampling sites

Degraded wood samples used in this study were collected from Sambhar Salt Lake, Rajasthan, India (26° 28' 43" N and 74° 35' 40" E) and Bichitrapur, Odisha, India (21° 36' 17" N, Longitude 87° 27' 45" E). Sambhar Lake is the largest inland hypersaline lake in Rajasthan, India with sediments of varying salinity reaching up to saturation (35%) (Pal et al 2019). On the other hand, Bichitrapur is a mangrove forest located in Balasore district of Odisha and is characterized by shallow and swampy muddy environment periodically flooded by seawater. The decomposed wood samples were named SLW and BPW, with the first two alphabets standing for the respective site and the third alphabet for wood. Wood particles from Sambhar Lake had clear deposition of salts. Samples were aseptically collected into sterile 50 mL falcon tubes and brought to the laboratory in ice buckets and stored at 4°C.

Physicochemical analysis

Organic carbon and available nitrogen content in the wood samples were measured respectively using Walkley-Black titration method (Walkley and Black 1934) and distillation method utilizing FOSS Kjeltek 8400 (Bremner 1960). Salinity and pH of the samples were measured on a portable multiparameter meter fitted with probes for pH and electrical conductivity (Hanna Instruments, USA). Moisture content was determined gravimetrically after drying samples in an oven at 105°C for 10 h.

Microbial growth conditions

All growth experiments were done on heterotrophic media that contain yeast extract 5 g, Tryptone 10 g, and NaCl 15 g or 2.5 g and pH adjusted to 9.0 and 7.5 respectively for SLW and BPW. For isolation of pure cultures, wood samples (1 g) were finely degraded and dissolved in saline water, and appropriate dilutions were spread on respective heterotrophic plates supplemented with 0.2% CMC and incubated at 30 ºC for 5 and 2 days respectively for SLW and BPW samples.

Enzyme assays

Plate-based endoglucanase and β-glucosidase activity screening was done respectively by congo red (Teather and Wood 1982) and esculin hydrate assay (Eberhart et al. 1964). In the former assay, colonies with halo-zone were considered as endoglucanase positive, while a black zone formation confirms β-glucosidase positive activity. Endoglucanase activity was further quantified by DNSA method (Ghose 1987). Briefly, 200 µL crude enzyme and 400 µL 1% CMC were dissolved in Tris-Cl buffer (0.05 M) and incubated at 30 ºC for 30 mins. Then 750 µL DNSA reagent was added and boiled for 10 min to stop the reaction. After cooling, absorbance at 540 nm was taken. Amount of reducing end released was measured from standard curve of glucose. One unit of endoglucanase activity was defined as the amount of enzyme required to release one micromole of reducing sugars from the substrate per minute (Gupta et al. 2011). For preparation of crude enzyme, 20 mL medium were inoculated with 1% overnight grown culture and incubated until OD_{600} reached to 0.8-1.0. Culture was centrifuged at 12,000 x g for 10 min and resultant supernatant was used as crude enzyme. In no enzyme control crude enzyme was replaced by same volume of fresh medium.

In situ saccharification

Rice straw (*Oryza sativa* L.), was collected from a local farm. It was washed 3–4 times with tap water, and then cut to 1–2cm. Chopped rice straw was air-dried and processed for alkali pretreatment as described earlier. In brief, 0.5 g rice straw was mixed with 4 mL 2% NaOH solution (i.e., solid to liquid ratio of 1:8) and incubated at 85 ºC for 1 h (Fig. S2) (Jeya et al. 2009). Sample was mixed by inverting tube every 15 min and after incubation solid residues were collected by filtration. Pretreated rice straw was washed six times with equal volume (4 mL) of different solvents. These include: (1) water (Wash [water; 6 times]); (2) seawater (Wash [seawater; 6 times]); (3) in another set [Alkali residual], entire solution after pretreatment was completely drained out and Tris-Cl buffer was added to rice straw to adjust the volume. (4) Fourth set contains rice straw from set (1) [i.e., six time water washed] but was incubated with fresh media instead of culture supernatant (no enzyme control). In another set, (5) during pretreatment rice straw was incubated with water alone (no pretreatment control) which was further used for enzymatic hydrolysis.

For ionic liquid pretreatment, 0.2 g air chopped, and dried rice straw was mixed with 30% aqueous solution of [Bmim][Cl] and incubated at 85 ºC for 20 h (Fig. S3) (Mesbah and Wiegel 2017). Incubations in different sets were performed as follows: (1) The entire aqueous solution of ionic liquid was completely drained out after pretreatment and volume was adjusted by adding Tris-Cl buffer, (Residual [Bmim][Cl]). (2) Final ionic liquid concentration of this set was adjusted to 5% [Bmim][Cl], and (3) third set include pretreated rice straw washed six times with 1 mL water and then used (Wash; water 6 times). (4) In another set, pretreated rice straw was incubated without enzyme and volume was adjusted with equivalent volume of fresh media (No enzyme control). In addition, a (6) no pretreatment control was also used with rice straw incubated similarly with water alone. Enzymatic hydrolysis was performed in triplicate in a final reaction volume of 5 mL containing 2.5 mL of crude enzyme.

Molecular identification

For molecular identification, genomic DNA of SL1, SL24 and BP6 was extracted and 16S rRNA gene was amplified with bacterial universal primers 8_F and 1492_R. PCR products were gel eluted and sequenced. Good quality sequences of SL1, SL24 and BP6 are submitted to GenBank under Accession No. MT271939, MT271940, and MT271941 respectively.

Result And Discussion

Physicochemical parameters of samples

Sambhar Lake (SLW) and Bichitrapur (BPW) wood particles had significant differences in their salinity and pH. While both values for SLW were high with 21.54% total salinity and pH 9.0, the same for BPW were 2.18% and 6.5. Organic carbon content in both wood particles was 43.25% and 41.98%, respectively. On the other hand, available nitrogen of these two samples was 0.83% and 0.59%, respectively. Thus,

although, the wood particles were collected from two drastically different saline environments, their total organic carbon and available nitrogen content were comparable.

Mangrove environment have more cellulolytic bacteria than Hypersaline Lake

To get an idea about the cellulolytic bacterial load associated with the two degraded wood samples, ten thousand colonies each were randomly picked from respective heterotrophic plates. Each colony was replica plated onto respective agar medium for screening endoglucanase and β-glucosidase activity using Congo red assay and esculin hydrate assay, respectively. Interestingly, BPW had 58% and 33% endoglucanase and β-glucosidase positive colonies respectively, as compared to only 31% and 21% for the same phenotypes in SLW (Fig. S1). Although both samples were degraded wood particles, the extreme condition in Sambhar Lake might be the reason behind low cellulolytic bacterial count in SLW. Bichitrapur, on the other hand, is a mangrove area, with moderate climatic conditions and abundant organic matter that favors microbial growth.

Endoglucanase of SLW isolates are stable and active in wide range of NaCl concentrations

Ten best performing and distinct endoglucanase positive colonies (hydrolysis zone of 3 mm or above in Congo red assay) from both SLW and BPW samples were selected for further characterization. Optimum heterotrophic growth was obtained for SLW isolates with 10–15% NaCl and pH 9.0 maintained in culture media, while BPW isolates require NaCl concentration (1–5%) and pH 7.0–8.0 at relatively lower amounts (Table S1). Thus, optimum growth conditions of all twenty isolates are in sync to their respective environmental parameters.

All twenty isolates produced endoglucanase extracellularly and activities were detected in spent media. Enzymes of SLW isolates were active in wide range of NaCl (0–25%) (Fig. 1a). On the other hand, endoglucanase of BPW isolates had optimum activity at 5% NaCl, but in higher concentrations, activity reduced drastically (Fig. 1b). Bacteria living in Sambhar Lake have to employ adaptive mechanisms and thus high salt requirements by SLW endoglucanases are justified. In a previous study by our laboratory, an endoglucanase from Sambhar Lake isolate, *Salisediminibacterium halotolerans* EN1, exhibited optimum activity at 25% NaCl (Sar et al. 2021). Another endoglucanase encoded by *Paenibacillus tarimensis*, which was isolated from an inland saline system in Tunisia, exhibited optimum activity at 29% NaCl (Raddadi et al. 2013). Endoglucanase from Yuncheng Salt Lake, China was highly active in wide range of NaCl concentration (7.5–17.5%) (Yu and Li 2015). On the other hand, endoglucanase secreted by *Bacillus agaradhaerens*, which was isolated from seawater showed optimum activity at 1.2% NaCl (Hirasawa et al. 2006). Altogether, the obtained results suggest that the optimum requirements by bacterial enzymes, particularly the extracellular ones correlate with the respective natural environments.

[Bmim][Cl] *tolerance potency correlates with NaCl tolerance potency*

Endoglucanase from all twenty isolates were assayed in presence of different concentrations of [Bmim][Cl]. Among the twenty selected isolates, only SL1, SL2, SL8, SL14 and SL24 retained more than 50% of its activity in 10% (v/v) [Bmim][Cl] (Fig. 2a) with maximum 72.9% retention by SL1. At 30% (v/v) [Bmim][Cl] concentration, SL1, SL8 and SL24 again retained a maximum of 33.89%, 38.43% and 38.88% activities respectively. SL14 and SL48 also retains 17.69% and 12.42% activity in 30% [Bmim][Cl]. Interestingly, all BPW endoglucanase lost their activity at 30% (v/v) [Bmim][Cl], and even with 10% of the IL, only BP6 and BP31, retained 49.39% and 45.64% activity, respectively (Fig. 2b). Thus, our results further support the idea of correlation of salt and ionic liquid tolerance of bacterial cellulase. A recent report by our group on Sambhar lake showed that bacteria which can grow in wide range of salt, also exhibit high ionic liquid tolerance potency (Pal et al. 2019). Recombinant endoglucanase NMgh45, with salt stability in 4M NaCl, also showed excellent ionic liquid tolerance with residual activities of 90% and 43% at 10% and 20% [Bmim][Cl], respectively (Zhao et al. 2018). Another ionic liquid tolerant cellulase CelA10 (Pottkamper et al. 2008) exhibited salt tolerance. *Paenibacillus tarimensis*, isolated from Tunisian salt lake, has ability to maintain 40 and 90% of its hydrolysis efficiency in 40% (v/v) [Bmim][Cl] and 20% (v/v) [Emim][Ac], respectively (Raddadi et al. 2013). It is logical to have ionic liquid tolerance properties of salt tolerant enzymes, because like inorganic salts ionic liquids also gets dissociated in respective ions in aqueous solution. However, based on the present results, it cannot be said that any halophilic bacteria or their extracellular enzyme would be tolerant to the IL.

In situ saccharification of lignocellulosic substrate with potent endoglucanases

Three of the best performing endoglucanase, two produced by Sambhar lake isolates SL1, and SL24, and one from the Bichitrapur isolate BP6 were used for in situ saccharification of alkali and [Bmim][Cl] pretreated rice straw. Based on 16S rRNA gene sequence analysis, SL1 and SL24 respectively showed 99.90 % and 100% sequence similarity to the single NCBI entry *Salipaludibacillus* sp. AK99 (Accession no. LT882622.1) that again was isolated from Sambhar Lake. Members of the genera *Salipaludibacillus*, belonging to class *Bacilli* are rod-shaped, non-motile, and form endospores (ellipsoidal or oval) at the sub-terminal position. Cells are aerobic or facultatively anaerobic (Sultanpuram and Mothe 2016). BP6 showed 100% sequence similarity with *Salinicola* sp. strain SB01-L0, which belongs to class *Gammaproteobacteria*. The genus *Salinicola* was proposed by Anan'ina et al. and member of this genus are rod shaped, non-spore-forming and motile by a single polar flagellum (Anan'ina et al. 2007).

Alkali pretreatment of lignocellulosic biomass with 2% NaOH solution has been suggested for effective removal of lignin (Jeya et al. 2009; Zhang and Cai 2008). Till date, several studies on saccharification have been reported (Hari Krishna and Chowdary 2000; Jeya et al. 2009; Sukumaran et al. 2009; Zhang and Cai 2008) using different lignocellulosic substrates. Rice straw is most preferred substrate for in situ saccharification experiments because of its abundant and cheap availability. After alkali pretreatment, pretreated rice straw was distorted and swelled which supports better accessibility of cellulose and efficiency of pretreatment. No reducing end was generated in 'no enzyme control' and 'no pretreatment

control' sets suggesting importance of the hydrolytic enzyme and the pretreatment process respectively for saccharification of lignocellulosic biomass (Fig. 3). Residual alkali strongly inhibited the activity of all three endoglucanases. Thus, washing off pretreated substrate with water to minimize further inhibition by residual alkali is needed. But global paucity of freshwater is another serious concern (Zhang et al. 2010), which can be overcome by replacing freshwater with seawater. Despite this considerable interest, no study has focused on enzymatic saccharification on seawater washed substrate. In distilled water and seawater washed condition, release of reducing end by all three endoglucanases was clearly detectable after 24 h of incubation and reached peak after 48 h. In all reactions, reducing end was not generated after 48 or 72 h incubation. It indicates that either reaction was completed after that particular time period or enzyme has lost its activity. After 48 h of incubation, reducing end generated from in situ saccharification of 'water washed' rice straw with SL1, SL24 and BP6 endoglucanase was 117.59, 114.9 and 124.12 μg mL^{-1}, respectively. Thus, these enzymes are excellent components for in situ saccharification of alkali pretreated biomass. In addition, reducing end generated by SL1 and SL24 endoglucanase from 'seawater washed' substrate was low, 106.06 and 110.2 μg mL^{-1}, respectively. Interestingly, BP6 endoglucanase exhibited 140% higher activity (173.7 μg mL^{-1}) in 'seawater washed' biomass as compared to 'water washed'. Even seawater washing steps can be included which makes these enzymes promising candidates for industrial use.

Applicability of these endoglucanase on [Bmim][Cl] pretreated biomass, was also tested in different concentration of residual or added or washed samples enzyme activity were assessed. Reducing end generated in washed substrate by SL1, SL24 and BP6 endoglucanase were 112.8, 92.4 and 119.9 μg mL^{-1}, respectively (Fig. 4). But, in presence of residual [Bmim][Cl], reducing end yield dropped to 48.0, 27.7 and 67.6 μg mL^{-1}, while in 5% [Bmim][Cl] yield by three endoglucanase was almost none (~ 2 μg mL^{-1}). A few studies on enzymatic saccharification of IL pretreated lignocellulosic material have been reported previously (Li et al. 2010; Wang et al. 2011; Xu et al. 2015). Although the saccharification rates was inhibited in 5% [Bmim][Cl], hydrolysis in residual IL conditions is advantageous, as it eliminates substrate washing step.

Conclusion

This study underlines the fact that endoglucanases, and possibly any other extracellular enzymes, produced by microorganisms from saline environments with fluctuating salinities (such as those from Sambhar Lake) exhibit excellent halotolerant property and remain active at wide range of salt concentrations. In addition, these enzymes are also active and stable at high concentrations of the IL, [Bmim][Cl] that is one of the favored pretreatment solvent for lignocellulosic biomass in biofuel industry. Thus, the general structural and biochemical features of these extracellular enzymes that help them to remain active in wide range of salt concentrations might help them to remain active in presence of ILs as well. On the other hand, enzymes from bacteria of saline environments without major fluctuations in salinities (like Bichitrapur) are not good target for IL tolerance capabilities. However these extracellularly produced salt stable enzymes by mangrove isolates can be exploited for use in industrial processes

where seawater can be thought of as a washing or basal reaction agent. Thus, further purification and commercialization of endoglucanase isolated in course of this study would really be a path breaking outcome in biofuel industry.

Declarations

Funding

This work was financially supported by UGC MRP [F. No. 43-462/2014(SR)] and WB-DST [1759(Sanc.)/ST/P/S& T/SG-18/2017]. Abhijit Sar and Srikanta Pal are thankful to UGC-BSR and CSIR, for their fellowships respectively.

Conflict of Interest

The authors declare that they have no conflict of interests.

Availability of data and materials

The datasets during and/or analyzed during the current study are available from the corresponding author on a reasonable request.

Code availability

Not applicable

Authors' Contributions

All authors contributed significantly to the work. Conceptualization: B. Dam and A. Sar. Methodology and data analysis: A. Sar, S. Biswas, R. Biswas and A. Misra. Writing-manuscript preparation: A. Sar. Writing-review and editing: B. Dam, S. Pal, A. Misra and R. Biswas. Supervision: B. Dam.

Ethical Approval

Not applicable

Consent to Participate

Not applicable

Consent to Publish

All authors are aware of the content and agree with the submission.

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Tables

Table 1 Physicochemical property of wood samples of Sambhar lake and Bichitrapur*

Sample	Moisture content (%)	pH	Salinity (%)	Organic carbon (%)	Available nitrogen (%)
SLW	14.0	9.0	21.54	43.25	0.83
BPW	55.5	6.5	2.18	41.98	0.59

*Values are mean of three independent readings

Figures

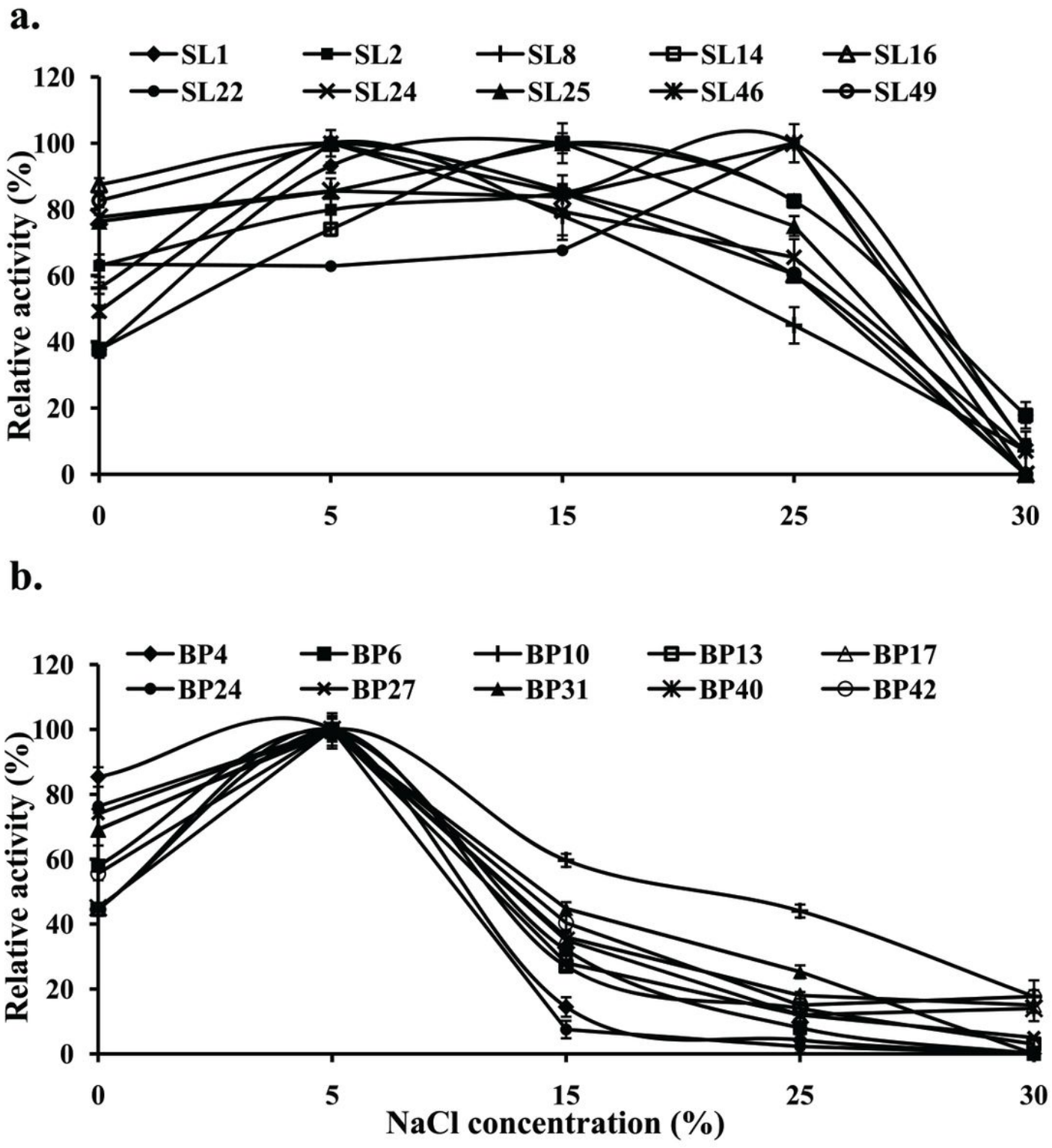

Figure 1

Effect of NaCl on endoglucanase activity of SLW (a) and BPW (b) isolates. 200 µL crude enzyme of respective isolate was mixed with 400 µL Tris-Cl buffer (0.05 M) with pH 9.0 and 7.0 respectively for SLW and BPW isolates. Reaction mixtures also contain 1% CMC, and 5, 10, 15, 20, 25 or 30% NaCl and were incubated at 30 ºC for 1 h and DNSA assay was performed. Relative activity was defined as the

percentage of activity retained as compared to the optimum condition. Each data represents mean value of three experiments with standard deviation shown by error bars.

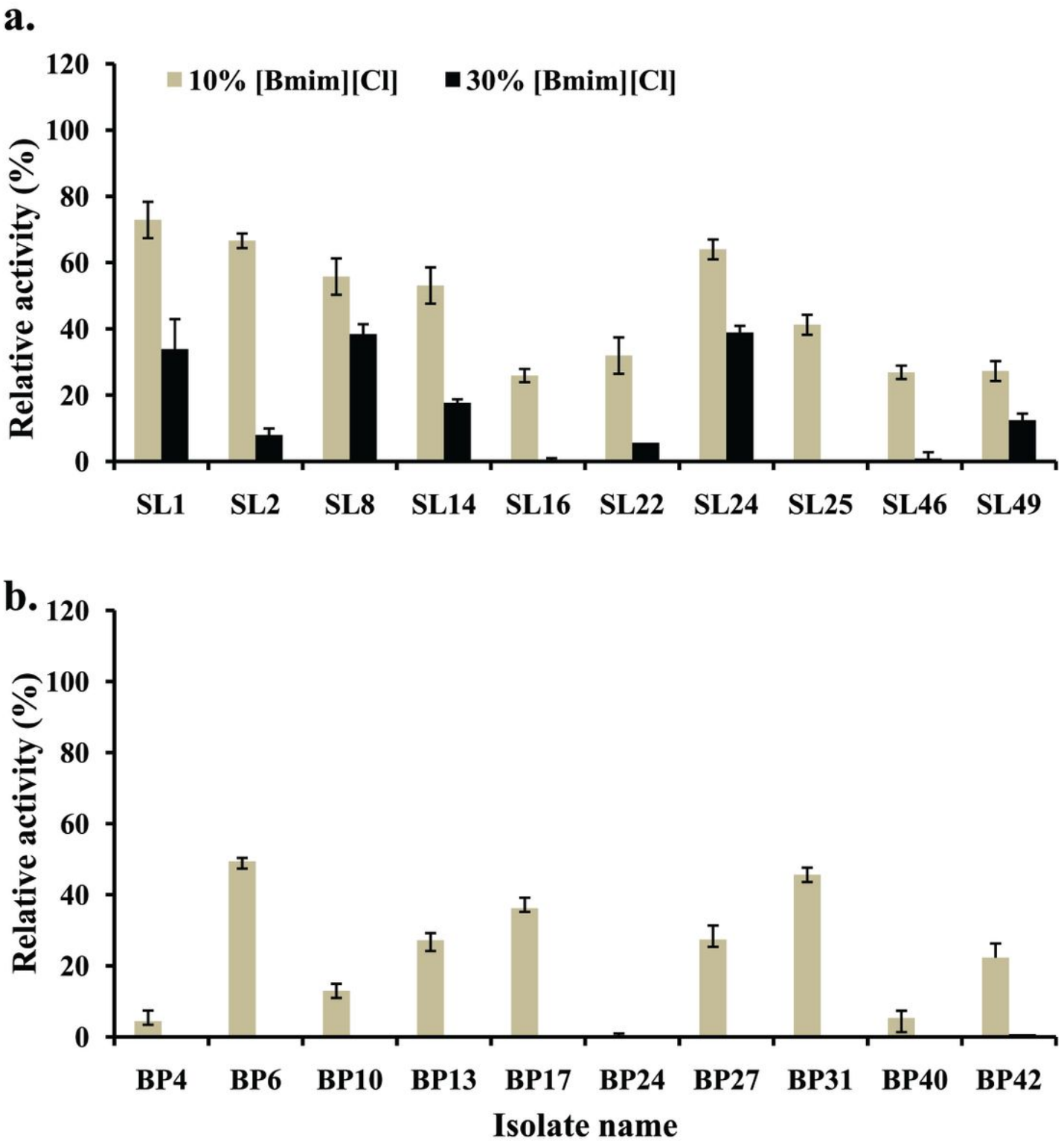

Figure 2

Effect of [Bmim][Cl] concentration on endoglucanase activity of SLW (a) and BPW (b) isolates. 200 µL crude enzyme of respective isolate was mixed with 400 µL Tris-Cl buffer (0.05 M) with pH 9.0 and 7.0 respectively for SLW and BPW isolates. Reaction mixtures also contain 1% CMC, and 10% or 30% (v/v)

[Bmim][Cl]. Reaction mixture was incubated at 30 ºC for 1 h and DNSA assay was performed. Relative activity was defined as the percentage of activity retained as compared to without [Bmim][Cl] condition. Each data point represents mean value of three experiments with standard deviation shown by error bars.

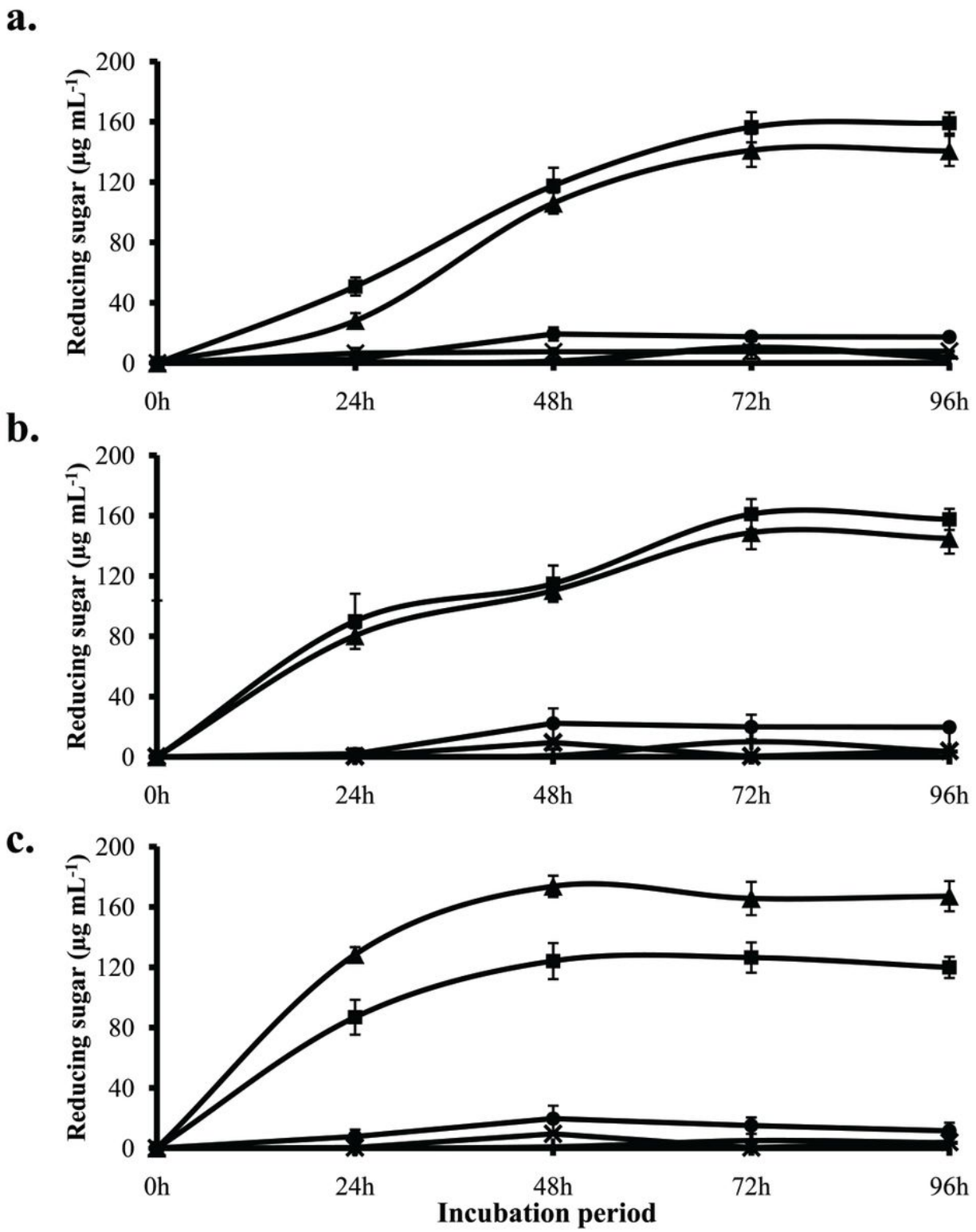

Figure 3

Release of reducing sugars from alkali pretreated rice straw by (a) SL1, (b) SL24, and (c) BP6 endoglucanase. Values for the five reaction set ups are plotted in five different symbols: wash; water 6

times (■), wash; seawater 6 times (▲), residual alkali (●), no enzyme control (+), and no pretreatment control (×).

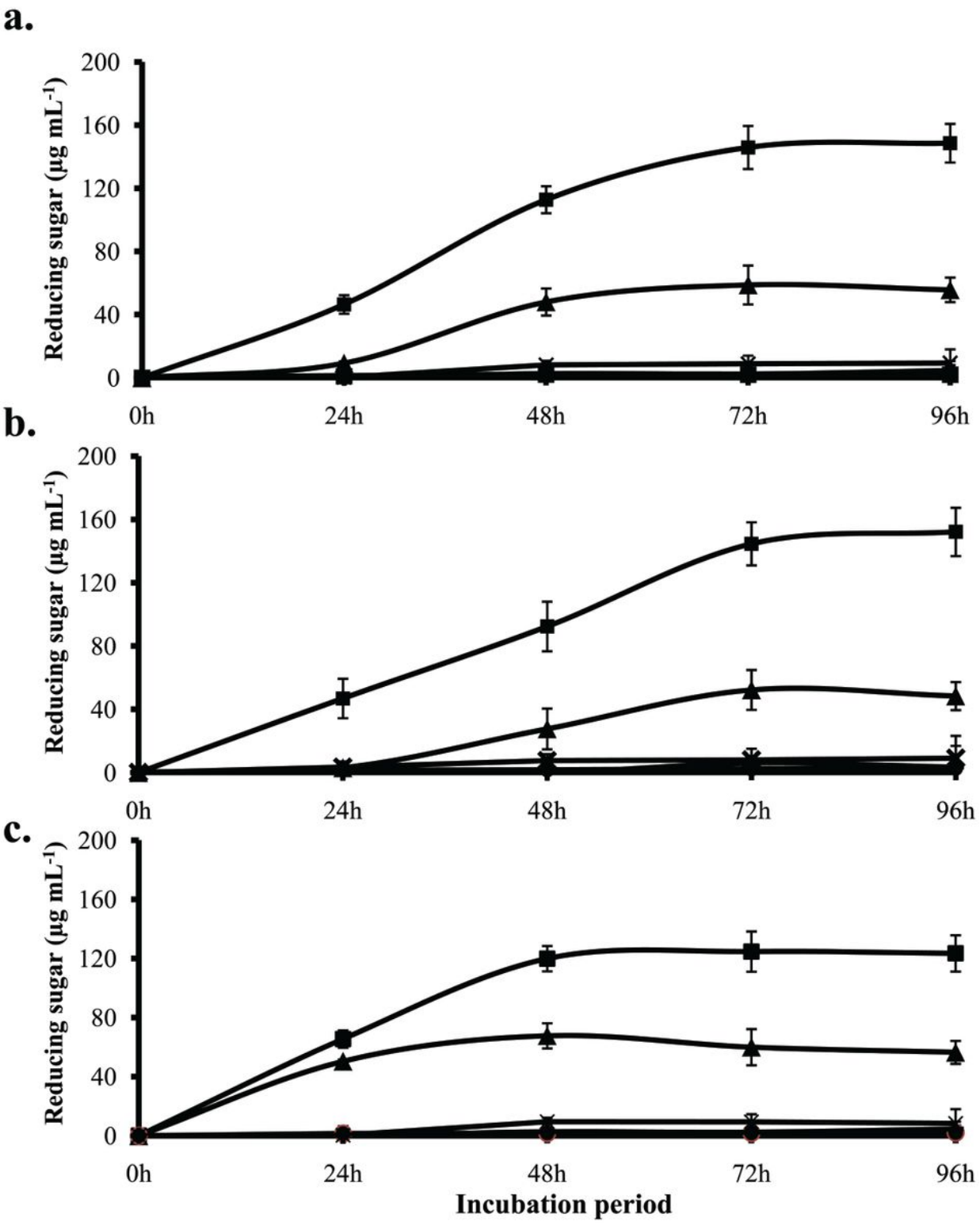

Figure 4

Release of reducing sugars from [Bmim][Cl] pretreated rice straw by (a) SL1, (b) SL24, and (c) BP6 endoglucanase. Values for the five reaction set ups are plotted in five different symbols: wash; water 6

times (■), 5% [Bmim][Cl] (●), residual [Bmim][Cl] (▲), no enzyme control (+), and no pretreatment control (×).

Supplementary Files

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