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PHYSICOCHEMICAL CHARACTERIZATION OF CELLULASE PRODUCED FROM *Kurthia gibsonii* (CAC1) ISOLATED FROM CASSAVA DUMPSITES IN IBADAN, NIGERIA

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Abstract

This experiment reports the physicochemical characterization of cellulase produced by Kurthia gibsonii CAC1 isolated from cassava dumpsites in Ibadan, Nigeria. Lineweaver-Burk plot of cellulase activities of K.gibsonii CAC1 was examined with K_{max} and V_{max} of the enzyme assayed. At optimal pH of 5.0, 10% increase in enzymatic activities was observed. The enzymatic activities of K. gibsonii CAC1 were optimal at 30°C and were still stable at 60°C with 50% reduction in cellulase activities. Cellulase activities of K. Gibsonii CAC1 showed significant difference ($p \geq 0.05$) with the different cations used. There was no significant difference ($p \leq 0.05$) observed with increased concentrations ($p \leq 0.05$) using two-way ANOVA. Ca^{2+} highly enhanced the cellulase activities of K.gibsonii CAC1 by 60% at 10mM. Highest inhibition by Hg^{2+} was observed at 20mM with 50% inhibition. At increasing concentration of the inhibitors, there was no significant difference ($p \leq 0.05$) in cellulase activities; although the effect of each inhibitor on the enzymatic activity was significantly different ($p \geq 0.05$). Benzoic acid gave the highest inhibition of the cellulase activities in K. gibsonii CAC1 by 30% at 20mM, while Ethylene diamine-tetraacetic acid (EDTA) boosted the enzymatic activities by 10% at 10mM. There was a significant difference ($p \geq 0.05$) in the effect of different surfactants on the enzymatic activity but no significant difference ($p \leq 0.05$) with the concentration of the surfactants on cellulase activity. Enzymatic activities of the bacterium were enhanced by Polyoxyethylenesorbitan mono-oleate (Tween 80) with a boost of 50%. Increasing the concentration of sodium dodecyl sulphate (SDS) and Polyethylene glycol p-isooctylphenylether (Triton X-100) caused a 70% increase in cellulase activities. Cellulase of K. gibsonii CAC1 had K_{max} of 7.4×10^{-2} mg/ml and V_{max} of 6.7×10^{-1} μ g/sec. The cellulase described in this work have many properties that are similar to those obtained from other microbial sources and may be useful for various industrial applications.

Key Words: *Kurthia gibsonii*, Cellulase activity, Substrate concentration, Cations, Inhibitors, Surfactants

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Introduction

Cellulase has attracted many scientific and industrial attention for its applications in many fields such as animal feed, textile, pulp and paper industries, ethanol production, laundry (Voragen, 1992; Godfrey and West, 1996; Tolan and Foody, 1999); de-inking of recycled paper (Smook, 1992; Moekerbak and Zimmermann, 1998). The importance of cellulase and allied enzymes is increasing in the global market. In the year 2000, the estimated world sale of industrial enzymes was put at 1.6 billion US dollars with cellulase occupying significant part (Demain, 2000). Microorganisms especially bacteria and fungi have played major roles in the production of enzymes such as cellulase and hemicellulase mainly due to low cost of production and the process is less expensive and not laborious (Karmakar and Ray, 2010).

Cellulase consists of three major components which are the endoglucanase, exoglucanase and β -glucosidase. Endoglucanase acts on carboxy methyl cellulose (CMC) and breaks the cellulose chains with resultant formation of glucose and cello-oligosaccharides; exoglucanase acts on microcrystalline cellulose (avicel), converting it to cellobiose as the primary product and beta-glucosidase causes the hydrolysis of cellobiose to glucose (Karmakar and Ray, 2011). The end product from this enzyme is glucose (Wood and McCrae, 1979).

Kurthia gibsonii CAC1 is a motile, aerobic, rod-shaped, non-pigmented and Gram positive bacterium which grow best at incubation temperature of 30°C and pH 5.5 with lactose and ammonium chloride supplemented medium as best carbon and nitrogen sources respectively (Adu et al., 2014). It has been investigated that *Kurthia gibsonii* CAC1 isolated from cassava dumpsites are capable of producing cellulase necessary to degrade cellulolytic

components of cassava (Adu et al., 2014). Hence, researching into factors that may influence enzymatic activity of the cellulase produced by *Kurthia gibsonii* CAC1 is the focus of this work.

Materials and Methods

Sample Collection

The bacterial isolate (*Kurthia gibsonii* CAC1) used for this study was collected from the culture collection centre of the Department of Microbiology, University of Ibadan and transported aseptically to the postgraduate laboratory of the department of Microbiology for further microbial analysis.

Cellulase Enzyme Production

This was done according to the modified method of Todar (2008). The composition of the optimized cellulase production medium was as follow: Lactose (10g/L), K_2HPO_4 (2g/L), KH_2PO_4 (2.5g/L), urea (1.0g/L), $MgSO_4 \cdot 7H_2O$ (0.2g), $FeSO_4 \cdot 7H_2O$ (0.01g/L) and $MnSO_4 \cdot 7H_2O$ (0.007g/L). Production medium was prepared by dissolving the above chemical constituents into conical flasks and then autoclaved at 121°C for 15min. The medium was later inoculated with 2ml of a 24hr old bacterial culture. A control experiment without bacterial inoculum was set up. They flasks were incubated for 24 to 72hr before harvesting the crude enzyme. The supernatant containing the crude enzyme was harvested by cold centrifuging at 5000rpm for 20min using a Himac CR21GII high speed refrigerated centrifuge. The harvested supernatant was stored at 4°C for further analysis.

Characterization of the Cellulase Enzymes

The activities of the cellulase enzyme from the bacteria were characterized considering the effects of temperature, pH, substrates, metal ions, inhibitors, and surfactants.

Assessment of the Optimum pH for Crude Cellulase Production

One millilitre each of 0.1M citrate-phosphate buffer adjusted to various pH values (4.0-9.0) was inoculated with 1ml of the enzyme and incubated at 30°C for 1hr. Volume of 30µL of each cellulase enzyme was inoculated aseptically into a 5mm well on CMC agar medium. The agar plate was then incubated at 30°C for 24hr. This was done according to the modified method of Bertrand *et al.* (2004). After the incubation period; Gram's iodine solution was used to flood the plates and allowed to stand for 30min. The diameter of the hydrolytic zone formed around the site of inoculation was measured and taken to represent the activity of the enzyme.

Determination of the Optimum Temperature for Crude Cellulase Production

One millilitre of the crude enzyme extract was introduced into four (4) test tubes. One test tube each was incubated at 25, 30, 37, and 45°C respectively for 15min. At the end of incubation, the crude enzyme extract was aseptically introduced into a 5mm well on CMC agar medium. The agar plates were then incubated at 30°C for 24hr according to the modified method of Bertrand *et al.* (2004). After the incubation period, Gram's iodine solution was used to flood the plates and was allowed to stand for 30min. The diameter of the hydrolytic zone formed around inoculation site, after the addition of iodine, was measured and taken to represent the activity of the enzyme. Each treatment was carried in three replicates.

Assessment of Temperature Stability of the Crude Enzyme

This was done according to the modified method of Bertrand *et al.* (2004). The enzyme sample was divided into four groups in glass test tubes and incubated at various temperatures of 25, 30, 37, and 45°C respectively before incubation for 24hr. Volume of 30µL of each crude

cellulase enzyme in the test tube was aseptically inoculated into a 5mm well on CMC agar plates. The agar plates were then incubated at 30°C for 24hr. Gram's iodine solution was used to flood the plates and allowed to stand for 30min. The diameter of the hydrolytic zone formed around inoculation site was measured and taken to represent the residual activity of the enzymes.

Investigation on the Effects of Cations, Inhibitors and Surfactants on Cellulase Activity

One millilitre of enzyme samples was introduced into varying concentration of the cations (5, 10, 15 and 20mM), inhibitors and surfactants (0.25, 0.5, 0.75, 1.0 and 1.25%) and incubated at 30°C for 1hr. Then 30µL of each cellulase enzyme was aseptically inoculated into a 5mm well on CMC agar medium. Control sample was inoculated with equal quantity of enzyme and buffer mixture. The agar plates were incubated at 30°C for 24hr according to the modified method of Bertrand *et al.* (2004). Gram's iodine solution was used to flood the plates and allowed to stand for 30min. The diameter of the hydrolytic zone formed around inoculation site was measured and taken to represent the residual activity of the enzymes. Chemical used include: NaCl, CaCl₂, HgCl₂, MgCl₂, NH₄Cl, Urea, Benzoic acid, EDTA (Ethylenediaminetetraacetic acid), SDS (sodium dodecyl sulphate), Triton X-100 (Polyethylene glycol p-isoctylphenylether), and Tween 80 (Polyoxyethylenesorbitan mono-oleate).

Assessment on the Effect of Substrate Concentrations on Crude Cellulase Activity

This was done according to the modified method of Bertrand *et al.* (2004). Increasing concentrations (0.5, 1, 1.5, 2.0 and 2.5% w/v) of carboxy-methyl cellulose was dissolved in 0.1M citrate phosphate buffer at pH 6.0 and used for enzyme assay

without increasing the enzyme volume. One millilitre of each solution was added to one millilitre enzyme sample and incubated at 50°C for 30 min. Quantities of reducing sugar released was measured according to Miller (1959). A reciprocal of quantity of reducing sugar recorded was plotted against a reciprocal of the substrate concentration. The V_{max} , K_{max} and the regression equation were determined from the line equation obtained.

Statistical Analysis

The analysis of variance (ANOVA) was carried out with 95% confidence level on the effect of physicochemical properties on the growth and cellulase activities of isolates CAC1 and CAC2.

Results

Effect of pH on cellulase activities

Figure 1 shows the effect of increasing pH on cellulase activity by *K.gibsonii* CAC1. As pH was increased from 5.0 to 8.0, there was a gradual decrease in the cellulase activity produced by *K.gibsonii* CAC1 from optimal pH of 5.0 until minimum pH was observed at 8.0. Increase in pH from 5.0 to 8.0 did not result in an increase in cellulase activity, rather a 40% reduction in activity was observed.

Effect of Temperature on Cellulase Activities

The effect of varying incubation temperature on cellulase activity by *K.gibsonii* CAC1 is illustrated in Figure 2. At 25°C, there was an increase in cellulase activity (100%). Highest activity (400%) was recorded at 30°C and this represented 300% increase in the cellulase activity of *K.gibsonii* CAC1 enzyme. Further increase in temperature to 37°C led to 250% increase in enzyme activity.

Effect of Temperature Stability on Cellulase Activities

Figure 3 illustrates the response of *K.gibsonii* CAC1 cellulase to incubation temperature relative to time. It was observed that the enzyme was stable at

high temperature of 60°C. When incubated for 30min at 30°C, there was a sharp increase in residual activity (100%) and further incubation resulted in the reduction of the activity. Incubation at 37°C initially resulted in increase in the residual activity (50%) but a slight reduction in activity (20%) was noted after 30 min of incubation before a gradual rise in activity was recorded.

Effect of Cations on Cellulase Activities

Figure 4 shows the effect of cations on cellulase activity of *K.gibsonii* CAC1. Addition of Ca^{2+} at increasing concentration (5,10,15 and 20mM) highly enhanced the cellulase activity with optimal enhancement at 10mM giving 60% increase in cellulase activity after which there was a decline in the boost. NH_4^+ also enhanced enzymatic activity of the cellulase, at lesser degree compared to Ca^{2+} . At 5Mm NH_4^+ , enzymatic activity was increased by 30% but only 10% increase was observed when concentration was increased to 10Mm. An increase of 20% was recorded as the concentration was further increased to 15Mm. At reduced concentration of 5mM, the enzymatic activities were boosted (10%) by the addition of Mg^{2+} after which further increase in concentration of the cations did inhibit the cellulase activities. The higher the concentration added, the higher the inhibition recorded. The highest inhibition (50%) was recorded with Hg^{2+} at 20mM. The peak enhancement concentration of Na^+ (10%) was observed at 15mM after which there was a decline, with lower concentrations giving a mild inhibition of cellulase activity.

Effect of Enhancers or Inhibitors on Cellulase Activities

Figure 5 shows the effect of enhancers/inhibitors on cellulase activity of *K.gibsonii*CAC1 at increasing concentration (5, 10, 15 and 20mM). Urea did not show any observable effect on the cellulase activities as the concentration

increased. Inhibition was recorded with benzoic acid as the concentration increased with highest inhibition (30%) observed at 20mM. Increase in the concentration of EDTA resulted in enhanced activities (10%) of the enzyme at 10mM.

Effect of Surfactants on Cellulase Activities

Figure 6 shows the effect of surfactants concentrations on cellulase activities of *K.gibsonii* CAC1. Increase in concentration of Tween 80 initially increased enzyme activities of *K. gibsonii* CAC1 until highest activity increase (40%) was observed at 0.5% after which further increase in concentration of Tween 80 caused a gradual decrease in cellulase activity. Increase in concentration of Triton X-100 resulted in gradual increase in cellulase activity of *K.gibsonii* CAC1 with peak increase (80%) recorded at 1.25%. There was an initial increase in cellulase activity (20%) of *K. gibsonii* CAC1 at 0.25% SDS after which further increase in concentration resulted in gradual decrease in enzymatic activity (10%) until least activity was recorded at 1.25%.

K_{max} and V_{max} Determination and Effect of Increasing Substrate Concentration

Effect of different concentrations of carboxymethyl cellulose on cellulase activity of *K.gibsonii* CAC1 was studied and the Michaelis-Menten kinetic constants K_{max} and V_{max} for the cellulase were determined. Cellulase of *K.gibsonii* CAC 1 showed maximum activity at 0.8% carboxymethyl cellulose concentration with a linear increase up to this concentration. Above this concentration, a decrease in the enzyme activity was observed. Line weaver-Burk plot for the enzyme activity is shown in Figure 7. Cellulase of *K.gibsonii* CAC1 had a Michaelis constant (K_{max}) of 7.4×10^{-2} mg/ml and a peak enzyme activity (V_{max}) of 6.7×10^{-1} μ g/sec.

Discussion

At increasing pH values, cellulase activities of *Kurthia gibsonii* CAC1 were not significantly different ($p \leq 0.05$) considering their two-way analysis of variance. Cellulase activity of *K. gibsonii* CAC1 however was optimum at pH 5.0 while, further increase in pH did not favour the enzymatic activities. This is similar to earlier report by Immanuel *et al.* (2006), who reported that *Micrococcus* sp, *Bacillus* sp, and *Cellulomas* sp had maximum activities at pH 7.0. Odeniyi *et al.* (2009) reported the cellulase activity of a *Bacillus coagulans* strain isolated from a fermenting palm-fruit residue to be pH-tolerant at 4.0 to 9.0. A similar report by Gautam *et al.* (2010) on a cellulase enzyme from *Pseudomonas* sp showed a broad range activity at pH optimum of 7.5.

There gradual increase in cellulase activities of *K.gibsonii* CAC1 at 25⁰C with optimal temperature observed at 30⁰C which lead to sharp decrease in enzyme activities is similar to the report given by Itoandon *et al.* (2011) which showed that *Aspergillus niger* had optimum temperature for cellulase activities at 30⁰C. Report by Otajevwo and Aluyi, (2010), showed that *Psuedomonas aeruginosa* had peak cellulase activities at 40⁰C and similarly, optimal temperature of 40⁰C was reported for *Aspergillus niger* Z10 strain by Gokhancoral *et al.* (2002). Cellulase activities from *Trichoderma* sp and other mesophilic cellulolytic fungi are at their optimum when assayed at about 50⁰C (Mandels *et al.*, 1974; Tangnu *et al.*, 1981 and Kawamori *et al.*, 1987). The least growth of *K. gibsonii* CAC1 was observed at pH 3.5. The cellulase activities of *K. gibsonii* CAC1 at various temperatures used were not significantly different ($p \leq 0.05$) when incubated at different incubation period.

Cellulase activities of *K.gibsonii* CAC1 showed a significant difference ($p \geq 0.05$) with different cations used but no

significant difference ($p \leq 0.05$) was observed with increasing concentration using two-way ANOVA. Of all the cations studied, it was discovered that the addition of Ca^{2+} resulted in highest cellulase activities of *K.gibsonii* CAC1 with optimal activity recorded at 10mM. Addition of Ca^{2+} at increasing concentration (5, 10, 15 and 20mM) highly enhanced the cellulase activities with optimal boost giving 60% increase. At reduced concentration of 5mM, the enzymatic activities were boosted (10%) by the addition of Hg^{2+} and Mg^{2+} . Increasing concentration of Hg^{2+} and Mg^{2+} resulted in inhibition of the activities of the cellulase but enhanced activities at low concentrations. The higher the concentration of Hg^{2+} and Mg^{2+} added; the higher the inhibition recorded. The highest inhibition (50%) was recorded with Hg^{2+} at 20mM. This was in contrast to the findings of Odeniyi *et al.* (2009) who recorded total inhibition of cellulase activities in *Bacillus coagulans* after the addition of 1mM HgCl_2 solution. Igbal *et al.* (2011) reported that Hg^{2+} had partial inhibition on purified cellulase activities from *Trichoderma viride* isolated from wheat straw.

At increasing concentration of the inhibitors, there was no significant difference ($p \leq 0.05$) in the cellulase activities of *K.gibsonii* CAC1 but the effect of each inhibitor on the enzymatic activity was significantly different ($p \geq 0.05$). Urea showed similar effect shown by the control on the cellulase activities of *K.gibsonii* CAC1 as the concentration increased. Benzoic acid showed pronounced inhibitory effect on the enzyme. Inhibition was recorded with benzoic acid as the concentration increased with highest inhibition (30%) observed at 20mM. Increase in EDTA concentration resulted in enhanced cellulase activities with highest boost recorded at 10mM after which there was decrease in activities of the cellulase enzyme.

At lower concentrations of Tween 80 up till 0.5%, cellulase activities of *K.gibsonii* CAC1 were enhanced, although this was followed by decrease in the enzymatic activities as the concentration was increased. Enzymatic activities of the bacteria were enhanced by Tween 80 with highest boost of 50% observed in *K.gibsonii* CAC1. There was 70% increase in cellulase activities when Triton X-100 was added. Igbal *et al.*, 2011, reported that SDS, and EDTA showed inhibitory effect on purified cellulase activity from *Trichoderma viride* isolated from wheat straw.

Using carboxy methyl cellulose as substrate, the enzyme showed maximum activity (V_{\max}) of $0.67 \mu\text{g}/\text{sec}$ with its corresponding K_{\max} value of $0.074 \text{ mg}/\text{ml}$ for *K.gibsonii* CAC1. In literature, different ranges of K_{\max} and V_{\max} for different microorganisms, especially fungal species have been reported. According to Ekperigin (2007), K_{\max} values for *A. anitratus* and *Branhamella* sp. were 0.32 and 2.54 mM respectively for cellobiose as substrate, while for CMC substrate the values recorded were 4.97 and $7.90 \text{ mg}/\text{mL}$ for the same species respectively. Similarly, K_m value of $3.6 \text{ mg}/\text{mL}$ for *Pseudomonas fluorescens* and $1.1 \text{ mg}/\text{mL}$ for *Trichoderma reesei* were reported by Bakare *et al.* (2005) and Cascalheira and Queiroz, (1999) respectively were reported. Odeniyi *et al.* (2009) reported the K_{\max} and V_{\max} of a carboxy-methyl-cellulase from *Bacillus coagulans* strain to be $0.65 \text{ mg}/\text{ml}$ and $1.36 \mu\text{g}/\text{sec}$ respectively.

Conclusion

Kurthia gibsonii CAC1 produced cellulase which is a thermostable enzyme whose activities are enhanced by some cations such as Ca^{2+} and Mg^{2+} and partially inhibited by Hg^{2+} . They were able to grow at pH range of 5-7 and optimum temperature of 30°C . The novel

microorganism can be used for the production of cellulase with various industrial applications and to aid in degradation of cellulolytic wastes.

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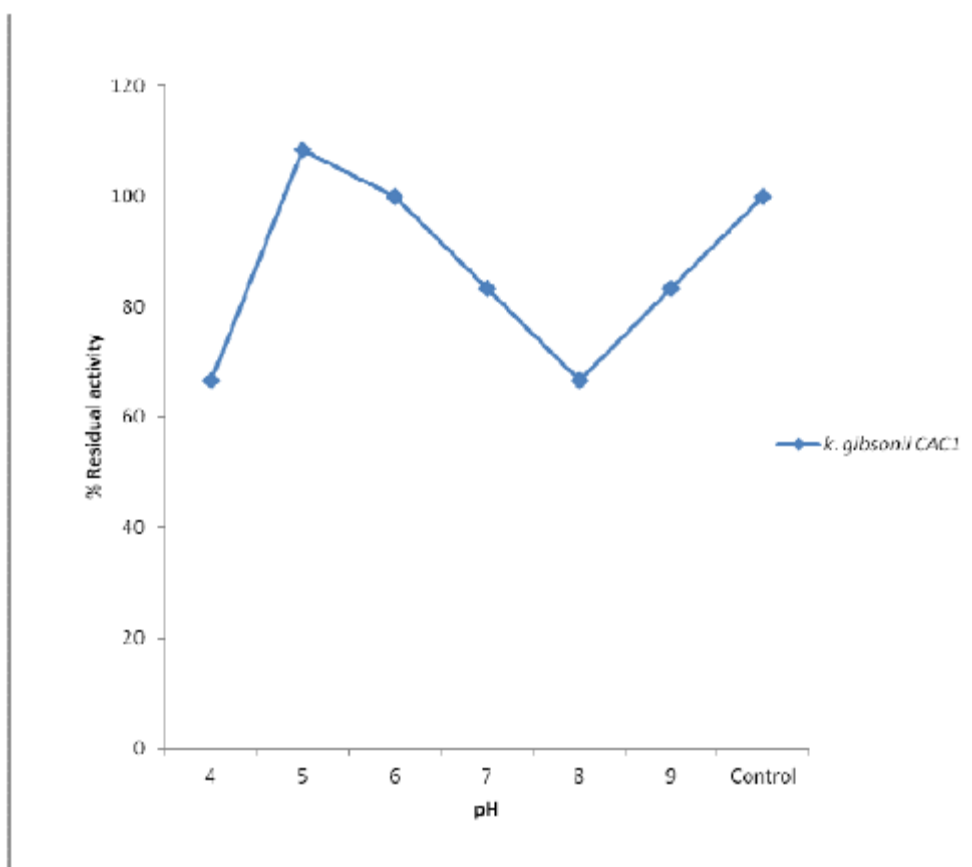


Figure 1: Effect of pH on the cellulase activities of *K. gibsonii* CAC1

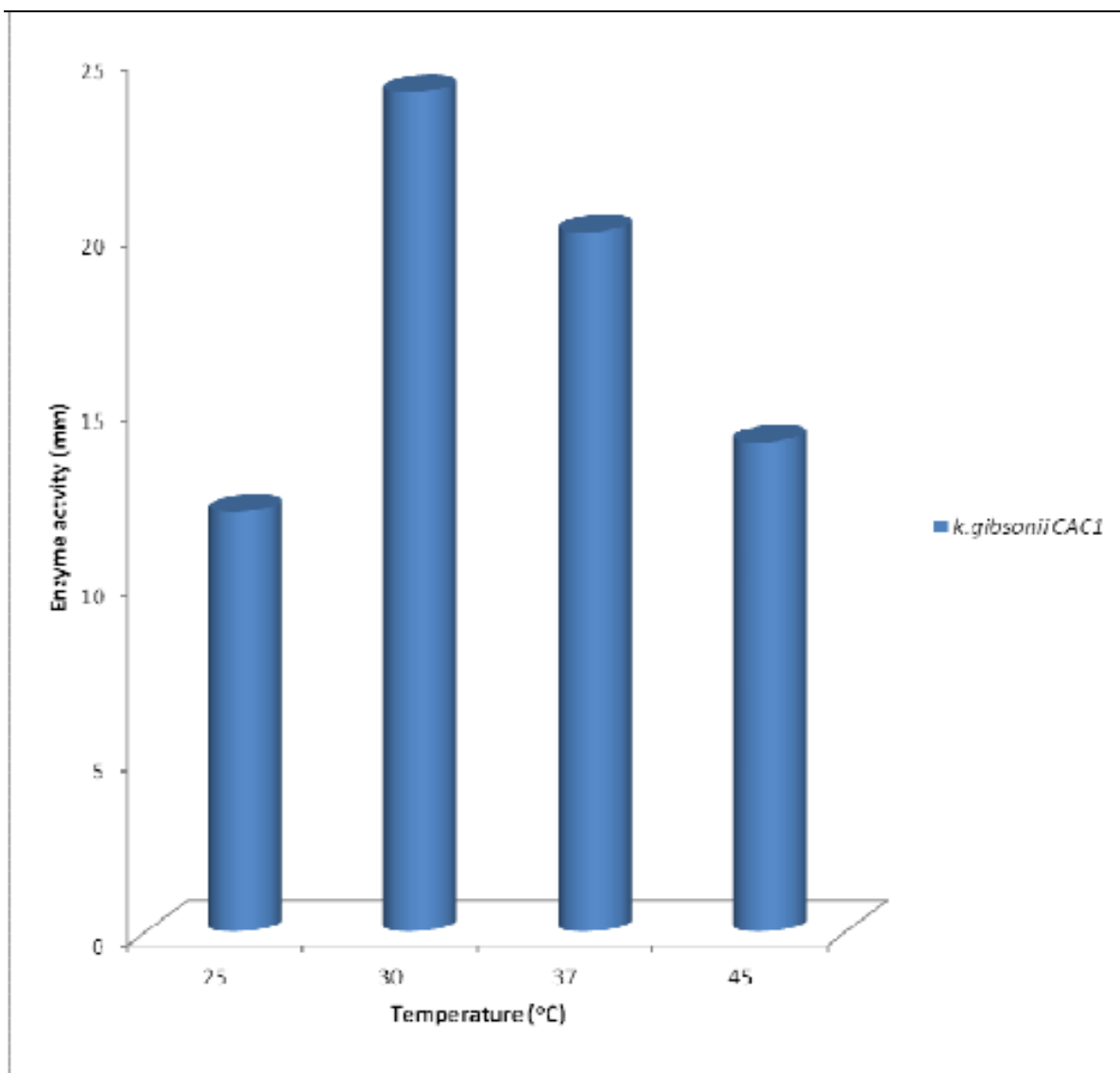


Figure 2: Effect of temperature on cellulase activities of *K. gibsonii* CAC1

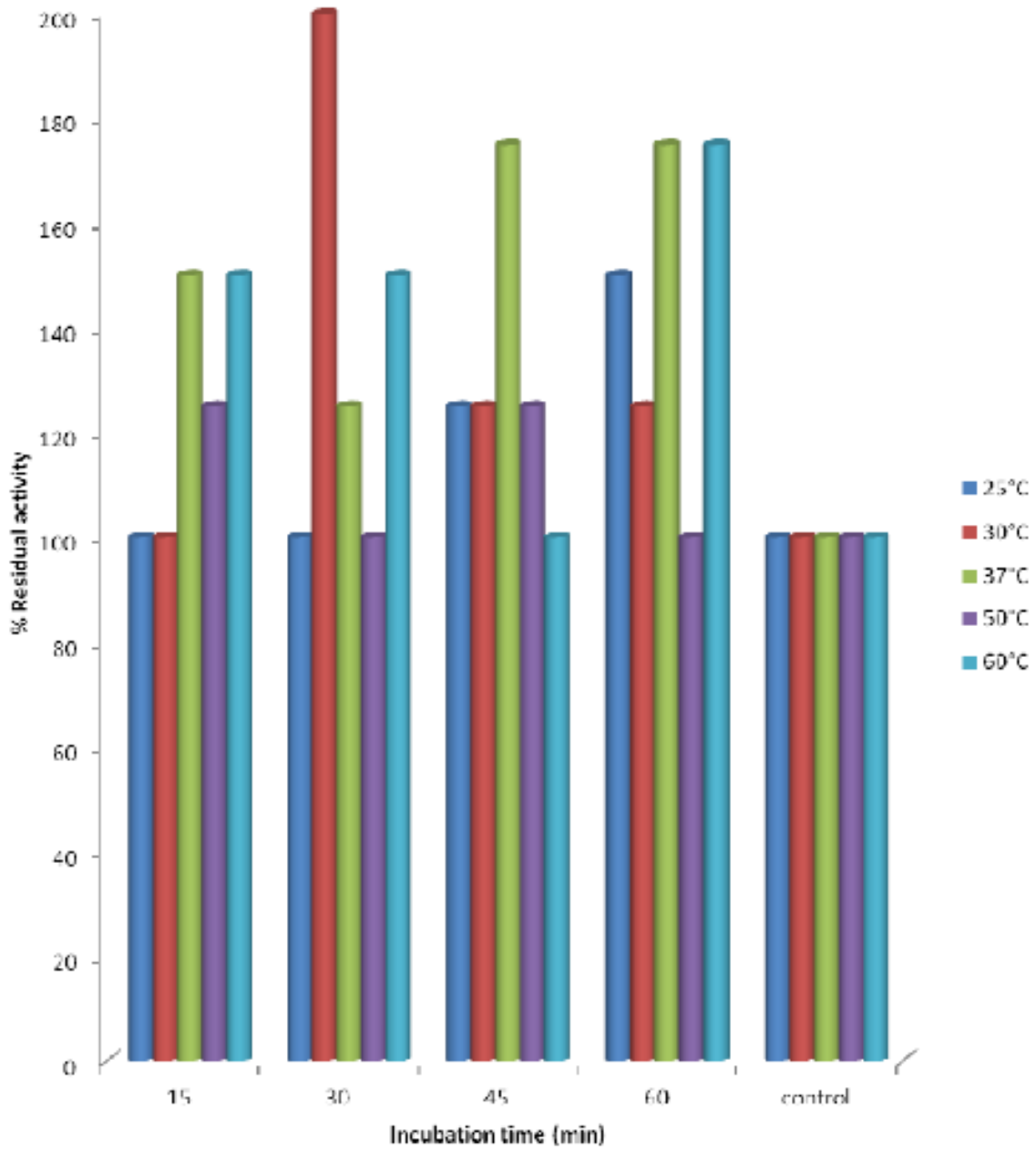


Figure 3: Response of *K. gibsonii* CAC1 cellulase to incubation temperature relative to time

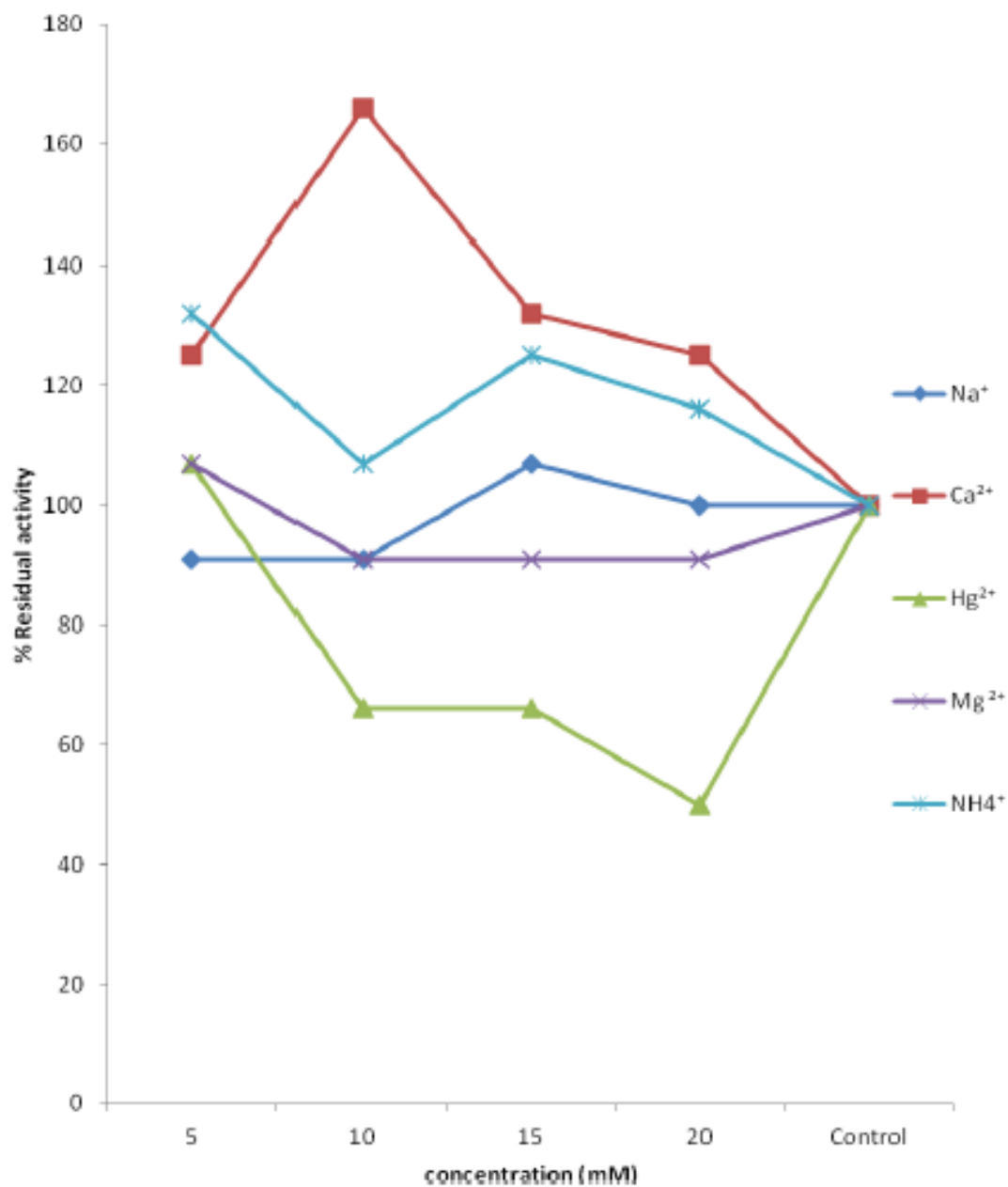


Figure 4: Effect of cations on cellulase activity of *K. gibsonii* CAC1

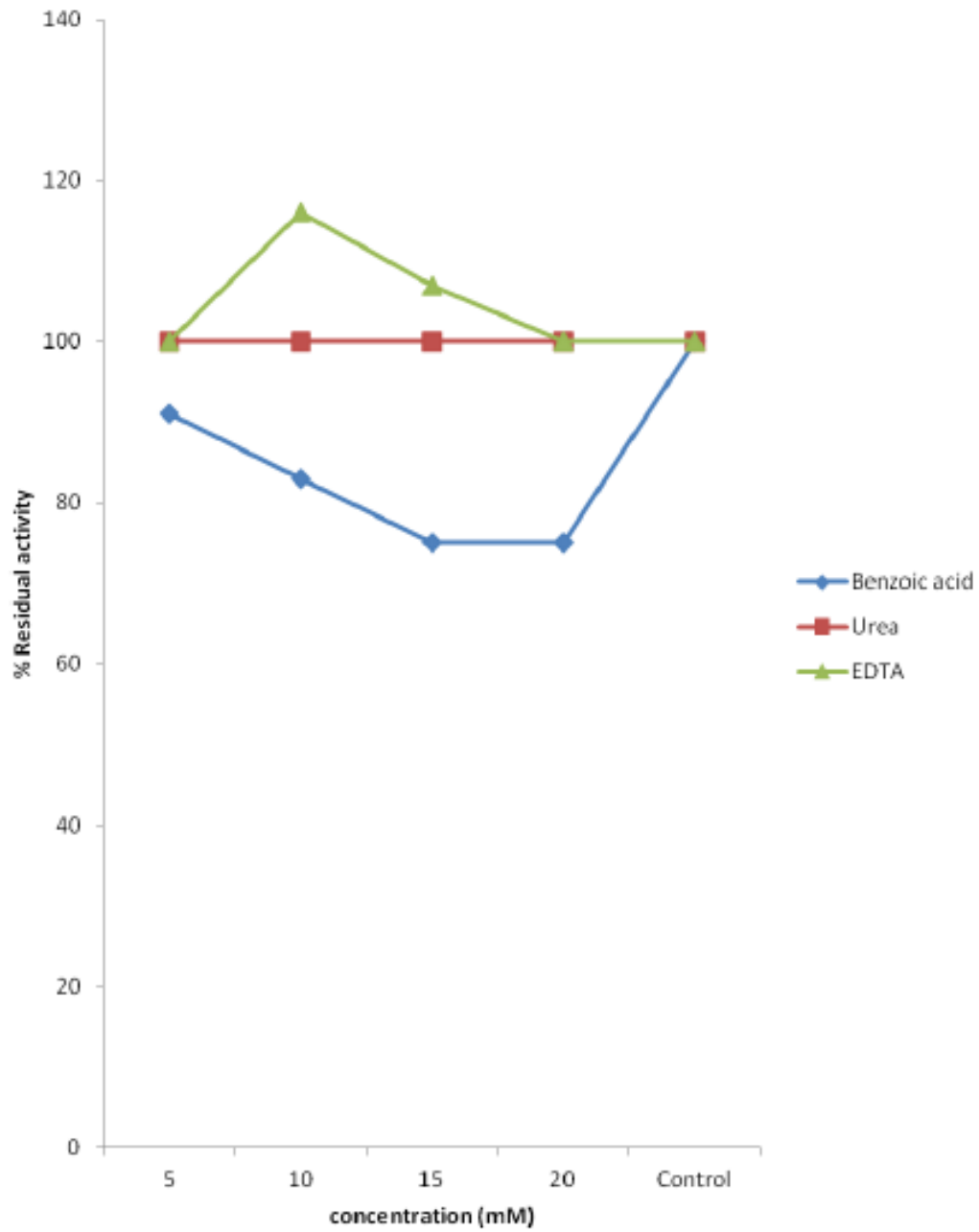


Figure 5: Effect of inhibitors/enhancers on cellulase activity of *K. gibsonii* CAC1

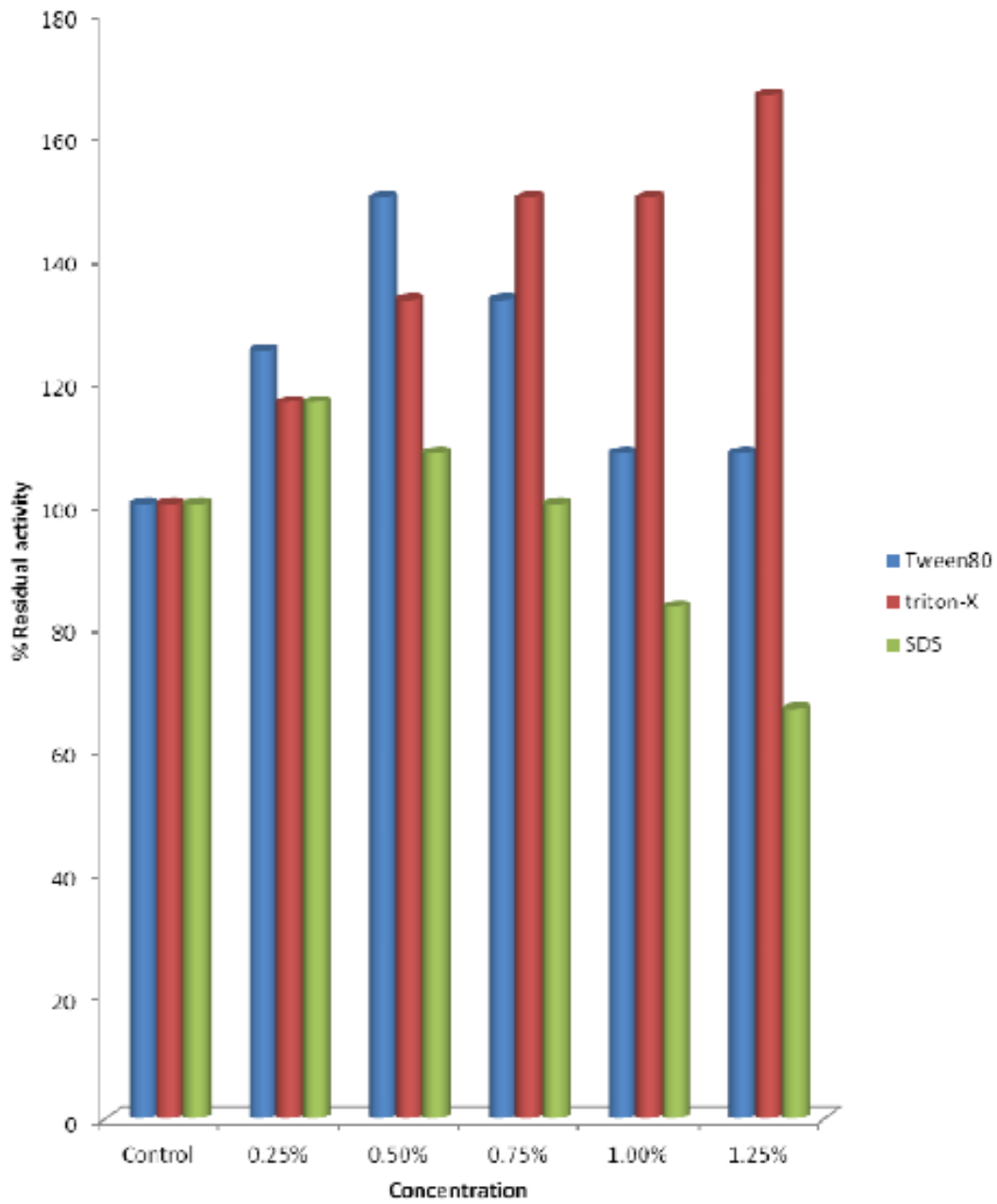


Figure 6: Effect of surfactants on cellulase activity of *K. gibsonii* CAC1

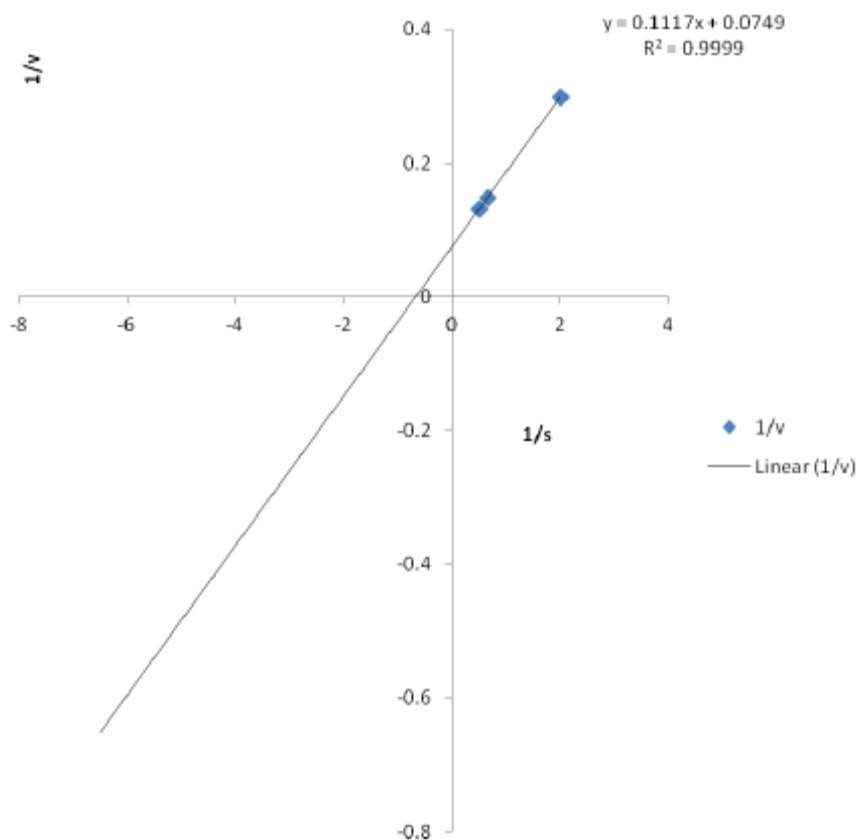


Figure 7: Lineweaver-Burk plot of CMCase activity of *K. gibsonii* CAC1

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