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***RESEARCH ARTICLE***

# MUTAGENESIS OF *KLEBSIELLA EDWARDSII* FOR MANNANASE SYNTHESIS

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**Abstract:** The aim of the present study was to produce mutants from *Klebsiella edwardsii* capable of producing large quantity of mannanase. Mutants of *K. edwardsii* were generated by subjecting the cell culture to nitrous acid (0.1 M sodium nitrite in phosphate buffer). The mutants and the wild type were screened quantitatively for mannanase synthesis in basal medium containing Locust Bean Gum (LBG) as inducer. Twenty mutants were generated and screened for mannanase production in comparison with the wild type in submerged state fermentation. The isolated mutants were screened in comparison with wild type for the isolation of carbon catabolite activation mutants in the presence of 0.1, 0.5 and 1.0% (w/v) glucose as energy source. The entire mutant strains showed higher mannanase activities than the wild type, with the highest mannanase activity lied on mutant designated HN02. The supplementation of 0.1% (w/v) glucose in the fermentation media caused repression of mannanase synthesis in 60% of the mutants, while only 20% of the mutant exhibited higher mannanase activities when compared with wild type. The supplementation of 0.5 and 1.0% (w/v) glucose in the fermentation media enhanced mannanase production in 80% and 60% of the mutants respectively when compared with the wild type.

**Keywords:** Chemical mutagenesis, catabolite repression, catabolite activation, *Klebsiella edwardsii*

## Introduction

Enzymes are considered as nature's catalysts. Microbial enzymes are often more useful than enzymes derived from plants or animals because of the great variety of catalytic activities available, the high yields possible, ease of genetic manipulation, regular supply due to absence of seasonal fluctuations and rapid growth of microorganisms or inexpensive media (Sharma *et al.*, 2001; Iftikhar *et al.*, 2007; Iftikhar *et al.*, 2008; Helen and Oliveira, 2009).

The exponential increase in the application of mannanase in various fields in the last few decades

demands extension in both qualitative improvement and quantitative enhancement. Quantitative enhancement requires strain improvement and medium optimization for the overproduction of the enzyme as the quantities produced by wild strains are usually too low (Haq *et al.*, 2010). The spectacular success examples of strain improvement in industry are mostly attributed to the extensive application of mutation and selection of microorganisms (Bapiraju *et al.*, 2004). UV irradiation (Haq *et al.*, 2010; Iftikhar *et al.*, 2010; Roja and Prasad, 2012), Ethyl Methyl Sulphonate (Bakare *et al.*, 2005; Femi-Ola, 2008; Haq *et al.*, 2010), N-methyl-N'-nitro-N-nitroso guanidine (Iftikhar *et al.*, 2010) and so on had been reported as effective mutagenic agents for strain improvement of certain microorganisms for enhanced productivity of relevant industrial enzymes.

The biotechnological potential of mannan-hydrolysing enzymes, in particular the mannanase, has been demonstrated within various industries. Industrially useful mannanase have recently attracted attention due to their role in the pulp and paper industry to remove the hemicelluloses from pulps (Gubitz *et al.*, 1996) and in pulp bleaching processes. This positive role has minimized the use of environmentally harmful bleaching chemicals in the pulp and paper industry (Moreira and Filho, 2008). Mannanases have potential application in animal feed production (Wu *et al.*, 2005; Lee et al. 2005; Sae-Lee, 2007) and laundry detergents (Norita *et al.*, 2010).

Bioconversion of agriculture waste containing mannan-based polysaccharides into valuable products such as animal feeds also required microorganisms capable of producing mannan degrading enzymes. Mannanases are also used for the extraction of vegetable oils from leguminous seeds and the clarification of fruit-juices in the food industry (Rattanasuk and Ketudat-Caims, 2009). They are useful in reducing the viscosity of extracts during manufacture of instant coffee, chocolate and cacao liquor (Francoise *et al.*, 1996) to lower the cost for subsequent evaporation and drying (Wong and Saddler, 1993). Mannanases are potentially used in the pharmaceutical industry for the production of physiologically interesting oligosaccharides (Olaniyi *et al.*, 2013).

The biosynthesis of enzymes is regulated by induction and repression. The inducer is usually the substrate for or some structurally related compound (Prescott *et al.*, 1999). In most organisms, enzyme production is repressed in the presence of high concentration of readily metabolized carbon source. For instance, the rate of enzyme-mediated hydrolysis of the cellulose has been reported to be inhibited by products of hydrolysis and fermentation products, particularly when hydrolysis and fermentation are carried out at the same time (Lynd *et al.*, 2002). As a result of presence of repressing substance and end product associated with the fermentation of wastes cellulose, this had led to an increase in the cost of enzymes production. Research articles had been published on catabolite repression of cellulase biosynthesis; however, there is no single research article on catabolite repression of mannanase synthesis in mannolytic microorganisms.

The challenge of catabolite repression of mannanase production in mannolytic microorganisms informed this study and series of efforts have been made to get mutant strains resistant to catabolite repression. It is believed that the use of catabolite repression insensitive mutants would circumvent the repression of mannanase synthesis and allow production of high yields of mannanase in direct microbial fermentation.

The aims of the present study were to produce mutants of *Klebsiella edwardsii* capable of producing large quantity of mannanase and screen the mutants for the isolation of catabolite activation mutants.

## Materials and Methods

### *Bacterial isolate*

The organism, *Klebsiella edwardsii* for this study was supplied from the culture collection of the Department of Microbiology, Federal University of Technology, Akure, Ondo State, Nigeria. The culture was originally obtained from the organic waste (pineapple peels), and screened for mannanase activity (Prescott *et al.*, 1999; Olaniyi and Arotupin, 2013). This strain was selected

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for this study because it gave highest mannanase activity out of the entire bacterial strains screened in our previous work (Olaniyi and Arotupin, 2013). The stock culture was maintained on nutrient agar slants and stored at 4°C in the refrigerator. Subculturing of the organism into new agar slants was carried out every month in order to have fresh culture of the organism throughout the duration of the study. Several preliminary tests including Gram, motility, sulphide indole motility, catalase, nitrate reduction and indole tests, were carried out on the organism in order to confirm the authenticity of the culture.

***Chemical mutagenesis***

The modified procedure of Shonukan and Nwafor (1989) was employed to mutagenize the cells of *K. edwardsii*. Nitrous acid (0.1M sodium nitrite in phosphate buffer, pH 5.0) was used as mutagenic agent. Broth culture of the organism was incubated for 18 to 24 h at 35°C. The culture was centrifuged at 6,000 g for 15 min and the sediment obtained was washed twice with normal saline. Then 0.08 ml of nitrous acid was added to 2.0 ml of the washed cells. The mixture of nitrous acid and the cell was incubated for 1 h at 35°C. At the end of the incubation period, the cell was washed twice with normal saline and diluted 1:10 in a minimal medium (MM) containing 1% LBG, 0.1% peptone, 0.1% yeast extract, 0.2% $NaNO_3$, 0.05% KCl, 1.4% $KH_2PO_4$, 0.06 % $MgSO_4.7H_2O$ and $FeSO_4.7H_2O$ traces, pH 6.8. The culture was incubated at 35 ℃ for 24 h to allow segregation of the mutants. Then, 0.2 ml of the growing cells was plated out on nutrient agar plate using sterile glass spreader. The plates were incubated at 35°C for 24 h to give rise to individual mutant colonies. Twenty isolated mutant colonies were identified properly and transferred to a fresh nutrient agar plate by replica plating technique. These mutants and the wild type strain of *K. edwardsii* were used throughout this work.

***Media preparation and enzyme production***

The mutants and the wild type of *K. edwardsii* were screened for their ability to produce mannanase under submerged state fermentation. Enzyme production was performed in 250 ml Erlenmeyer flask containing 50 ml of enzyme producing medium (PM) modified method of Phothichitto *et al.* (2006). The composition was as followed: 1% LBG, 0.1% peptone, 0.1% yeast extract, 1.4% $KH_2PO_4$, 0.06 % $MgSO_4.7H_2O$, and 1% inoculums, pH 6.8. The flasks were incubated at 35$^oC$ for 24 h on a rotary shaker (Gallenkamp) at 120rpm. Then, the culture broth was centrifuged at 6,000 rpm, 4 ºC for 15 min. The supernatant was collected and kept at -20ºC for further study.

***Assay for mannanase activity***

Mannanase activity of supernatant collected at the end of incubation period was determined using Spectrophotometer (Lab-Tech Digital Colorimeter) by the method of Phothichitto *et al.* (2006). The reaction mixture contained 1ml of 1% LBG dissolved in 50 mM phosphate buffer pH 7.0 and 1 ml enzyme solution. The control tube contained the same amount of substrate and 1ml of the enzyme solution heated at 100$^oC$ for 15 min. Both the experimental and control tubes were incubated 40$^oC$ for 5 min. At the end of the incubation period, tubes were removed from the water bath (Lamfield Medical England Model DK-600), and the reaction was stopped by the addition of 2 ml of 3, 5-dinitrosalicylic acid (DNSA) reagent per tube (Phothichitto *et al.*, 2006). The tubes were incubated for 10 minutes in a water bath for colour development and were cooled rapidly. The activity of reaction mixture was measured against a reagent blank at 540 nm. The released mannose due to mannanase activity was determined by DNS method (Miller, 1959). One unit of mannanase activity is defined as the amount of enzyme, which produced 1 µmol/ml of mannose under the assay conditions.

***Protein Determination***

The amount of protein liberated in the fermentation media was evaluated according to the method designed by Lowry *et al.* (1951) using Bovine Serum Albumin (BSA) as a standard.

***Screening for catabolite repression resistant mutants***

All the mutants and wild type of *K. edwardsii* were cultured in the basal medium containing different concentrations of glucose as energy source (0.1, 0.5 and 1% w/v glucose). The cultures were incubated for

24 h has described above under enzyme production. Mannanase synthesis by each mutant was induced by using 1% (w/v) LBG. After incubation, mutants which exhibited appreciable enzyme activity were referred to as catabolite repression resistant mutants.

***Statistical analysis***

Data presented on the average of three replicates (±SE) are obtained from there independent experiments.

## Results and Discussion

***Morphological and biochemical reaction of the tested culture***

The colonies of culture appeared creamy on nutrient agar. A microscopic examination of the isolate revealed that it was a Gram-negative bacterium with long rod and produced catalase enzyme. Furthermore, the isolate displayed positive reaction on indole test and nitrate reduction while negative reaction was displayed towards sulphide indole motility (Table 1). From these morphological and biochemical reactions, the identity of the culture was authenticated (Olaniyi and Arotupin, 2013).

***Table 1.*** *Morphological and biochemical reaction of the tested culture*

| Biochemical tests | Result |
|---|---|
| Cell shape | Long rod |
| Gram reaction | Negative |
| Motility | Non-motile |
| Sulphide indole motility | Negative |
| Catalase | Positive |
| indole | Positive |
| Nitrate reduction | Positive |

***Mannolytic activity of wild and mutant strains (HN01-HN020) of Klebsiella edwardsii***

In the present investigation, chemical mutagenesis: a conventional approach for mutant generation was adopted to develop mutant strains of *K. edwardsii* for enhanced mannanase biosynthesis. A total of twenty mutant strains were developed from *K. edwardsii* after nitrous acid treatment. Mannanase was synthesized by the mutants at varying quantities (Table 2).

All the mutant strains generated from *K. edwardsii* showed higher specific enzyme activities when compared with the parent strain.

However, of all the mutants generated, mutant designated HN02 had the highest specific enzyme activity of 1005.952 U/mg with approximately 10.2 fold higher than the parent strain. The entire mutant strains of *K. edwardsii* (HN01-HN020) generated by subjection to chemical mutagen, nitrous acid produced promising amounts of mannanase and can therefore be utilized at bioreactor level for commercial production. The variation in mannanase production between mutant strains could be due to some factors like damaged DNA and differences in their ability to repair damaged genes (Narasimha *et al.*, 2012).

The improvement in few of the mutants for mannanase production could be attributed to ease of re-constitution of damaged genes by these strains with improved properties. Improvement of microbial strains for overproduction of industrial bio-products has been the hallmark of all commercial fermentation processes (Iftikhar *et al.*, 2010). Strain improvement is an essential part of process development for fermentation products.

Developed strains can reduce the costs with increased productivity and can possess some specialized desirable characteristics. Such improved strains can be achieved by inducing genetic variation in the natural strain and subsequent screening. The use of mutation and selection to improve the productivity of cultures has been strongly established for over fifty years and is still recognized as a valuable tool for strain improvement of many enzyme producing organisms.

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***Table 2.*** *Mannolytic activity of wild and mutant strains (HN01-HN020) of K. edwardsii*

| Mutants | Mannanase activity (U/ml) | Protein content (mg/ml) | Mannanase activity per mg protein (U/mg protein) |
|---|---|---|---|
| HN01 | $33.306^{m}$±0.10 | $0.250^{k}$±0.10 | $133.224^{n}$±0.10 |
| HN02 | $42.250^{w}$±0.12 | $0.042^{a}$±0.10 | $1005.952^{y}$±0.12 |
| HN03 | $33.111^{l}$±0.12 | $0.074^{b}$±0.10 | $447.446^{z}$±0.12 |
| HN04 | $41.778^{t}$±0.15 | $0.310^{l}$±0.15 | $134.768^{o}$±0.10 |
| HN05 | $34.639^{n}$±0.12 | $0.157^{f}$±0.10 | $220.630^{t}$±0.12 |
| HN06 | $34.917^{o}$±0.12 | $0.199^{h}$±0.12 | $175.462^{q}$±0.10 |
| HN07 | $38.361^{r}$±0.10 | $0.482^{n}$±0.10 | $79.587^{c}$±0.12 |
| HN08 | $24.278^{f}$±0.12 | $0.523^{o}$±0.10 | $46.421^{b}$±0.12 |
| HN09 | $35.083^{p}$±0.1 | $0.329^{m}$±0.1 | $106.635^{k}$±0.10 |
| HN010 | $38.806^{s}$±0.12 | $0.472^{n}$±0.12 | $82.216^{e}$±0.10 |
| HN011 | $36.250^{q}$±0.13 | $0.148^{e}$±0.10 | $244.932^{w}$±0.13 |
| HN012 | $31.750^{k}$±0.14 | $0.324^{m}$±0.10 | $97.994^{j}$±0.12 |
| HN013 | $24.222^{e}$±0.12 | $0.255^{k}$±0.10 | $94.988^{i}$±0.10 |
| HN014 | $27.250^{j}$±0.12 | $0.227^{j}$±0.10 | $120.044^{l}$±0.12 |
| HN015 | $23.194^{d}$±0.10 | $0.153^{e}$±0.12 | $151.595^{p}$±0.12 |
| HN016 | $25.000^{g}$±0.12 | $0.134^{d}$±0.10 | $186.567^{r}$±0.10 |
| HN017 | $16.806^{b}$±0.12 | $0.208^{i}$±0.10 | $80.798^{d}$±0.12 |
| HN018 | $25.417^{h}$±0.12 | $0.125^{c}$±0.10 | $203.336^{s}$±0.12 |
| HN019 | $22.472^{c}$±0.12 | $0.185^{g}$±0.10 | $121.470^{m}$±0.12 |
| HN020 | $25.778^{i}$±0.10 | $0.310^{l}$±0.10 | $83.155^{f}$±0.10 |
| Wild type | $15.806^{a}$±0.10 | $1.708^{p}$±0.12 | $9.254^{a}$±0.10 |

In recent years, new procedures such as rational screening and genetic engineering have begun to make a significant contribution to this study but mutagenesis and selection- so-called random screening is still cost effective procedure, and reliable short term strain development is frequently the method of choice.

Means with the same superscript letters down the column are not significantly different (P>0.05) (Bapiraju *et al.*, 2004). Mutation induction and/or selection techniques together with cloning and protein engineering strategies have been exploited to develop enzyme production (Roja and Prasad, 2012).

The current practice of strain improvement by mutagenesis and selection is a highly developed technique drawing on the latest advances from a wide range of scientific and technical disciplines. Mutagenic procedures can be optimized in terms of type of mutagen and dose. Ultraviolet radiation is one of the well known and most commonly used mutagen. It is universally used to induce genetically improved strains.

There have been many efforts to screen large array microorganisms with ability to produce substantial volume of mannanase for industrial application (Sae-

Lee, 2007; Mabrouk and El Ahwany, 2008). Series of research had been conducted globally to improve microbial strains for enhanced enzyme production; however there is no information on improved microbial strains for enhanced mannanase production. Improved strains could be achieved via physical and chemical mutagenesis (Roja and Prasad, 2012).

***Screening for catabolite repression resistant mutants***

The supplementation of different concentrations of glucose (0.1, 0.5 and 1.0% (w/v)) in the production media had varied influence on the mannolytic activity of the mutants and wild type (Table 3, 4 and 5).

Mannanase production was repressed to varying degrees in 85 percent of the mutants generated in comparison to the wild type (Table 3). However, mannanase biosynthesis potential was completely repressed in the mutants coded HN01, HN02, HN03, HN04, HN05, HN06, HN07, HN08, HN09, HN011, HN012 and HN016 when 0.1 w/v % glucose was supplemented in production media. There was an improvement in mutants HN015, HN017 and HN019 for mannanase production when compared with the wild type.

***Table 3****. Influence of 0.1% (w/v) glucose supplementation on mannanase production potential of mutants of K. edwardsii*

| Mutants | Mannanase activity (U/ml) | Protein content (mg/ml) | Mannanase activity per mg protein (U/mg protein) |
|---|---|---|---|
| HN01 | $1.120^{i}$±0.10 | $1.322^{c}$±0.10 | $0.847^{d}$±0.12 |
| HN02 | $1.116^{i}$±0.10 | $1.353^{c}$±0.10 | $0.825^{d}$±0.15 |
| HN03 | $0.847^{f}$±0.10 | $1.295^{c}$±0.10 | $0.654^{b}$±0.10 |
| HN04 | $0.907^{h}$±0.10 | $1.317^{c}$±0.10 | $0.689^{b}$±0.10 |
| HN05 | $0.884^{g}$±0.12 | $1.351^{c}$±0.10 | $0.654^{b}$±0.10 |
| HN06 | $1.338^{m}$±0.10 | $1.303^{c}$±0.10 | $1.027^{f}$±0.12 |
| HN07 | $1.208^{l}$±0.10 | $1.319^{c}$±0.10 | $0.916^{e}$±0.14 |
| HN08 | $1.167^{jk}$±0.10 | $1.331^{c}$±0.10 | $0.877^{d}$±0.13 |
| HN09 | $1.189^{l}$±0.10 | $1.126^{b}$±0.10 | $1.056^{f}$±0.10 |
| HN010 | $1.954^{o}$±0.10 | $1.352^{c}$±0.10 | $1.445^{g}$±0.10 |
| HN011 | $0.796^{e}$±0.10 | $1.135^{b}$±0.10 | $0.701^{c}$±0.12 |
| HN012 | $1.171^{k}$±0.10 | $1.348^{c}$±0.10 | $0.869^{d}$±0.12 |
| HN013 | $1.218^{l}$±0.10 | $1.389^{d}$±0.10 | $1.140^{g}$±0.10 |
| HN014 | $1.153^{j}$±0.10 | $1.944^{e}$±0.10 | $1.686^{h}$±0.10 |
| HN015 | $1.736^{n}$±0.10 | $12.944^{h}$±0.10 | $7.456^{k}$±0.10 |
| HN016 | $0.255^{a}$±0.10 | $1.342^{c}$±0.10 | $0.190^{a}$±0.12 |
| HN017 | $1.171^{k}$±0.10 | $12.944^{h}$±0.10 | $11.054^{l}$±0.12 |
| HN018 | $0.403^{c}$±0.10 | $0.806^{a}$±0.10 | $2.000^{i}$±0.12 |
| HN019 | $0.616^{d}$±0.10 | $15.583^{i}$±0.10 | $25.297^{n}$±0.12 |
| HN020 | $0.319^{b}$±0.10 | $7.500^{f}$±0.10 | $23.511^{m}$±0.12 |
| Wild type | $2.181^{p}$±0.12 | $10.306^{g}$±0.10 | $4.725^{j}$±0.12 |

Means with the same superscript letters down the column are not significantly different (P>0.05)

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Table 4 revealed the specific enzyme activities of mutants generated from *K. edwardsii* in media supplemented with 0.5% glucose. There was an improvement in terms of mannanase biosynthesis in approximately in 80% of the mutants generated, while the rest exhibited varied degrees of repression when compared with the wild type. The specific enzyme activity exhibited by mutant HN011 (highest mannanase producer) was 157.27 % higher than the value obtained for the wild type.

***Table 4.*** *Influence of 0.5% (w/v) glucose supplementation on mannanase production potential of mutants of K. edwardsii*

| Mutants | Mannanase activity (U/ml) | Protein content (mg/ml) | Mannanase activity per mg protein (U/mg protein) |
|---|---|---|---|
| HN01 | $103.500^{o}$±0.10 | $2.042^{k}$±0.10 | $50.686^{l}$±0.12 |
| HN02 | $41.334^{a}$±0.11 | $1.713^{h}$±0.12 | $24.130^{a}$±0.10 |
| HN03 | $131.832^{w}$±0.12 | $1.352^{e}$±0.10 | $97.509^{w}$±0.10 |
| HN04 | $99.168^{j}$±0.10 | $1.079^{a}$±0.10 | $91.907^{t}$±0.10 |
| HN05 | $101.334^{l}$±0.10 | $1.611^{g}$±0.10 | $62.901^{o}$±0.15 |
| HN06 | $84.834^{e}$±0.10 | $1.700^{h}$±0.10 | $49.902^{j}$±0.10 |
| HN07 | $103.002^{n}$±0.10 | $1.273^{b}$±0.10 | $80.913^{q}$±0.10 |
| HN08 | $99.666^{k}$±0.11 | $1.977^{j}$±0.12 | $50.413^{k}$±0.10 |
| HN09 | $102.666^{m}$±0.10 | $2.310^{n}$±0.10 | $44.444^{g}$±0.10 |
| HN010 | $112.998^{q}$±0.10 | $1.310^{d}$±0.10 | $86.258^{s}$±0.13 |
| HN011 | $129.168^{t}$±0.10 | $1.278^{c}$±0.10 | $101.07^{z}$±0.10 |
| HN012 | $119.166^{s}$±0.10 | $1.810^{i}$±0.10 | $65.838^{p}$±0.10 |
| HN013 | $105.000^{p}$±0.12 | $2.347^{o}$±0.10 | $44.738^{h}$±0.15 |
| HN014 | $74.334^{b}$±0.11 | $2.107^{l}$±0.10 | $35.280^{c}$±0.10 |
| HN015 | $79.500^{c}$±0.4 | $2.519^{q}$±0.10 | $31.560^{b}$±0.10 |
| HN016 | $80.832^{d}$±0.12 | $1.546^{f}$±0.10 | $52.285^{m}$±0.12 |
| HN017 | $91.002^{f}$±0.10 | $2.181^{m}$±0.10 | $41.725^{f}$±0.10 |
| HN018 | $91.836^{h}$±0.10 | $2.403^{p}$±0.10 | $38.217^{d}$±0.10 |
| HN019 | $98.664^{i}$±0.10 | $2.130^{l}$±0.10 | $46.321^{i}$±0.10 |
| HN020 | $118.332^{r}$±0.10 | $2.181^{m}$±0.10 | $54.256^{n}$±0.10 |
| Wild type | $91.500^{g}$±0.12 | $2.329^{n}$±0.12 | $39.287^{e}$±0.10 |

Means with the same superscript letters down the column are not significantly different (P>0.05)

The isolated mutants and the wild type of *K. edwardsii* were subjected to high glucose concentration (1%) as shown in Table 5.

It could be observed that mannanase biosynthesis was improved in approximately 60 percent of the mutants generated when compared with the wild type, while varied degrees of repression occurred in 40 percent of the mutants. The highest specific enzyme activity of 574.846 U/mg was observed in mutant designated HN03.

***Table 5.*** *Influence of 1.0% (w/v) glucose supplementation on mannanase production potential of mutants of K. edwardsii*

| Mutants | Mannanase activity (U/ml) | Protein content (mg/ml) | Mannanase activity per mg protein (U/mg protein) |
|---|---|---|---|
| HN01 | $165.139^{b}$±0.12 | $1.394^{g}$±0.12 | $118.464^{j}$±0.10 |
| HN02 | $385.556^{w}$±0.12 | $1.056^{b}$±0.10 | $365.109^{w}$±0.10 |
| HN03 | $455.278^{z}$±0.12 | $0.792^{a}$±0.10 | $574.846^{z}$±0.15 |
| HN04 | $295.278^{p}$±0.12 | $1.171^{c}$±0.10 | $252.159^{q}$±0.13 |
| HN05 | $138.333^{a}$±0.12 | $1.602^{i}$±0.10 | $86.350^{b}$±0.10 |
| HN06 | $271.806^{k}$±0.12 | $1.468^{h}$±0.10 | $185.154^{n}$±0.10 |
| HN07 | $358.611^{t}$±0.12 | $1.324^{e}$±0.10 | $270.854^{t}$±0.10 |
| HN08 | $343.333^{s}$±0.12 | $1.352^{f}$±0.11 | $253.945^{s}$±0.10 |
| HN09 | $277.778^{m}$±0.12 | $1.185^{d}$±0.13 | $234.412^{p}$±0.13 |
| HN010 | $292.917^{o}$±0.10 | $3.597^{w}$±0.12 | $81.434^{a}$±0.11 |
| HN011 | $210.972^{g}$±0.10 | $1.718^{k}$±0.10 | $122.801^{k}$±0.12 |
| HN012 | $248.194^{i}$±0.10 | $1.917^{o}$±0.10 | $129.470^{l}$±0.41 |
| HN013 | $303.611^{q}$±0.12 | $1.384^{g}$±0.10 | $219.372^{o}$±0.10 |
| HN014 | $206.528^{f}$±0.10 | $2.162^{q}$±0.10 | $95.526^{d}$±0.10 |
| HN015 | $194.583^{e}$±0.12 | $1.773^{l}$±0.10 | $109.748^{h}$±0.10 |
| HN016 | $285.000^{n}$±0.12 | $2.611^{t}$±0.10 | $109.154^{g}$±0.10 |
| HN017 | $275.833^{l}$±0.12 | $1.829^{m}$±0.10 | $150.811^{m}$±0.10 |
| HN018 | $178.333^{d}$±0.12 | $1.963^{p}$±0.10 | $90.847^{c}$±0.10 |
| HN019 | $265.972^{j}$±0.10 | $2.444^{s}$±0.10 | $108.827^{f}$±0.10 |
| HN020 | $175.417^{c}$±0.10 | $1.676^{j}$±0.15 | $104.664^{e}$±0.10 |
| Wild type | $222.639^{h}$±0.10 | $1.884^{n}$±0.12 | $118.174^{i}$±0.10 |

Means with the same superscript letters down the column are not significantly different (P>0.05)

Enzyme production in microorganisms was regulated by induction and repression mechanisms (Bakare *et al.*, 2005; Femi-Ola, 2008). Certain numbers of *K. edwardsii* mutants were sensitive to catabolite repression at the addition of 0.1% glucose concentration to the production medium. The mutagenic agent may not have affected the regulatory gene.

Low enzyme production is expected because the organisms (wild type and mutants) already have the simple sugar, glucose, in their media and hence do not need to secrete the hydrolytic enzymes (proteins) in which mannanase is one. It has been reported by Brock and Madigan (Brock and Madigan, 1991) that cyclic AMP plays a crucial role in catabolite repression mechanisms of exoenzyme production.

This molecule exerts its influence through an allosteric protein known as catabolite gene activation protein (CAP) or cyclic AMP receptor protein (CRP). The cyclic AMP-CRP complex facilitates binding of RNA polymerase to the promoter site of catabolite sensitive operon which alleviates the repression state.

As long as the glucose is available as an energy source, the cyclic AMP concentration is low, thereby preventing the synthesis of enzymes capable of utilizing other energy sources through CRP (Brock and Madigan, 1991; Bakare et al. 2005). This

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observation may also be applicable to mannanase production in mutants of *K. edwardsii*, since mannanase activity was completely lost or reduced in some mutant strains in the presence of different glucose concentrations. Regulation of cellulase production had been well researched and reported (Moreno et al. 2001; Kotchoni et al. 2003; Zang and Lynd, 2005); however, no information has been reported on regulation of mannanase production in microorganisms to date.

Some mutants incubated in media supplemented with 0.5 and 1.0 % glucose could be regarded as catabolite activation mutants because of their ability to produce substantial mannanase in the presence of high glucose concentration. The regulatory gene in these mutants might have lost its regulatory role thereby allowing the catabolite activation mutants to produce mannanase without inhibition by glucose.

## Conclusions

There have been efforts to generate improved microorganisms with high ability to produce microbial enzymes that can meet up with industrial demand of these enzymes. However, no potential mannanase producers have been developed through either chemical or physical mutagenesis till date. All mutants generated from *K. edwardsii* in this study produced promising amounts of mannanase, and some were activated by supplementation of high glucose concentrations. It is unusual behavior that glucose at low concentrations inhibits mannanase activity, but at higher concentrations stimulates it. It is believed that the use of catabolite activation mutants would circumvent the repression of mannanase biosynthesis and allow production of high yields of mannanase in direct microbial fermentation. How nitrous acid triggered catabolite activation in these mutants is not yet elucidated. Therefore, further molecular studies on catabolite activation mutants will be necessary to reveal the mutation.

## Limitations of the study

The wild type of *K. edwardsii* was presumptively identified by means of morphological examination and some biochemical characterizations (Olaniyi and Arotupin, 2013). The molecular studies on the wild type and mutant strains of *K. edwardsii* could not be carried out because of lack of fund and equipments. The work was self-sponsored.

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