Characterization of Crude Cellulases From a Bacillus Sp. Isolated From Lake Bogoria, Kenya

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Short report

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Abstract

Background

The demand for a carbohydrate driven economy coupled with the abundant cellulosic biomass has driven the search for cellulases that can be applied for the utilization of the cellulosic biomass.

Context of the study

Of special focus for our study is cotton, which is the preferred textile fiber given its favorable high quality. In addition, sugar, which is an important feedstock for several chemical industries, can be readily produced from cellulosic biomass.

Purpose of the study

We therefore sought to characterize cellulases from favorable ecosystems in order to gauge the validity of their inclusion in cellulosic biomass industrial utilization.

Conclusion

We have shown the production of saccharides from cellulosic biomass (cotton and filter paper). Further, we established the stability of the cellulases in 40% (v/v) organic solvents; propanol, methanol, ethanol. In addition, the enzymes showed tolerance to the accumulation of sugars. Moreover, the cellulase enzymes were most stable at pH 6 and demonstrated a wide activity temperature range of 20°-80°C. Optimal cellulase activity was recorded at 60°C confirming the thermophilic nature of the enzymes.

Brief Summary

This validates and shows the potential of these enzymes in co-expression bio-factories and applications requiring high sugar yields

Potential Implications

This work adds to the study on the activity of the pure extracted cellulases from this microorganism under review at the journal of applied Biochemistry and Biotechnology. These findings substantiate the potential inclusion of either this microorganism or its isolated/extracted enzymes in the industrial processing of cellulosic biomass including but not limited to cotton and paper.

Introduction

Previously, chemical methods of cotton treatment were employed in the textile industry. However, these ancient methods have presented with problems of environmental pollution, high temperature requirements, high alkaline pH and excessive energy costs involved in the maintenance of the high
temperature and in the removal of the vast residual wastes during wastewater treatment (Verenium-
cottonase ® and Novozymes).

This escalated production cost due to huge alkali chemical requirements, water requirements and energy
costs as well as taxing decontamination processes and the poor fabric quality obtained at the end of the
process, have driven the need for alternative processes that can alleviate the environmental exertions,
reduce processing costs and minimize chemical requirements hence making the processes more
sustainable.

Biological solutions in the form of enzymes have been found to alleviate the inconveniences associated
with chemical treatments as they work optimally under mild conditions with minimal residual wastes.
Moreover, the fabric obtained at the end of the process, is of good quality.

In nature, multiple enzymes are employed by microorganisms to efficiently degrade plant cell wall
cellulosic polysaccharide material. In fact, microorganisms employ three principal systems for enzymatic
breakdown of cellulosic plant cell wall. These work either in combination or in separation and include;
free enzymes, multifunctional enzymes and multi enzyme complexes for example, cellulosome
(Mcclendon et al., 2012).

Applications of cellulases have increased considerably especially in the textile industry during the last
two decades. These include bio processing of natural bers, bio polishing of cotton fabrics in order to
enhance the softness and feel, appearance and treatment of recycled bers to restore ber texture and
flexibility lost during operations (Karmakar & Ray, 2011).

It is therefore vital to bioprospect for and characterize novel cellulases as well as novel cellulase
producers. This study aimed at characterizing new cellulase enzymes from the thermophilic, alkalophilic
and saline Lake Bogoria found in the Rift valley region of Kenya.

**Materials And Methods**

**Chemicals**

G-75 sephadex (1.7 X 30cm, Pharmacia Fine chemicals, Sweden), salts used in making of microbial
media were of analytical grade, TLC plates, TLC sprayer (CAMAG), sugars used here were of analytical
grade, cotton, and p-anisaldehyde for TLC visualization.

**Strains and media**

Luria Bertani (LB) medium was used for *Bacillus sp. LAF-A8* (gb|MT682523), cell growth, Mineral salt
Medium (MSM) supplemented with 0.5% sodium carboxymethylcellulose (Na-CMC) was used for
production of cellulase enzymes.

**Enrichment medium**
Bacillus sp. LAF-A8 were grown in Luria bertani (LB) nutrient medium (10g/l tryptone, 5g/l yeast, 10g/l NaCl). This was done in order to enrich the culture before sub-culturing in supplemented MSM with Na-CMC as the sole carbon source.

**Enzyme Production**

LB overnight culture (1ml) was inoculated into MSM made of tryptone 0.5%, yeast extract 0.1% , Na-CMC 0.5% , K₂HPO₄ 0.08% , KH₂PO₄ 0.06% , (NH₄)₂SO₄ 0.1% , MgSO₄.7H₂O 0.02% , CaCl₂.2H₂O 0.005% , NaCl 0.3% , FeCl₃ 0.00001% and supplemented with 0.5% Na-CMC to induce the expression of cellulase enzyme genes. The medium was incubated for 5 days at 37°C, 180rpm (Anish, Rahman & Rao , 2007).

**Enzyme Purification**

**Concentration of the protein**

Following growth, the supplemented MSM culture medium was centrifuged at 10,000rpm, 4°C for 20mins. The clarified supernatant containing the secreted cellulase enzyme proteins was then concentrated by precipitation in 50% ice-cold acetone overnight at -20°C. The precipitate was thereafter pelleted by centrifugation at 15,000xg, 4°C for 15mins. The pellet was subsequently resuspended in 20mM Tris-HCl pH8.8.

**Gel filtration**

A sephadex column (G-75, 1.7cm X 30cm, Pharmacia fine chemicals, Sweden) was used to fractionate the concentrated crude cellulase protein based on molecular weight. The sample buffer used was phosphate buffer with 0.15M NaCl, whereas the eluent buffer was phosphate buffer with 0.5M NaCl. Thirty fractions of 1.5ml each were collected and monitored at 280nm (UV-mini 1240, UV-Vis spectrophotometer, SHIMADZU). The fractions were tested for protein content using biuret assay.

Fractions that peaked at 280nm were then assayed for cellulase activity. Those showing the highest activity corresponded to elution fractions 8 to 16 (LB pH7, chromatogram) and elution fractions 40 and 45 (Na-CMC, MSM pH10, chromatogram).

**Cellulase enzyme assays:**

**Cotton assay**

A preliminary continuous assay was conducted using sodium acetate buffer (50mM, pH5), cotton (0.1%w/v) and crude enzyme concentrate. This cellulase activity assay using cotton was done over a 10-day incubation period. Aliquots of 1ml each were collected in time constant time intervals, and tested for enzymatic activity by checking for reducing sugars using DNS assay (Fig.6).

**Cellulase activity assays**
Cellulolytic activity was quantified using DNS assay (Miller, 1959; Dashtban et al. 2010). Due to the analytical complexities presented with pure crystalline cellulose substrates, we used Na-CMC; a cellulose derivative with a higher degree of polymerization and with better solubility for these analysis.

**Dinitrosalicylic acid (DNS) assay**

Gel filtration fractions, were tested for cellulase activity using the 3,5 dinitrosalicylic acid method (Miller 1959). DNS reagent (750µl), was added to a 1ml cellulase reaction test tube containing 100µl of the gel purification fractions. 40% sodium potassium tartrate (250µl) was added to the mixture and heated at 100°C for 5minutes. Optical density (O.D) was then recorded at 550nm using a spectrophotometer (UV, SHIMADZU). Using a predetermined glucose standard curve, the glucose concentrations of the samples were obtained and used to determine enzymatic activity.

**Thin Layer Chromatography (TLC) assays**

TLC was used to follow the cotton and filter paper cellulase hydrolysis. The spotted plate, were developed in acetonitrile: water (v/v) . The plates were then air dried and sprayed ( TLC sprayer, MERCK) with visualization solution (1ml P-anisaldehyde, 1ml 97%H$_2$SO$_4$ in 18ml ethanol) . This was followed by heating at 110°C for 30 minutes for staining.

**Optimization of cellulase activity**

Cellulase enzymatic activity parameters (temperature, pH and time), were determined.

**Temperature Optimum**

The temperature profile was determined by recording the cellulase activity between 20°C -100°C.

**pH Optimum**

The optimum pH was also determined by recording the cellulase activity between pH 2-14. Different solutions with potent buffering capacities at various points were used; Glycine-HCl buffer, pH2-3 , sodium acetate buffer pH 4-5, phosphate buffer pH 6-7, Tris-HCl buffer pH 8-9, Sodium bicarbonate –NaOH buffer pH 10 and Glycine –HCl buffer pH 11-14.

**Reaction times**

Optimum reaction/ incubation times was determined by recording the cellulase activity in 1-hour intervals over an 8-hour period. This was preceded by an initial 30minutes interval reading.

**Enzyme Stability assays**

pH and temperature stability was done by determining the residual enzymatic activity following pre-incubation at pH 2-14 and 20°-100°C.
Effect of different compounds on cellulase activity

The effect of various sugars (monosaccharides, disaccharides, polysaccharides), alcohols, chemical reagents (ions, metal chelators surfactants and detergents) on cellulase activity was also determined.

Cellulase substrate specificity assays

Cellulase substrate specificity was also determined on a number of soluble and insoluble substrates (Avicel, Na-CMC, cellobiose and cotton).

Results And Discussion

RESULTS AND DISCUSSION

Production and Purification of cellulase enzyme proteins

The microorganism was inoculated into LB medium and minimal salt medium supplemented with 0.5% sodium carboxymethylcellulose (Na-CMC) as the main carbon source. The role of Na-CMC was to induce the expression of the cellulase gene and cellulase production (Sang-Mok & Koo, 2001; Kubicek, 1993). The secreted cellulase enzymes were harvested by centrifugation at 4°C, 10,000 rpm for 20 mins (Schallmey et al. 2004). The harvested proteins were purified on a shorter column (Fig. 2) (production in a LB medium), then a final run (production in MSM) as shown in Fig. 1. The fractions corresponding to C10 and C16 (Fig. 2), Fraction 41, and Fraction 45 that showed the highest reading using UV were further analysed for enzyme activity using pNp-β-cellobioside as a substrate. They recorded enzyme activity of $4.93 \times 10^{-6}$ U and $7.01 \times 10^{-6}$ U respectively.

Optimization of cellulase activity

Reaction temperature

Bacterial cellulases have been shown to have optimal activity in the temperature range (35°-50°C) (Aygan et al. 2011). For this study, there was a significant change in enzyme activity at the various temperatures within the tested temperature range of 20°-100°C (p<0.05) (ANOVA). Further, the enzyme showed a working temperature range between 20°-80°C. Moreover, the highest enzymatic activity was recorded at 60°C (Fig 9). This confirms and validates the therophilic nature of the microorganism which was isolated from Lake Bogoria; a hot water lake in the Rift valley region of Kenya. This is of interest particularly for industry as these enzymes could find application in both ambient and temperature intensive applications. Further, they could be used to substitute the temperature intensive processes by conducting the processes at the ambient conditions; subject to process optimization and cost/ benefit analysis.
Further the recorded optimum temperature is similar to those reported by Lima et al. 2005 but higher than those reported by Balasubramanian & Simoes, 2013.

**Optimum pH**

There was a significant effect on enzyme activity with change in pH (p<0.05) (ANOVA). The crude enzymes showed dual peaks of activity between pH3-7 and pH8-11 (Fig.9). The highest activity was observed at pH5 and pH 10.

**Optimum reaction time**

There was a significant effect on the enzyme activity with different reaction times. (p<0.05) (ANOVA). Significant reaction products were observed after a reaction time of 4hrs, 6hrs and reaction seemed to reach completion at 8hrs. The highest enzyme activity was recorded following a 4hour reaction incubation period (Fig.11).

**Cellulase stability assays**

**Temperature stability**

The crude cellulase enzymes showed triple peaks of thermal stability (Fig. 12) with a sharp peak at 40°-70°C and flanking peaks at 20°-40°C and 70°-90°C. Moreover, it would be interesting to further test temperature ranges lower than 20°C as well as higher temperature ranges greater than 100°C due to the observed continued peaking at these points (Fig12). The enzymes retained 9.50-99.99% of activity between 40°-60°C and 5.97-39.55% of activity between 70°-90°C. With the enzymes showing preference for the temperature range 40°-60°C, in which they retained the highest activity. This is an important characteristic of these enzymes, which would be of interest in synthetic biology/industry where high reaction temperatures (50°-60°C) for prolonged periods are compulsory (Lima et al.2005).

**pH stability**

The crude enzymes showed stability peak between pH4-8 and was most stable at pH 6 (Fig 12). This is consistent with the results on the pure glycosyl hydrolase9 (GH9) enzyme we extracted from this microorganism (Under review)

**Cellulase stability in various compounds**

The effect of various compounds (saccharides, alcohols and chemical reagents; detergents/surfactants, ions and inhibitors). This is important especially in co-fermentation in bio-factories where cellulases could be applied. In addition, it is important to study for these because the cellulosic biomass is normally not pure and in some cases, pre-processing is needed. And to determine reagents to use in enzyme preparations. There was a significant effect on enzyme activity with the different chemical reagents (p<0.05) (ANOVA) (Table1) (Fig. 13).
Stability in Chemical reagents

Native crude cellulase activity was greatly inhibited by 10mM EDTA, urea, β-mercaptoethanol and moderately by 10mM PMSF, triton 1% (v/v), SDS 1% (w/v) and 10mM CaCl₂. Moreover, native crude cellulase activity was moderately enhanced by 10mM: CuSO₄, Imidazole and tween-20 1% (v/v). Native crude cellulase activity was greatly enhanced by 10mM: NaCl, MgSO₄, MnCl₂, MnSO₄, ZnSO₄, CoCl₂, KCl, DTT and FeSO₄. Similar effect on cellulase activity profiles have been previously reported (Balasubramanian & Simões, 2014).

Stability in Sugars

Effect of different saccharides on native crude cellulase activity was tested (Fig.14). There is a significant effect on enzyme activity with different saccharides (p<0.05) (ANOVA). Cellulase activity was enhanced by mannitol, starch, inositol, sucrose, sorbitol, D-xylose, raffinose, lactose, maltodextrin, trehalose, D-glucose and cellobiose. This is contrary to previous studies that showed significant inhibitory effects on cellulase activity during cellulose hydrolysis by sugars (Xiao et al. 2004). This profile is however slightly similar to those reported by (Nigam & Prabhu, 1991) who showed that glucose, xylose and sucrose enhanced cellulase activity while cellobiose had severe inhibitory effects. The stability and activity in the presence of these sugars could be attributed to the low levels of sugars utilized (0.2µg/µl). Further, the stability and activity observed can be attributed to the presence of cellulase cocktail in the crude enzyme extract thus enabling a high substrate consistency and minimizing the classical product (sugar) inhibition.

Stability in alcohols

Effect of alcohol on native crude cellulase enzyme was tested. There is a significant effect on enzyme activity with different alcohols (p<0.05). 40% (v/v) ethanol, 2-propanol and methanol enhanced native cellulase activity (Figure. 20)

Conclusions

The findings from this study show cellulase enzymes stable over a wide range of processing conditions and stability in organic solvents as well as a high tolerance for sugar accumulation. In addition, the cellulase activity on cotton with the release of shorter saccharides has been demonstrated. It would therefore be important to test for the quality (functional and physical properties) of the cotton fibre achieved after this processing.

Abbreviations

TLC – Thin layer chromatography

LB-Luria Bertani
MSM-Minimal salt medium
Na-CMC – sodium carboxymethylcellulose
UV-ultraviolet
DNS-dinitrosalicylic acid
OD-optical density
Rpm-revolutions per minute
ANOVA- analysis of variance
GH9-glycosyl hydrolase 9
PMSF – phenylmethylsulfonyl fluoride
SDS-sodium dodecyl sulfate
DTT-dithiothreitol

Declarations

Ethics approval and consent to participate - N/A
Consent for publication – N/A
Availability of data and materials - data in report
Competing interests - N/A

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Author's Contribution

LAO- conceived and designed research, conducted experiments and data analysis and interpretation and wrote initial draft. EKM – revised initial draft. MFJ, EKM, BMW and CT-Supervised the work. All authors read and approved the manuscript

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Tables

Table 1: Effect of chemical reagents on crude cellulase enzymatic activity
| Reagent          | Concentration | Relative activity (%) |
|------------------|---------------|-----------------------|
| EDTA             | 10mM          | -425.1396648          |
| NaCl             | 10mM          | 334.6368715           |
| MgSO4            | 10mM          | 692.1787709           |
| CuSO4            | 10mM          | 44.13407821           |
| MnCl2            | 10mM          | 831.8435754           |
| Tween 20         | 1%            | 88.82681564           |
| MnSO4            | 10mM          | 513.4078212           |
| SDS              | 1%            | -67.59776536          |
| Imidazole        | 10mM          | 60.89385475           |
| Triton           | 1%            | -62.01117318          |
| CaCl₂            | 10mM          | -84.3575419           |
| β-mercaptoethanol| 10mM          | -291.0614525          |
| ZnSO₄            | 10mM          | 206.1452514           |
| CoCl₂            | 10mM          | 1329.050279           |
| KCl              | 10mM          | 256.424581            |
| Control (without chemical) |         | 100                   |
| DTT              | 10mM          | 2485.47486            |
| PMSF             | 10mM          | -134.6368715          |
| UREA             | 10mM          | -386.0335196          |
| FeSO₄            | 10mM          | 1083.240223           |

**Table 3:** Substrate specificity of crude native cellulase
### Figures

![Graph](image)

**Figure 1**

Gel filtration chromatographic purification of cellulase by Sephadex G-75 using a 5day Na-CMC MSM growth culture at 37°C, 180rpm at pH 10.
Figure 2

Gel filtration chromatographic purification of cellulase by Sephadex G-75 using an overnight LB growth culture at 37°C, 180rpm at pH 7.
Figure 3

Protein (Bovine serum Albumin) standard for Biuret assay

\[ y = 0.1058x + 0.0175 \]
\[ R^2 = 0.9847 \]
Figure 4

Glucose standard curve for reducing sugar (DNS) assay test

\[ y = 0.8017x + 0.0581 \]

\[ R^2 = 0.9643 \]
Figure 5

Para nitrophenol standard assay

\[ y = 0.01x + 0.0063 \]
\[ R^2 = 0.9952 \]
Figure 6

Specific activity; mg/ml glucose released per minute per mg/ml of concentrated crude cellulase protein, on cotton.
Figure 7

Cotton cellulase hydrolysis product profiling by TLC (Spot 1: Glucose; Spot 2: Na-CMC; Spot 3: Cellobiose; Spot 4: Control Spot 5: Enzyme cotton; Spot 6: Acid; cotton; Spot 7: Control filter; Spot 8: Enzyme filter; Spot 9: Acid filter)
Figure 8

Effect of temperature on crude native cellulase enzymatic activity
Figure 9

Effect of pH on crude native cellulase enzymatic activity (Glycine – HCl (pH 2-3); Sodium acetate (pH 4-5); Phosphate buffer (pH 6-7); Tris-HCl (pH 8-9); Sodium bicarbonate-NaOH (pH 10); Glycine-HCl (pH 11-14)
Figure 10

Effect of reaction time on crude native cellulase enzymatic activity
Figure 11

Effect of temperature on crude native cellulase enzyme stability
Figure 12

Effect of pH on crude native cellulase enzyme stability
Figure 13

Effects of chemical reagents on cellulase enzymatic activity
Figure 14

Effect of saccharides on crude native cellulase enzyme activity
Figure 15

Effect of alcohol on crude native cellulase activity