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DEVELOPMENT OF *ENTEROBACTER CLOACAE* IP8 MUTANT STRAIN PRODUCING THERMOSTABLE CELLULASE

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ABSTRACT

Mutagenesis of *Enterobacter cloacae* IP8 for enhanced cellulase production was carried out using ultraviolet (UV) irradiation and ethyl methanesulphonate (EMS) treatment. The mutant strain exhibited cellulolytic activity which was 2.18-fold higher than that of the wildtype strain. The optimal conditions for cellulase production were an incubation period of 28 h, a temperature of 45 ºC, and pH 7.0, using CMC and peptone as carbon and nitrogen sources, respectively. The cellulases from both strains were purified by using ammonium sulfate precipitation, CM Sephadex C-50, and Biogel P-100 column chromatography. The specific activity of the purified cellulase from the mutant strain was 29.47 U/mg while that of wildtype cellulase was 21.5 U/mg. Biochemical characterization of the purified enzyme revealed the optimum pH and temperature of 8.0 and 65 ºC, respectively, for the cellulase from the mutant strain, and 7.0 and 60 ºC for the wild-type cellulase. The mutant cellulase was thermally stable up to 70 ºC retaining 86.5% of its original activity after 180 h. Metal ions Na^+ and Ca^{2+} remarkably enhanced the activity of the cellulase from both strains while Al^{3+} and the chelating agent, EDTA, strongly inhibited the activity. Mutagenesis of *E. cloacae* IP8 using combined UV and EMS treatment led to the development of mutant strain with enhanced capacity for the production of cellulase exhibiting novel properties such as thermostability, alkalinity, low $K_{m,}$ and high V_{max} values. Therefore, the enzyme from the mutant strain of *E. cloacae* IP8 has the potential for broad industrial applications.

Keywords: Cellulase, *Enterobacter cloacae*, Characterization, Mutagenesis, Production, Purification, Thermostability

INTRODUCTION

Cellulose is a linear polysaccharide molecule composed of D-glucose subunits linked together by β-1,4-glycosidic linkages **(Islam and Roy, 2018)**. It is the most abundant polymer in the biosphere constituting major structural units of plant biomass. Cellulases hydrolyze the β-1,4-glycosidic bonds in cellulose resulting in the release of oligosaccharides, cellobiose, and glucose. The complete hydrolysis of cellulose to glucose requires the synergism between three enzyme systems: β-1,4-endoglucanase (EC 3.2.1.4), which randomly attacks the internal glycosidic bonds, resulting in glucan chains of different lengths; β-1,4-exoglucanase (EC 3.2.1.91), which attacks the reducing or non-reducing ends of cellulose chains liberating glucose and cellobiose and, β-glucosidase (EC 3.2.1.21), which hydrolyses cellobiose to glucose from the non-reducing ends **(Patel *et al*. 2019)**.
The use of fungi for cellulase production has received more attention due to the high expression of the enzyme which is often simple as compared to bacterial cellulases. This allows for easy extraction and purification **(Sadhu and Maiti, 2013)**. However, the isolation and characterization of novel cellulases from bacteria are now becoming widely explored. Bacteria often have higher growth rates than fungi allowing higher production of enzymes **(Acharya and Chaudhury, 2012)**. Also, bacteria inhabit a wide variety of environmental and industrial niches leading to the development of cellulolytic strains that are extremely resistant to environmental stresses **(Maki *et al*. 2009)**. These strains can survive and produce cellulolytic enzymes in harsh conditions, which are found to be stable under extreme conditions and may be used in bioconversion processes **(Maki *et al*. 2009)**. This has the advantage of increased enzymatic hydrolysis, fermentation, and product recovery rates. The wide variety of bacteria in diverse environmental niches thus permits the screening for more efficient cellulases to help overcome challenges in the biotechnological applications of the enzyme.
Several bacterial species have been implicated in cellulase production such as *Bacillus licheniformis* **(Shah and Mishra, 2020)**, *Paenibacillus* sp. **(Islam and Roy, 2018)**, *Bacillus* sp. **(Ogonda *et al*. 2020)**, *Clostridium cellulovorans* **(Tsai *et al*. 2015)**, and *Thermotoga naphthophila* **(Khalid *et al*. 2019)**.
The enzymatic conversion of cellulosic biomass is a potentially sustainable and efficient approach for the development of novel bioprocess systems and products. The abundance of cellulosic wastes in the environment has led to the search for bacteria with improved cellulolytic activities from their natural environment, whose enzymes could be explored for important applications in various bioconversion processes of lignocellulosic biomass in the biofuel and bioproduct industries.
Cellulase has diverse industrial applications such as in fruit juice, textile, animal feed, detergent, pharmaceutical, paper and pulp, and biofuel production **(Menendez *et al*., 2015; Patel *et al*. 2019; Ladeira *et al*. 2019; Ejaz *et al*. 2021)**. Thus, cellulases are ranked among the top two enzymes in the global industrial enzyme market based on volume **(Patel *et al*. 2019)**.
Despite the utilization of cellulases for diverse industrial applications, the problems of low yield and stability have limited their comprehensive applications. Thus, there is a necessity to improve the production of cellulase to make the process more economically viable. Enhanced expression of bacterial cellulolytic enzymes could be achieved through strain improvement to yield an improved mutant strain, as well as by fermentation medium optimization. Strain improvement may also lead to the production of mutants capable of expressing enzymes with novel properties such as improved activities and stabilities over a broad range of environmental conditions. Mutagenesis using chemical agents such as ethyl methanesulphonate (EMS) and N-methyl-N-nitrosoguanidine (MNNG), and or physical mutagens such as ultraviolet (UV) and microwave (MW) irradiation is a strategy that can be effectively applied for the improvement of enzyme yields from mutant microbial strains **(Ali and Munir, 2017; Khambhala *et al*., 2017; Peng *et al*., 2021)**. The use of a single agent for mutagenesis has been reported by several authors **(Liu *et al*., 2020; Wang *et al*., 2020)** whereas there are few reports of mutagenesis using combined mutagenic treatments.
This study involves the development of a higher cellulase-producing mutant strain of *Enterobacter cloacae* IP8 using the combined effect of UV irradiation and EMS treatment while the biochemical characterization of the mutant strain cellulase is compared with that of the wild type (WT).

MATERIAL AND METHODS

Bacteria strain

The wild-type *Enterobacter cloacae* IP8 was previously isolated from decayed plant leaf litter of *Lagerstroemia indica* Linn in the botanical garden at Obafemi Awolowo University, Ile-Ife, Nigeria (7º 31′ 14.76″ North, 4º 31′ 49.13″ East) **(Akintola *et al*., 2019)**. It was observed to exhibit the ability to produce a high amount of cellulase, under submerged fermentation conditions, and was identified molecularly based on the 16S rRNA gene sequencing and analysis. The bacterial strain was maintained on nutrient agar slants at 4 ºC, and simultaneously as 50% glycerol stocks stored at -80 ºC.

Mutagenesis of *Enterobacter cloacae* IP8

The WT *E. cloacae* IP8 was subjected to sequential mutation by exposure to ultraviolet (UV) irradiation followed by ethyl methanesulphonate (EMS) treatment. The nutrient broth culture of *E. cloacae* IP8 was incubated at 37 ºC for 24 h and standardized to 0.5 McFarland standards equivalent to 1.0×10^8 cells/mL. A 0.5 mL standardized inoculum of the bacterium was spread-plated on the 0.5% w/v carboxymethyl cellulose (CMC) agar plates under aseptic conditions and each plate was exposed to UV irradiation (20 W lamp, 254 nm) at a distance of 15 cm. The plates were exposed for different time intervals 15, 30, 45, and 60 min. Thereafter, the plates were incubated at 37 ºC for 24 h under dark conditions. The survival colonies after UV treatment were then selected for cellulase production based on their relative zones of clearance on the CMC agar medium. The selected mutant strains were further screened for cellulolytic activity by being inoculated onto fresh CMC agar plates, which were incubated at 37 ºC for 24 h. Nutrient broth cultures of mutant strains with high cellulolytic activity were incubated at 37 ºC for 24 h. The cultures were centrifuged at 6,000 rpm for 5 min and the sediments obtained were washed twice with phosphate-buffered saline. To 2.0 mL of the cell, suspension was added 80 µL of sterile ethyl methanesulphonate and the mixtures were incubated at 37 ºC at different time intervals of 15, 30, 45, and 60 min. At the end of incubation, cells were withdrawn and washed twice with phosphate-buffered saline. It was then diluted 1:10 in minimal salt medium containing (g/L): $MgSO_4.7H_2O$, 0.2; $Fe_2(SO_4)_3$, 0.5; $CaCl_2$, 5.0; $(NH_4)_2SO_4$, 2.0 and Glucose, 2.0 and the culture were incubated at 37 ºC for 24 h to allow for aggregation of the mutants. Thereafter, 0.2 mL of the culture was plated out on fresh nutrient agar and incubated at 37 ºC for 24 h for isolation of distinct mutant colonies.

Screening of WT *E. cloacae* IP8 and mutant strains for cellulolytic activity

The WT *E. cloacae* IP8 and mutant strains were screened for cellulolytic activity on CMC agar plates composed of (g/L): Yeast extract, 2.0; KH_2PO_4, 1.0; $MgSO_4.7H_2O$, 0.5; NaCl, 0.75; CMC, 5.0 and bacteriological agar, 15.0. The cultures were incubated at 37 ºC for 24 h. The mutant strain exhibiting the most appreciable cellulolytic activity potential was selected based on the cellulolytic index as expressed by zones of clearance around colonies which were measured and recorded. The secondary screening was carried out by the use of the submerged fermentation technique in a basal medium containing 0.2% w/v CMC as the sole source of carbon. At the end of incubation, the mutant strain with the highest cellulolytic activity was selected and its stability was studied for nine generations by successive inoculations on the same cellulase fermentation medium.

Submerged fermentation for cellulase production

Cellulase production from wild type and mutant strains of *E. cloacae* IP8, under submerged fermentation technique, was carried out in Erlenmeyer flasks (250 mL) containing 100 mL basal medium composed of (g/L): peptone, 20.0; K_2HPO_4, 3.0; $MgSO_4.7H_2O$, 1.0; NaCl, 0.75 and CMC, 2.0 **(Kotchoni and Shonukan 2002)**. The medium was inoculated with an aqueous suspension of the organism from 24 h old culture, standardized to 0.5 MacFarland standard (1.0×10^8 cells). The culture was incubated at 45 ºC for 48 h on a rotary shaker at 150 rpm. At the end of incubation, the culture was centrifuged at 6,000 rpm for 20 min at 4 ºC and the cell-free supernatant was used as crude enzyme for subsequent analysis.

Cellulase assay and protein determination

Cellulase activity towards carboxymethyl cellulose (CMC) was determined by estimating the amount of reducing sugars released by the action of the enzyme on the substrate using the modified method of **Nelson (1944)** and **Somogyi (1952)**. One unit (U) of cellulase activity was expressed as the amount of enzyme that liberated reducing sugar equivalent to 1.0 µmol of glucose per milliliter per minute under standard assay conditions. The specific enzyme activity was expressed as the unit of enzyme activity per milligram protein. Protein concentration was assayed according to the method of **Bradford (1976)** using bovine serum albumin (BSA) as standard protein against a blank that was set up with only distilled water.

Optimization of fermentation parameters for cellulase production

Incubation period

The growth and cellulase production profile of the WT *E. cloacae* IP8 and mutant strain were determined by inoculating the fermentation medium in several Erlenmeyer flasks with standardized bacterial inoculum (1.0×10^8 cells/mL). The culture flasks were incubated at 45 ºC for 48 h with agitation at 150 rpm. A flask was taken out at 2 h intervals for a period of 48 h. The cell optical density of the culture was measured at 680 nm using the Spectrum Lab 23A Spectrophotometer. The culture was then centrifuged at 6,000 rpm for 30 min and the crude enzyme extracted was assayed for cellulase activity.

Initial pH

The production medium was adjusted to different pH conditions 4.0 to 10 each of which was inoculated with 0.5 mL standardized bacterial inoculum and the culture was incubated at 45 ºC for 48 h with agitation speed to 150 rpm. After incubation, the crude enzyme extract was assayed to estimate the amount of cellulase present.

Temperature

The influence of incubation temperature on cellulase production was determined by varying the incubation temperature from 35 to 60 ºC, at pH 7.0, and agitation speed to 150 rpm. After incubation, cells were removed by centrifugation and the amount of cellulase present in the supernatant was quantified.

Carbon sources

The effect of various carbon sources glucose, fructose, galactose, lactose, maltose, starch, and CMC on cellulase production was determined. The fermentation medium was inoculated with standardized bacterial cell suspensions and the culture was incubated at 45 ºC for 48 h with agitation at 150 rpm. The supernatant was then evaluated for cellulase production.

Different concentrations of carboxymethyl cellulose (CMC)

Different concentrations of CMC (0.5, 1.0, 1.5, 2.0, 2.5%, and 3.0%, w/v) and their effect on cellulase production from both strains were studied. Incubation was at 45 ºC for 48 h with agitation at 150 rpm. The cellulase in the supernatant was then estimated.

Nitrogen sources

Different nitrogen sources peptone, ammonium sulfate, potassium nitrate, sodium nitrate, yeast extract, and urea were studied for their effect on cellulase production. The production medium was incubated at 45 ºC for 48 h with agitation at 150 rpm. Then, the supernatant was assayed for cellulase production.

Purification of cellulase from WT *E. cloacae* IP8 and mutant strain

The lyophilized cell-free supernatant powder was re-dissolved in 10 mM phosphate buffer, pH 7.0, and analyzed for cellulase and protein concentration. The crude cellulase enzyme was loaded into an ion-exchange chromatography column (1.0 cm x 10.0 cm), packed with CM Sephadex C-50 resin which had been washed and equilibrated with 10 mM phosphate buffer, pH 7.0. Protein fractions were eluted from the column using a linear concentration gradient of NaCl (0-1.0 M) in 10 mM phosphate buffer, pH 7.0, at a flow rate of 20.0 mL/h (Wang et al 2009). Fraction tubes were assayed for cellulase activity and protein concentration. Active fractions were pooled together and further concentrated using lyophilization. This was re-dissolved in 10 mM phosphate buffer, pH 7.0, and 1.0 mL of this was layered on the gel filtration column (1.0 cm x 40.0 cm) containing Biogel P-100 resin which was washed with the equilibration buffer (10 mM phosphate buffer, pH 7.0). Elution was carried out using the same buffer at a flow rate of 10.0 mL/h and 0.5 mL fractions were collected **(Zhou *et al*. 2008)**. Cellulase activity and protein profile of the fractions were determined. Fractions with high cellulase activity were pooled together and their enzyme activity and protein concentration were determined.

Sodium dodecyl polyacrylamide gel electrophoresis

For the estimation of the molecular weight of the purified cellulase from mutant strain *E. cloacae* IP8, the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out following the method of **Weber and Osborn (1969)**. Pre-stained protein marker (Thermo Fisher, USA) comprising proteins with molecular weights ranging from 20 to 100 KDa was used as standard. Protein bands formed were visualized after staining with Coomassie Brilliant Blue R-250.

Biochemical characterization of cellulase

Determination of kinetic parameters

The apparent kinetic parameters K_m and V_{max} of the purified cellulase from wild and mutant *E. cloacae* IP8 were determined by incubating aliquots of the enzyme with different concentrations of carboxymethyl cellulose ranging from 0.05 to 0.5 mg/mL, in 10 mM phosphate buffer solution, pH 7.0. The apparent kinetic parameters K_m and V_{max} values were estimated from the Lineweaver-Burk plot **(Lineweaver and Burk, 1934)**.

Influence of pH on cellulase activity

The effect of pH on cellulase activity was determined by assaying for enzyme activity at different pH values using various buffer solutions of pH 3.0 to 9.0. The following 10.0 mM buffer solutions at the indicated pH values were used: sodium citrate (pH 3.0), sodium acetate (pH 4.0-5.0), potassium phosphate (pH 6.0-7.0), and Tris HCl (pH 8.0-9.0).

Influence of temperature on cellulase activity and thermostability

The influence of temperature on cellulase activity and stability was determined by measuring enzyme activity at various temperatures ranging from 35 to 80 ºC. The thermal stability of purified cellulase was determined by incubating the enzyme in 10 mM phosphate buffer solution, pH 7.0, at different temperatures of 50-80 °C for 240 min. Aliquots were then withdrawn at 30 min intervals and assayed for residual enzyme activities.

Influence of metal ions and chelating agent on cellulase activity

The influence of metal ions (Na^+, $Ca^{2+}$and Al^{3+}) and the chelating agent (ethylenediamine tetraacetic acid (EDTA)) on cellulase activity was measured by adding the metal ions and EDTA in the assay buffer at concentrations varying from 0 to 50 mM. Thereafter, the individual residual activity was expressed as a percentage of the activity at zero time which was taken to be 100%.

Statistical analysis

All experiments were carried out in triplicates and measurements were expressed as means ± standard deviation using SPSS version 16.

RESULTS AND DISCUSSION

Screening of WT *E. cloacae* IP8 and mutants for cellulolytic activity

A total of twenty-one mutants were obtained from the first mutagenesis step using UV irradiation. These were screened for cellulase production based on their relative zones of inhibition on the CMC agar medium. Twelve mutant strains exhibiting high cellulolytic activity were then subjected to further mutagenic treatment with EMS. Table 1 presents the results of the primary screening (cellulolytic plate assay) and secondary screening (submerged fermentation) on the WT *E. cloacae* IP8 and mutant strains. The mutant strain MT12, exhibiting the most appreciable cellulolytic activity in both cases, was selected for further studies (Table 1).

Table 1 Screening of mutant and wildtype strains of *E. cloacae* IP8 for cellulolytic activity

Isolate code	Zone of hydrolysis (mm)	Cellulase activity (U/mL)
WT	18.5 ± 0.71	19.25 ± 0.92
MT1	15.5 ± 0.71	15.05 ± 1.20
MT2	12.5 ± 0.71	11.45 ± 0.50
MT3	16.5 ± 0.71	14.85 ± 0.80
MT4	15.5 ± 0.71	13.35 ± 0.80
MT5	17.5 ± 0.71	11.30 ± 0.57
MT6	12.5 ± 0.71	14.20 ± 0.57
MT7	16.5 ± 0.71	17.50 ± 0.85
MT8	11.0 ± 0.00	14.70 ± 0.71
MT9	17.5 ± 0.71	16.60 ± 1.41
MT10	16.0 ± 0.00	18.35 ± 0.35
MT11	14.5 ± 0.71	18.35 ± 0.78
MT12	22.5 ± 0.71	42.00 ± 0.85

Values are means of three replicate determinations ± standard deviation

The high cost of production as a result of low yield and stability limits the comprehensive application of cellulases in industries **(Jana *et al*. 2013)**. The development of mutant strains with the potential for overexpression of cellulase with novel properties will go a long way in alleviating these challenges. In our previous study, the bacterial strain *E. cloacae* IP8 which was isolated from the decayed plant leaf litter of *Lagerstroemia indica* Linn was observed to produce an appreciable quantity of thermostable cellulase **(Akintola *et al*. 2019)**.

There are several reports of the development of microbial mutants with the capability for improved enzyme yield using conventional physical and chemical mutagenic treatment **(Zhang *et al*. 2006; Khambhala *et al*., 2017; Ire *et al*. 2021)**. In the present study, mutagenesis of *E. cloacae* IP8 was carried out using a combination of UV irradiation and the chemical ethyl methanesulphonate (EMS) treatment. The resulting mutants were screened for their cellulase activity using the cellulolytic plate test on CMC agar plates followed by submerged fermentation. The mutant, MT12 was observed to exhibit the most appreciable cellulase production ability among the mutant strains of *E. cloacae* IP8. Therefore, it was selected for further studies. Similar results were reported for the observed increased yield of cellulase from N-methyl-N-nitro-N-nitrosoguanidine (NTG)-treated mutant strains of *Cellulomonas* sp. TSU-03 **(Sangakharak *et al*. 2012)** and *Bacillus* sp. C1 **(Sadhu *et al*. 2014)**.

Influence of fermentation parameters for cellulase production from WT *E. cloacae* IP8 and mutant strain

Enzyme production is influenced by various cultural parameters and growth factors such as pH, temperature, and media substrate composition **(Nandimath *et al.* 2016)**. These physicochemical parameters must be combined in the appropriate manner for optimal enzyme synthesis.

Effect of incubation period on bacterial growth and cellulase production

Cellulase production was observed to increase with the incubation period reaching the maximum at 28 h and 32 h for mutant and WT strains, respectively (Figures 1A and 1B). For both strains, cellulase production increased with an increase in bacteria cell growth reaching a maximum at the late exponential to the stationary phase of growth. Thereafter, cellulase production decreased as the cell growth enters the stationary phase (Figures 1A and 1B). The decline in cellulase production may be due to nutrient depletion or accumulation of toxic metabolites in the medium. It could also be a result of catabolite repression by monosaccharides such as glucose and fructose **(Prakash *et al*., 2009)**.

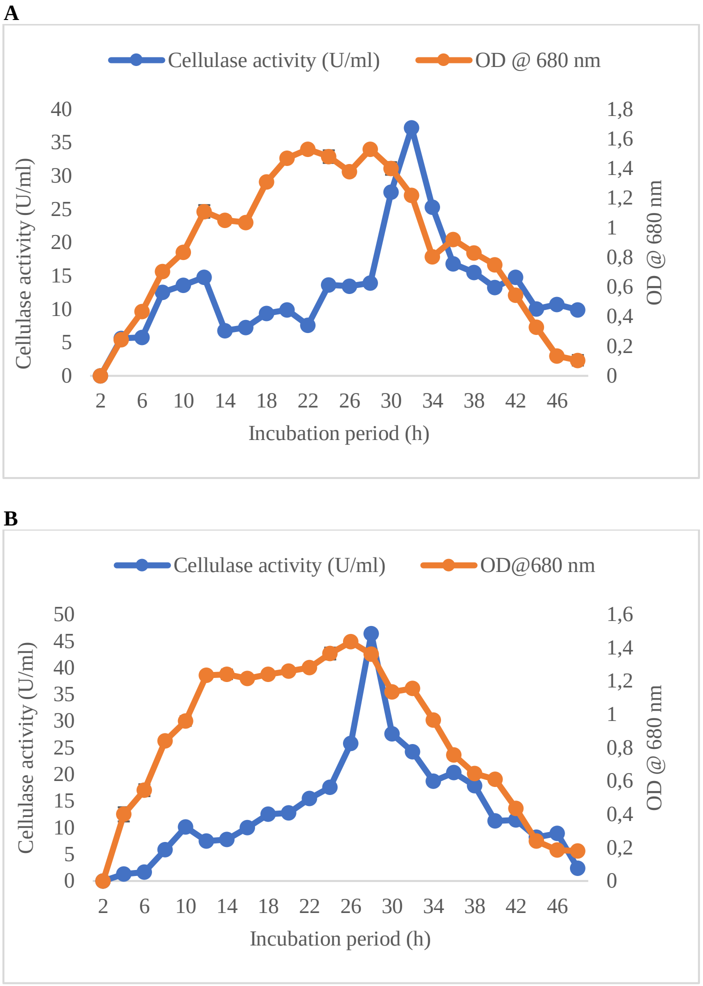


Figure 1 Effect of incubation period on growth and cellulase production from **A** WT *E. cloacae* IP8 and **B** mutant. Values are means of three replicate determinations ± standard deviation

Effects of pH on cellulase production

The best pH for cellulase production from mutant *E. cloacae* IP8 was 7.0 (24.82 ± 0.84 U/mL) while the optimum pH for cellulase production from the WT *E. cloacae* IP8 was 6.0 (22.73 ± 0.30 U/mL) (Figure 2). Although, a similar pH of 7.0 was reported for the production of cellulase from *Paenibacillus* sp. **(Islam and Roy 2018)** and *Bacillus* sp. C1 **(Sadhu *et al*. 2013)**, the pH 5.0 was reported to be the best for cellulase production from *E. cloacae* WPL 214 **(Lokapirnasari *et al*., 2015)**. The initial pH of a culture medium is important as it promotes and regulates the synthesis of the enzyme by microorganisms **(Liang *et al*., 2009; Rafique *et al*., 2022)**.

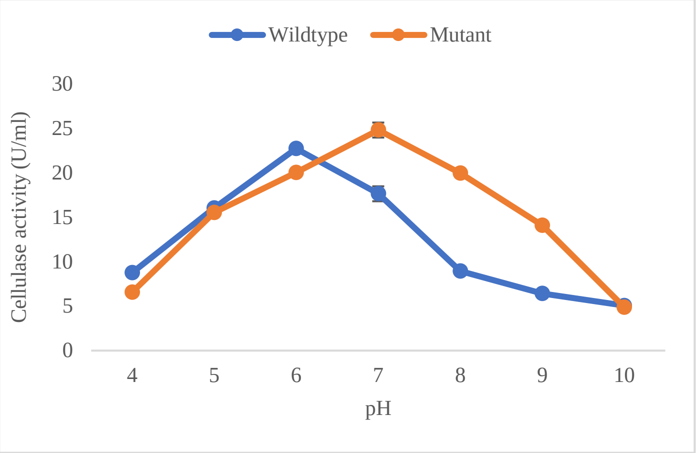


Figure 2 Effect of pH on cellulase production from mutant and WT *E. cloacae* IP8. Values are means of three replicate determinations ± standard deviation

Effect of temperature on cellulase production

The maximum production of cellulase from both the WT *E. cloacae* IP8 and mutant strains was at 45 ºC with 24.16 ± 0.75 U/mL and 17.64 ± 0.17 U/mL, respectively (Figure 3). Temperature is a vital factor that controls microbial growth and metabolite production **(Zhou *et al.*, 2018)**. However, **Sami *et al*. (2008)** and **Islam and Roy (2018)** reported the temperature of 40 ºC as being optimum for cellulase production from strains of *E. cloacae* and *Paenibacillus* sp., respectively.

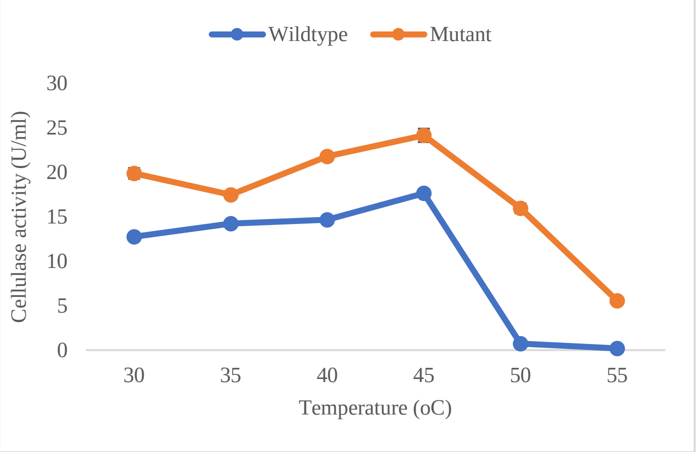


Figure 3 Effect of temperature on cellulase production from *E. cloacae* IP8 and mutant strain. Values are means of three replicate determinations ± standard deviation

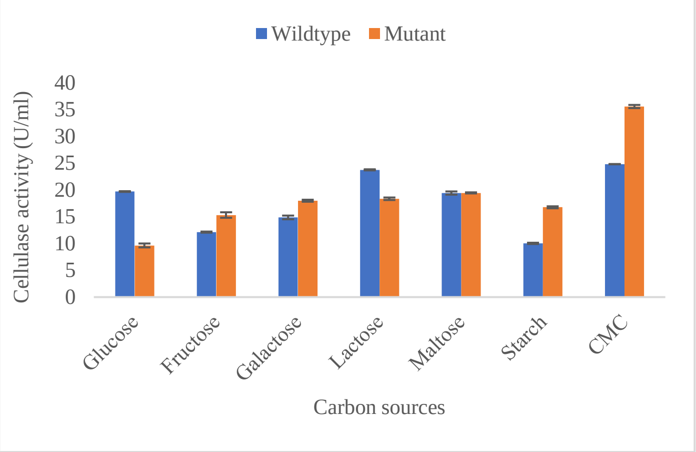


Figure 4 Effect of carbon sources on cellulase production from WT *E. cloacae* IP8 and mutant strains. Values are means of three replicate determinations ± standard deviation

Effect of carbon sources on cellulase production

Carboxymethyl cellulose was the best carbon source for cellulase production from both the WT *E. cloacae* IP8 (25.06 ± 0.33 U/mL) and mutant (35.57 ± 0.30 U/mL). This was followed by the use of maltose and lactose in the case of the mutant cellulase production and lactose and glucose, in the case of the WT cellulase production (Figure 4). Available carbon sources are one of the basic requirements for microbial growth and enzyme synthesis. The maximum cellulase production from *Bacillus* sp. Y3 **(Lugani *et al.*, 2015)** and *Enhydrobacter* sp. ACCA2 **(Premalatha *et al.*, 2015)** also occurred when CMC was used as the sole source of carbon in the basal media. This shows that the enzyme is inducible rather than constitutive.

Effects of different concentrations of carboxymethyl cellulose (CMC) on cellulase production

Carboxymethyl cellulose (CMC), at the concentration of 1.5% w/v, was the best for cellulase production from both the WT *E. cloacae* IP8 (32.91 ± 0.30 U/ml), and the mutant (51.76 ± 0.56 U/ml) (Figure 5). As the concentration of CMC increased beyond this level, cellulase production decreased in both cases.

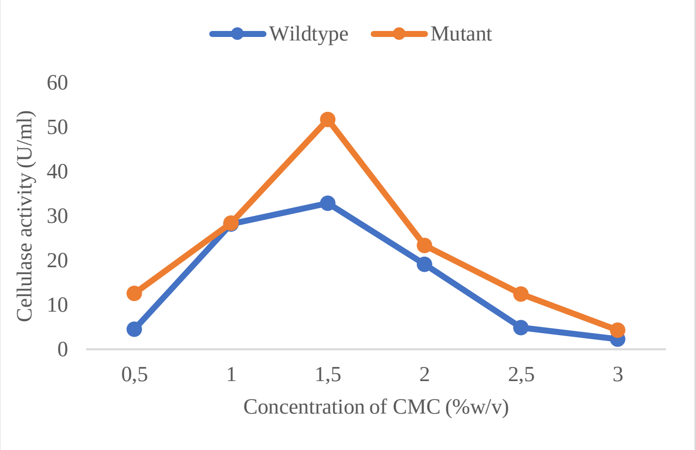


Figure 5 Effect of different concentrations of carboxymethyl cellulose on cellulase production from WT *E. cloacae* IP8 and mutant. Values are means of three replicate determinations ± standard deviation

Effect of nitrogen sources on cellulase production

Peptone, an organic nitrogen source, was observed to be the most suitable for cellulase production from both the WT *E. cloacae* IP8 and mutant (Figure 6). This was followed by the use of sodium nitrate in the case of mutant cellulase production and, ammonium sulfate in the case of the wildtype strain cellulase production. Nitrogen sources are metabolized within the cell to produce amino acids, nucleic acids, proteins, and cell wall components **(Akcan 2011)**. Peptone was reported to be a good nitrogen source for cellulase production owing to the fact that it is an enzymatic digest of other proteins, which makes it readily available for microbial metabolism **(Das *et al*., 2010)**.

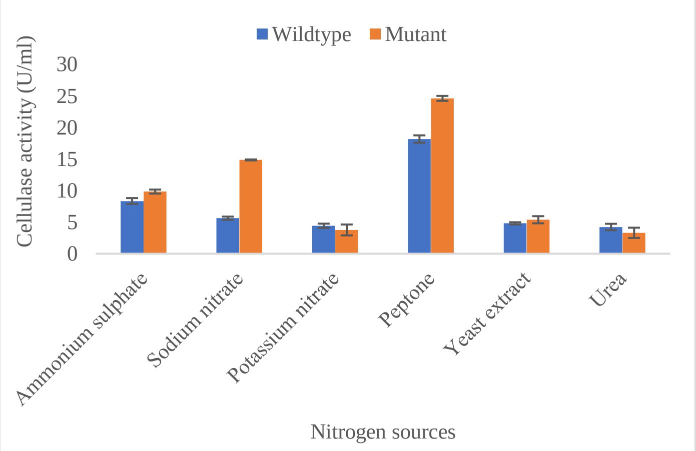
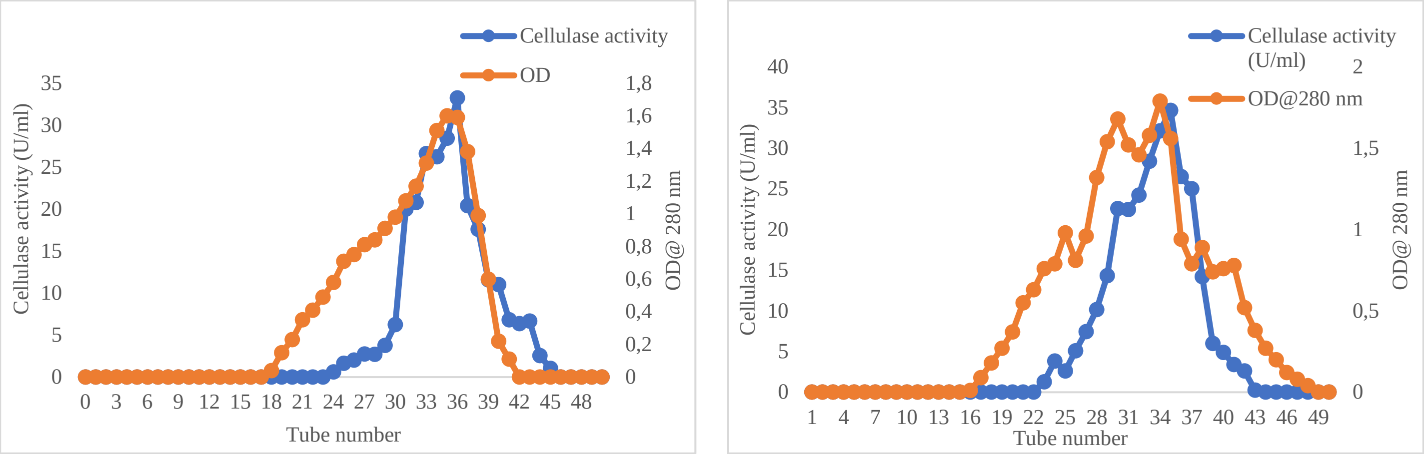


Figure 6 Effect of nitrogen sources on cellulase production from WT *E. cloacae* IP8 and mutant. Values are means of three replicate determinations ± standard deviation

Purification of cellulase from WT *E. cloacae* IP8 and mutant

Ion exchange chromatography, on CM Sephadex C-50 column, of lyophilized crude cellulase from both strains gave a single peak of cellulase in each case. A purification fold of 2.15 and specific activity 42.99 U/mg with 42.99% yield was obtained for the cellulase from WT strain while the purification fold of 3.19 and specific activity 51.43 U/mg with 30.42% yield was achieved for the mutant cellulase. Gel filtration chromatography, on the Biogel P-100 column, of the partially purified cellulase from the ion exchange chromatography also gave a single peak of cellulase activity for both strains in the elution profile (Figures 7A and 7B). A purification fold of 1.06 with a specific activity of 21.50 U/mg and 16.04% yield was obtained for the wildtype strain cellulase while a purification fold of 1.83 with a specific activity of 29.47 U/mg and 17.28% yield was achieved for the mutant strain cellulase (Table 2).



A **B**

Figure 7 Purification profile of cellulase from **A** WT *E. cloacae* IP8 and **B** mutant *E. cloacae* IP8 by Biogel P-100 column chromatography showing protein and enzyme activity

Table 2 Summary of purification protocol of cellulase from WT *E. cloacae* IP8 and mutant

Purification step	Strain	Volume (mL)	Enzyme activity (U/mL)	Total activity (U)	Protein concentration (mg/mL)	Total protein (mg)	Specific activity (U/mg protein)	Yield (%)	Purification fold
Crude enzyme	WT	10	13.40	134.00	0.66	6.60	20.30	100	1.00
	MT	10	14.20	142.00	0.88	8.80	16.14	100	1.00
Ion exchange	WT	6.0	9.60	57.60	0.22	1.32	43.63	43	2.15
	MT	4.0	10.80	43.20	0.21	0.84	51.43	30	3.19
Gel filtration	WT	5.0	4.30	21.50	0.20	1.00	21.50	16	1.06
	MT	5.0	5.60	28.00	0.19	0.95	29.47	17	1.83

SDS-PAGE Analysis

The purified cellulase from the mutant *E. cloacae* IP8 strain had a single band on sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel, with an apparent molecular weight of 54.5 KDa, according to denaturation electrophoresis (12.0% SDS-PAGE) (Figure 8). This result is similar to the molecular weight of 54.4 KDa reported for the purified cellulase from *Bacillus licheniformis* Z9 isolated from the soil **(Elsababty *et al.*, 2022)**.

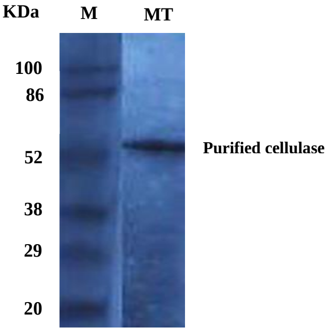


Figure 8 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of cellulase from mutant *E. cloacae* IP8. Lane M – Prestained Protein Ladder of 10-100 KDa range. MT – Mutant purified enzyme from Biogel P-100 column

Biochemical characterization of purified cellulase

Kinetic parameters

The kinetic parameters K_m and V_{max} of the purified cellulase from the WT *E. cloacae* IP8, as deduced from the Lineweaver-Burk plot, were 0.15 mg/ml and 54.64 U/ml, respectively (Figure 9A). The K_m and V_{max} of the purified cellulase from the mutant strain were 0.29 mg/ml and 53.76 U/ml, respectively (Figure 9B).

A

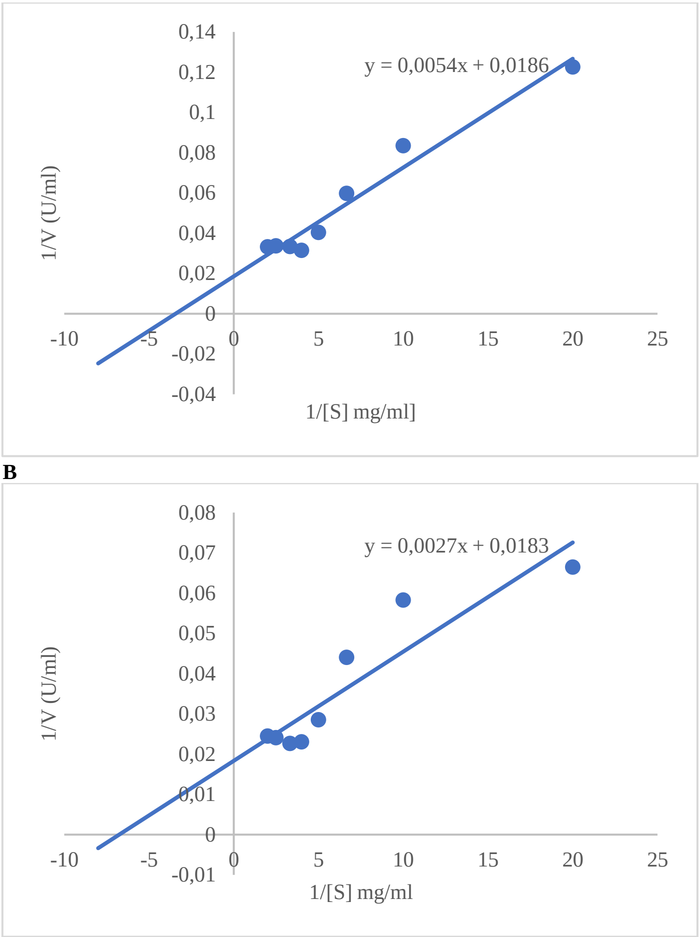


Figure 9 Kinetic parameters (K_m and V_{max}) of the purified cellulase from **A** WT *E. cloacae* IP8, and **B** mutant *E. cloacae* IP8

Optimum pH

The optimum pH for the activity of the purified mutant and WT cellulase were 8.0 and 7.0, respectively (Figure 10). A similar optima pH of 7.4 and 7.5 were observed for cellulase from *B. licheniformis* Z9 **(Elsababty *et al.*, 2022)** and *E. cloacae* **(Muslim and Zaki, 2009)**. The mutant cellulase can thus be described as being neutral to alkaline.

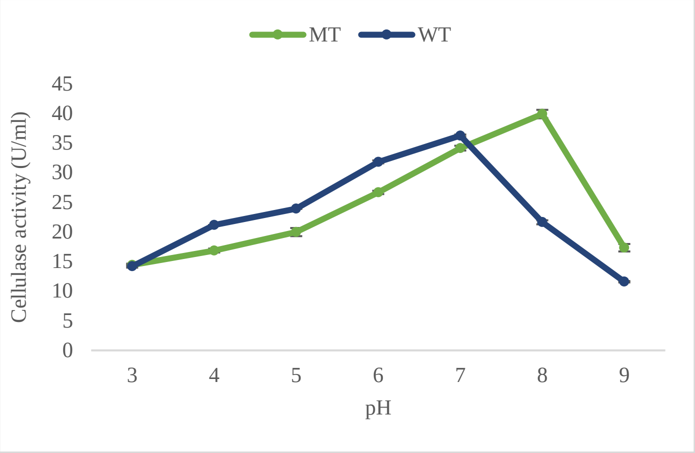
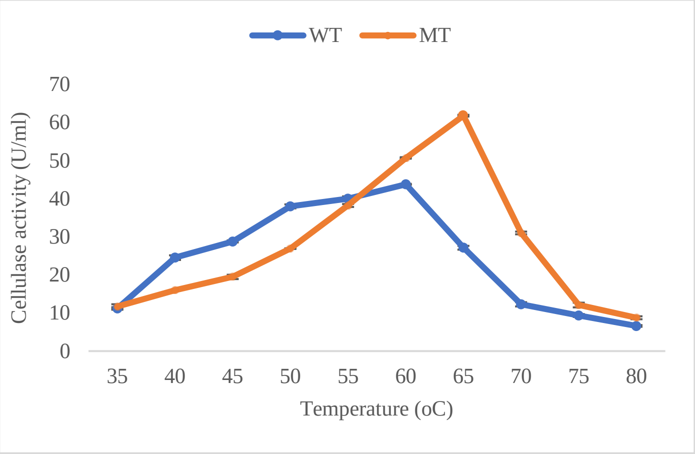
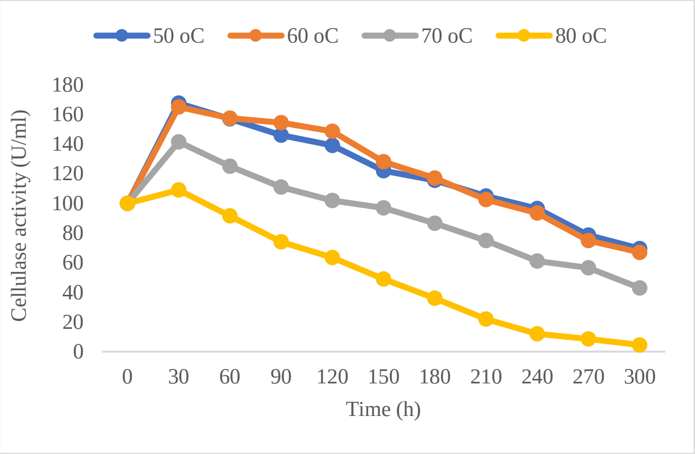


Figure 10 Optimum pH for the cellulase from mutant and WT *E. cloacae* IP8. Values are means of three replicate determinations ± standard deviation

A



B



C

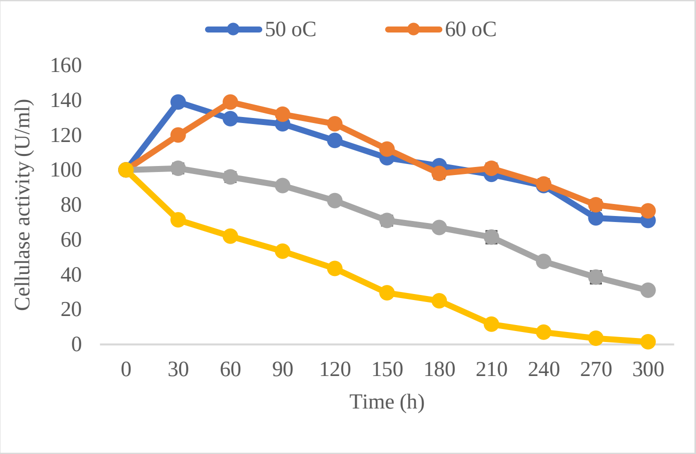


Figure 11. **An o**ptimum temperature of the cellulase from WT and mutant strains of *E. cloacae* IP8. Thermal stability of cellulase from **B** Mutant *E cloacae* IP8 **C** WT *E. cloacae* IP8. Values are means of three replicate determinations ± standard deviation

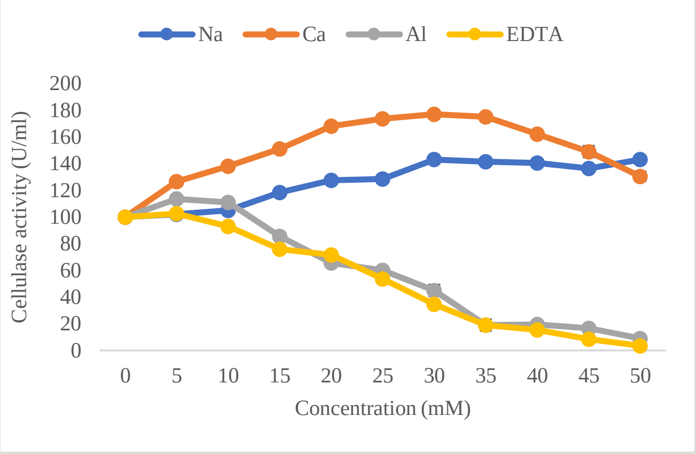
Optimum temperature and thermostability

The temperature of 65 ºC was the optimum for the activity of the cellulase from the mutant strain while the temperature of 60 ºC was optimum for the WT cellulase (Figure 11A). This shows that the mutant cellulase was able to act at higher temperatures which has implications for industrial applications requiring high temperatures such as in the biorefinery industry for the production of biofuel products **(Bhardwaj *et al.*, 2021)**. Also, the enzyme exhibited good thermal stability than the wildtype cellulase. At 70 ºC, it retained about 97% of its original activity after 150 h (Figure 11B) unlike the 71% activity retained by the wildtype cellulase at the same temperature condition and exposure time (Figure 11C). Thermostability is a much sought-after characteristic of cellulases for extensive industrial applications at elevated temperatures such as in the biofuel, textile, and detergent industries **(Kinet *et al.*, 2015; Chang *et al.*, 2016; Ejaz *et al.*, 2021)**.

Influence of metal ions and chelating agent

The metal ions Na$^+$ and Ca^{2+} enhanced the activity of the mutant cellulase at all concentration ranges (5 to 50 mM) used. At concentrations 5.0 to 10.0 mM, the metal ion Al^{3+} and EDTA slightly enhanced mutant cellulase activity but strongly inhibited it at concentrations above 10 mM (Figure 12A). For the WT cellulase, while the metal ions Na$^+$ and Ca^{2+} enhanced the enzyme at all the concentrations used, there was the inhibition of the enzyme activity moderately by Al^{3+} but strongly by EDTA across all ranges of concentrations used. (Figure 12B). Previous research had revealed that most cellulases, being metalloenzymes, require divalent metals such as Ca^{2+} for activation **(Femi-Ola and Olowe 2011; Zin *et al.*, 2014)**. Also, it was reported to enhance the substrate affinity of cellulase by stabilizing the conformation of the catalytic site **(Zin *et al.*, 2014)**. The chelating agent, EDTA, acts by reducing the concentration of free metal ions in the solution and could inhibit cellulase by binding inside the enzyme as a ligand **(Zeng *et al.*, 2014)**. Thus, EDTA could be responsible for the inhibitory effect on cellulase from the mutant by chelating Ca^{2+} leading to loss of activity.

A



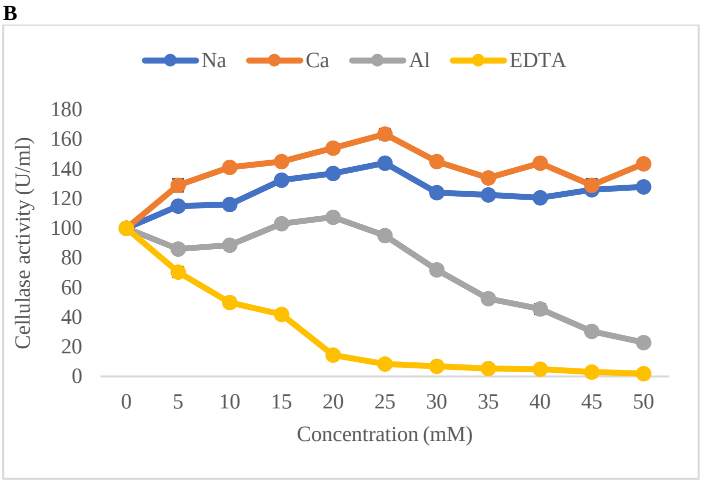


Figure 12 Influence of metal ions and chelating agent on the activity of cellulase from **A** mutant and **B** WT *E. cloacae* IP8. Values are means of three replicate determinations ± standard deviation

CONCLUSION

In this study, a cellulase hyperproducing mutant was developed from the wildtype *Enterobacter cloacae* IP8. This established the combined UV irradiation and ethyl methanesulphonate treatment as a viable mutagenic technique for the development of an efficient cellulase-producing mutant of the isolate. Maximum cellulase production from the mutant was at a 28 h incubation period, pH and temperature conditions 7.0 and 45 ºC, and with the use of CMC and peptone as carbon and nitrogen sources, respectively. The mutant enzyme exhibited optimum activity at pH 8.0 and temperature 65 ºC As a result of its thermostability and activity at neutral to alkaline conditions, it has the potential for utilization for several industrial and biotechnological processes.

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