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Production, Characterization and Optimal Performance Studies of Glucose Isomerase by *Achromobacter xylosoxidans* mck-4 Isolated from Starch Milling Wastes

M. C. Mbagwu[1], E. J. Egong[2*] and O. D. Akan[3]

[1]International Breweries PLC, Gateway Plant, Sagamu, Ogun State, Nigeria.
[2]Department of Microbiology, University of Nigeria, Enugu State, Nigeria.
[3]College of Food Engineering, Central South University of Forestry and Technology, Changsha, Hunan, China.

Authors' contributions

This work was carried out in collaboration between all authors. Author MCM designed the study, performed the statistical analysis, managed literature searches, wrote the protocol and initiated the study and wrote the first draft of the manuscript. Authors EJE and ODA contributed to the analyses of the study. Author EJE complimented the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Seventy-five (75) bacterial isolates from starch milling wastes in Nsukka, Nigeria were screened for their ability to produce glucose isomerase (GI). The isolate *Achromobacter xylosoxidans* Mck-4 gave the highest activity of intracellular GI using starch casein agar (SCA) medium supplemented with nystatin to eliminate fungi contaminations, incubated at 30°C for 48 hours and pH 7. The SDS PAGE showed that the enzyme was partially purified and the band was near the protein marker of 43 KDa. The enzyme was purified 4.7 fold with a specific activity of 6.4±0.52 U/mg of protein and

**Corresponding author: E-mail: contactegongegong@gmail.com;*

30.9% enzyme recovery in the final desalted sample. The Km value calculated for glucose by Lineweaver Burk plot is 0.89 moles and Vmax was 6.4±0.52 U/mg. The highest activity of the enzyme was at a temperature of 70˚C and pH 7 and maximally activated by the cations; magnesium and cobalt with magnesium having the highest effect followed by manganese and cobalt in individual effects. These parameters for the production of GI by *Achromobacter xylosoxidans* mck-4 are well in the range of economic feasibility; therefore such a process has good chances for industrialization. It is recommended that the gene from our isolate should be isolated and cloned in GRAS status organism to scale up enzyme production and optimize industrial production of high fructose corn syrup (HFCS) in Nigeria using agro-residues.

Keywords: D-glucose/Xylose isomerase; Achromobacter xylosoxidans; fructose; Km (Michaelis Menten constant); agro-residues; enzyme activity.

1. INTRODUCTION

D-glucose/Xylose isomerase (D-Xylose ketolisomerase; EC 5.3.1.5) commonly referred to as glucose isomerase (GI), catalyzes the reversible isomerization of D-glucose and D-xylose to D-fructose and D-xylulose respectively. Interconversion of xylose to xylulose serves a nutritional requirement in saprophytic bacteria that thrive on decaying plant materials and also aids in the bioconversion of hemicellulose to ethanol [1]. The major use of glucose isomerase in commercial production of high fructose corn syrup (HFCS) as a sweetener of choice in the beverage industry is as a consequence of the high cost of production and awareness of the adverse effects of sucrose and invert sugar consumption on human health [1]. A large number of non-calorific and non-carbohydrate artificial sweeteners such as saccharine, cyclamate, acesulfame-K, aspartame and thaumatin have been discovered and dismissed on the basis of health concerns or other drawbacks [1].

Glucose isomerase (GI) is widely distributed in microorganisms mainly as an intracellular enzyme. It has been reported to be produced by *Lactobacillus bifermentans* [2], also extracellularly by *Streptomyces* spp. [3].

The cost of production of the enzyme is an important factor in the evaluation of its suitability for the industrial process application. Elimination of expensive xylose (inducer) and hazardous cobalt from the production media is crucially required [1]. Searching for a strain which can use crude sources of xylose, independent of cobalt ions is a challenge for developing an indigenous technology. There is a need to find a sustainable agro-residue which can substitute expensive xylose.

The intracellular enzyme can be extracted from the cells by treatment with detergents like CTAB, SDS and physical methods like sonication, osmotic shock, applying high pressure as in X-press or French press. Ultrasonication for 30 minutes and treatment with CTAB are efficient methods for extraction according to [3] and [4].

In Nigeria, glucose isomerase and subsequently high fructose corn syrup (HFCS) is being imported up to a tune of about 200 million naira [5] as well as in other African countries and developing nations because they are not locally produced and often patented because of its great commercial importance. Thus, this research was aimed at the adding value to production, characterization and selection of optimal production process for glucose isomerase from a best-producing isolate.

2. MATERIALS AND METHODS

2.1 Isolation of Bacteria from Soil Samples and Optimization of Culture Conditions for GI Production

2.1.1 Isolation of bacteria from soil samples

Bacterial isolates were gotten from soil at a starch milling wastes in Nsukka Local Government, Enugu State, Nigeria and placed in sterile disposable Petri-dishes with pH of the soil acidic and moderately dry. Isolation was by soil dilution plate technique [6] using starch casein agar (SCA) medium (g/L: starch-10, casein-0.3, KNO_3-2, NaCl-2, K_2HPO_4-2, $MgSO_4.7H_2O$-0.05, $CaCO_3$-0.02, $FeSO_4.7H_2O$-0.01 and Agar-18) supplemented with nystatin to eliminate fungi contaminations. Bacteria colonies were marked from two days onwards, and identical colonies were scored out and the selected colonies were subcultured on SCA Petri-plates until clear isolates were gotten.

2.2 Optimization of Culture Conditions for GI Production

2.2.1 Effect of incubation time

The effect of incubation time was determined by inoculating 5.0 mL of inoculum into 500 mL of sterile production medium and incubated at 30˚C on shaker rotary incubator at 120 rpm. 20 mL of culture sample was aseptically collected at 6 hours interval starting from 12 hours up to 96 hours from the fermenting medium. The enzyme extract was examined for the protein content and glucose isomerase activity [7].

2.2.2 Effect of pH

The effect of pH on production was determined by adjusting the pH with a dilute H_2SO_4 and NaOH. The following pH ranges: 4, 5, 6, 7, 8, 9, 10, and 11 were investigated. The enzyme extract was examined for protein content and glucose isomerase activity.

2.2.3 Effect of temperature

The effect of temperature was assessed by incubating the flasks at 20˚C, 25˚C, 30˚C, 35˚C, 40˚C, 45˚C and 50˚C [8] with modification. The enzyme extract was examined for protein content and glucose isomerase activity.

2.3 Screening for Best Glucose Isomerase Producing Bacterial Isolate

The purified isolates (marked Mck-1 to Mck- 75) were inoculated into test tubes containing 10 mL of production culture medium (peptone 1.0 g, yeast extract 0.5 g, $K_2HPO_4$0.3 g, $MgSO_4.7H_2O$ 0.1 g, xylose 1.0 g, distilled water 100 mL), incubated at room temperature for 72 hours [8]. The culture supernatant after centrifugation was assayed for extracellular glucose isomerase production. The intracellular enzyme was obtained by permeabilizing the cell pellet with detergent as described by [3]. Cell pellets were suspended in 0.1% cetyl-trimethyl ammonium bromide (CTAB) at a ratio of 1 mL packed cells to 3 mL of the CTAB solution and permeabilization was carried out for 24 hours at room temperature. After removing cell debris by centrifugation at 10,000 x g for 20 minutes, the enzyme extract obtained was used to assay for GI activity by the method described by [9]. The reaction mixture contained 500 µL of 0.2M phosphate buffer, 200 µL of 1 M glucose, 100 µL of 0.1 M $MgSO_4.7H_2O$, 100 µL of 0.01 $CoCl_2$ and 200 µL of enzyme extract. The volume was made up to 2 mL. Isomerization was carried out at 70°C for 1 hr and the reaction stopped by adding 2 mL of 0.5 M perchloric acid. The amount of fructose formed was determined using Seliwanoff's reaction. An observation was made for substrate utilization and formation of cherry red colour indicating the presence of fructose (ketohexose). The best isolate (Mck-4) was stored in slants, identified on the basis of 16S rRNA gene sequencing by Inqaba Biotechnology Pty South Africa and used for further study.

2.4 Production of Glucose Isomerase

The enzyme production was carried out using the method described by [10]. The production medium (peptone 1.0 g, yeast extract 0.5 g, $K_2HPO_4$0.3 g, $MgSO_4.7H_2O$ 0.1 g, xylose 1.0 g, pH 7.O per 100 mL) was prepared and 20 mL of the medium was dispensed into 50 mL Erlenmeyer flask. This was sterilized, allowed to cool and inoculated with 1.0 mL of the seed culture and incubated at 30°C for 48 hours. The medium was agitated at 120 rpm for better aeration and growth of the organism. Thereafter, the enzyme was extracted according to the method of [3] as earlier described.

2.5 Enzyme Assay

Glucose isomerase activity was assayed by the method described by [9]. The reaction mixture contained 500 µL of 0.2 M phosphate buffer, 200 µL of 1 M glucose, 100 µL of 0.1 M $MgSO_4.7H_2O$, 100 µL of 0.01 M $CoCl_2$ and 200 µL of enzyme extract. The volume was made up to 2 mL. Isomerization was carried out at 70°C for 1 hr and the reaction stopped by adding 2 mL of 0.5 M perchloric acid. The amount of fructose formed was estimated by the [11]. Different concentrations of fructose (2.0-12.0 µmol) were prepared and 0.1 mL of the different concentrations were mixed with 200 µL of 1.5% cysteine hydrochloride, 6.0 mL of 70% H_2SO_4 and 200 µL of 0.12% alcoholic carbazole. Reaction media was read using spectrophotometer at 560 nm. One unit of glucose isomerase activity was defined as the amount of enzyme that produced 1 µmol/mL of fructose per minute under the assay conditions described.

2.6 Determination of Protein Content of Enzyme

The protein content of the enzyme was determined by Bradford method [12] using bovine serum albumin as standard. Exactly 940 µL of Bradford reagent was mixed with 60 µL of the enzyme extract and incubated at room temperature for 5 minutes. The absorbance was measured at 595 nm against a blank that was composed of 60 µL of water and 940 µL of Bradford reagent.

2.7 Partial Purification of Glucose Isomerase

Achromobacter xylosoxidans (Mck-4) was grown in the production medium as described above at 30°C and 120 rpm for a period of 48 hours. The partial purification was started using 1000 mL of enzyme extract obtained as described in the production of the enzyme. Glucose isomerase acitivity and protein content were determined as described above.

2.8 Ammonium Sulphate Precipitation of Crude Enzyme Extract

The crude enzyme extract was chilled by keeping it in the refrigerator for 2 hrs. The extract was saturated to 70% with solid ammonium sulphate crystals. The precipitation was done according to the method by [13]. The precipitates formed were collected by centrifuging the saturated extract at 10,000 x g for 15 minutes. The pellets were dissolved in 0.05 molar phosphate buffer and used to carry out the enzyme assay.

2.9 Gel Filtration for Removal of Salt on Sephadex G-50

A Sephadex G-50 column with a height of 60 cm for high resolution fractionation using 0.05 M Sodium phosphate buffer was used. The enzyme yield from ammonium sulphate precipitation was loaded on a column of Sephadex G-50 as described by Ogbo and Odibo [13]. Enzyme precipitation of ammonium sulphate was applied to the column three times equilibration to the 35 mL buffer in the column before application. Elute fraction labeled G-1 was first collected bearing in mind that molecules with partial access to the pores of the matrix elute from the column in order of decreasing size. Small molecules such as salts that have full access to the pores more than the column but do not separate from each other usually elute just before one total column volume (G-1) of the buffer has passed the column. Thereafter, further fractions G-2, G-3 and G-4 were collected and assayed for GI activity.

2.10 Molecular Weight Determination by Sodium Dodecylsulphate (SDS) Polyacrylamide Gel Electrophoresis (PAGE)

A Sephadex G-50 column with a height of 60 cm for high-resolution fractionation using 0.05 M Sodium phosphate buffer was used for SDS PAGE to determine the molecular weight of the enzyme. The gel (10%) used for electrophoresis was prepared according to the details given by Laemmli [14] for gel cast for electrophoresis. The comb was placed between the glass plates. The gel was left for polymerization for 45 minutes at room temperature. The gel of size 17 by 17.4 cm was prepared. The running buffer was poured in upper and lower chamber of the unit. The desalted sample and protein marker used was loaded in different wells. The marker composed of phosphorylase B of molecular weight 97.4 KDa, bovine serum albumin 66 KDa, ovalbumin 43 KDa, carbonic anhydrase 29 KDa, lactoglobulin 18.4 KDa and aprotonin 6.5 KDa. A constant voltage of 150 volts was applied and the current was run for 4hrs as described by Laemmli [14].

2.11 Characterization of Partially Purified Glucose Isomerase

2.11.1 Influence of substrate concentration on partially purified glucose isomerase activity

The effect of substrate concentration on glucose isomerase activity was determined by varying the glucose concentration in a series of 20 test tubes from 0.1 moles to 2 moles. All the other components of the assay were kept constant. The enzyme assay was done and used to determine the Vmax and Km of the GI and Lineweaver-Burk plot was also gotten for the enzyme.

2.11.2 Influence of temperature on partially purified glucose isomerase activity

The influence of varying temperature on GI activity was determined in a series of test tubes at a wide temperature range. The selected

temperature were; 10°C, 20°C, 30°C, 40°C, 50°C, 60°C, 65°C, 70°C, 75°C, 80°C and 90°C.

2.11.3 Influence of pH on partially purified glucose isomerase activity

The pH was varied in different test tubes using different pH buffers as well as their control. Acetate buffer was used for pH 4 and 5 and phosphate buffer was used for pH 6 and 7. Tris buffer was used for pH 8 and 9 and glycerol buffer for pH 10 and 11.

2.11.4 Influence of metal ions on glucose isomerase activity

Selected metal salts used were magnesium sulphate, cobalt chloride, calcium chloride, copper sulphate, zinc sulphate, barium chloride, nickel chloride, manganese sulphate, iron sulphate, mercuric chloride and silver nitrate. The metal salts were tested by replacing magnesium sulphate from the enzyme assay mixture. All of them were added as 100 µL of 0.1 M concentration in the assay mixture as described by Chen et al. [9].

2.12 Statistical Analysis

SPSS statistical software, version 21 (IBM cooperation, New York, USA) was used to in evaluating the results. Analysis of variance (ANOVA), tables, line plot diagrams, graphs, charts etc was used to present results in a simple, lucid format and each reaction was carried out in triplicates.

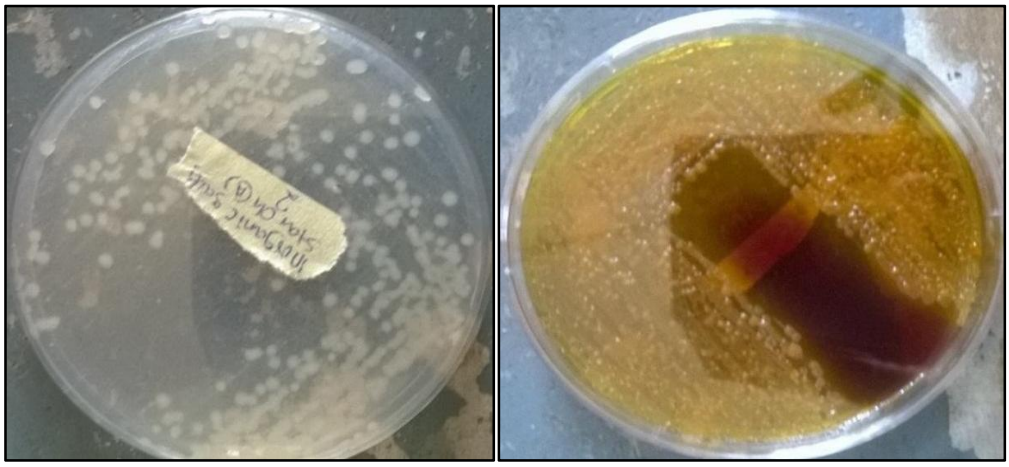
3. RESULTS

3.1 Isolation and Screening for Glucose Isomerase (GI) Production by the Bacteria Isolates

A total of 75 bacterial isolates were gotten from different starch mill waste soil samples, designated Mck 1-75. The bacterial count was about $7.99\pm0.45x10^{7}$cfu/mL. This count carried out to ascertain the actual viable cells involved in the fermentation process. After preliminary screening of the isolates for intracellular GI as well as extracellular GI production using Seliwanoff reaction, 34 isolates gave a colour change while five isolates gave a highly significant positive colour change (cherry red) indicating the production and activity of GI and these were stored in slants. There was no significant result for extracellular GI production by the isolates. The best isolate (Mck-4) for intracellular GI production was characterized biochemically and molecularly and was identified as *Achromobacter xylosoxidanz*.

3.2 Molecular Identification Results

The phylogenetic tree was inferred using the Neighbor-Joining method [15]. The optimal tree with the sum of branch length = 1.55658972 is shown in Fig. 3.



A **B**

Fig. 1. Isolated Culture of *Achromobacter xylosoxidans*Mck-4 (A=on inorganic salts starch agar, B= on carbohydrate assimilation agar (xylose)

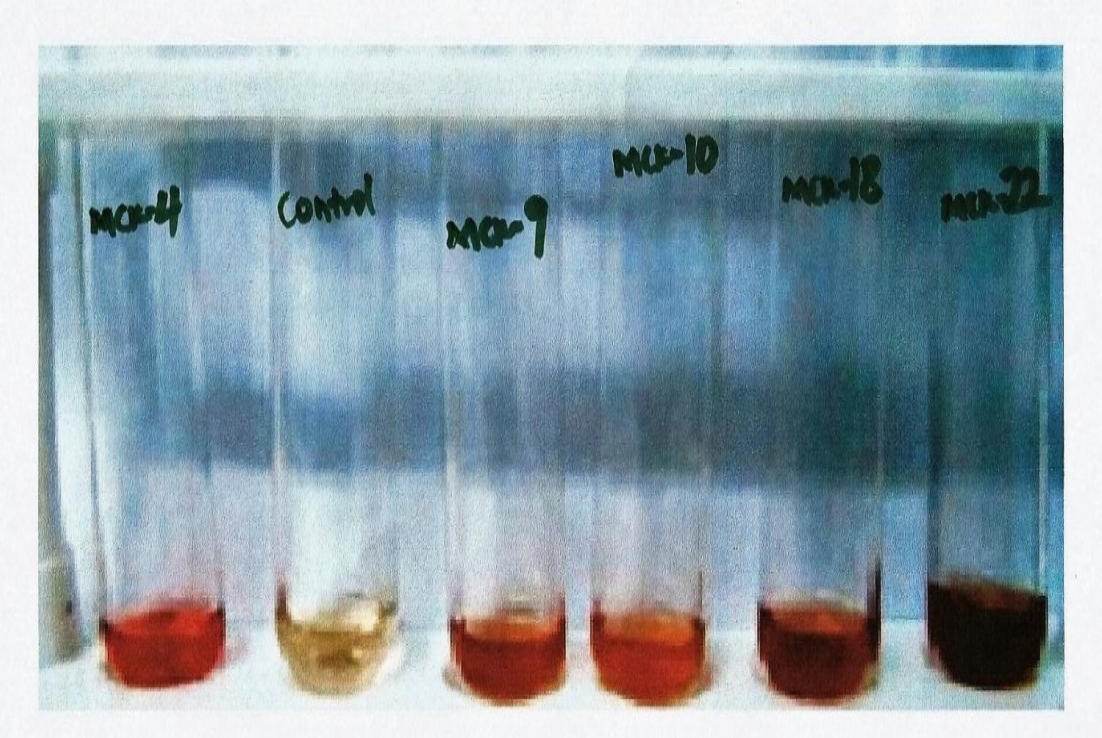


Fig. 2. Preliminary screening of glucose isomerase activity.

Table 1. Molecular identification results

S/N	Lab code	Sample type	DNA type	Primer for sequencing	Accession number	ID of isolate
1	Mck4	2CJ	'	16S	JQ724537	*Achromobacter xylosoxidans*

2CJ_16S 1492-R_G08_20
CAGTGGTAKCGCSCYYCTTGCGGTTAGGCTARCTRCTTCTGGWRAAACCCACTCCCATGGKGT
GACGGGCGGTGTGTACAAGACCCGGGAACGTATTCACCGCGACATGCTGATCCGCGATTACT
AGMGATTCCGACTTCACGCASTCGAGTTGCAGACTGCGATCCGRACTAYGATSGGKTTTSTGG
AATTGGTTCCCCCTCGSGGGTCGSTGACCCTTTGSACCAACCATTGKATGACGGGKGAASCCC
WACCCATAAGGGCCATGAGGACTTGACGYCWTCCCCMCCTTCCTCCGGTTTATCACCGGCAG
TCTCCTTARAGTGCCCTTCCGAASCRAGTAATGACAAGGGTAGGGTTCGTTGCGGGACTTAAC
CCAACCTCTCACTACCCAAGCTGACGATAGCCATGCAKCCCCTGTGTTCGGGTTCTCTTGCCC
GAACTGATAAATCTCTCTCTGATAACAKACATGSCAAGGGAAGGSAMGGAAWGTCSTTTTGCA
TSSATTTAATTWAAACCCCCCACCCCTTGYGCGGGGGGGCGCCCCTTCATTTCCTTTTTAWTTT
TGCTTACCGYACTCCCTCKCCGGTGGGCTTCATTCGTGAGCTGCGTTCCGAAGGKASGAATGA
YCYAWCAGMTAGYTGACATMGWTTGTGTAGRGGGCTGACTACMMAKCTATCTATGYCTGCTC
GCTCCCCACKCTTGCATGACTGASMGTGWTAKCYYGKGCRGCTGACTSCCTTATCCACGGGYG
TYCCTCCATCTATSTATGTATCTGACACATRCRCAATRCATTCTACCCCTCWCTSMCAMAATCT
AGACAGGCAMAAYGAAGGYAKATCSYCRGTGGATSSCMGYTGATTTCWYMTCTGWCTKAWAC
AGMCCGCCTRCGCKCGMTKTWCWCSCAYTAAKAYYRAYKAAYGCWCKCYTMCCSTMYKAWAT
KAYCGCGSKKGCTGGGTACGWMAGATAAGTCGGTGTTTYTTTMTGYAGYTGAACAGWCATKW
TWGCTMGYRYTKRATKMAATGAACRTATTWCTWTACTGACTAGAACKWGATTAAKSATAYCAG
MMGACKGGCAATCGATCMTGCMGSCGASTGMTGCATSATGCACKCMTGGCCTSCSATTCTRA
CAKACAATTMCTGACCTGGCTGGCCKTCACGGCAGAAGGATSTGAAGTGCATGSRGTTCTCAC
CWG

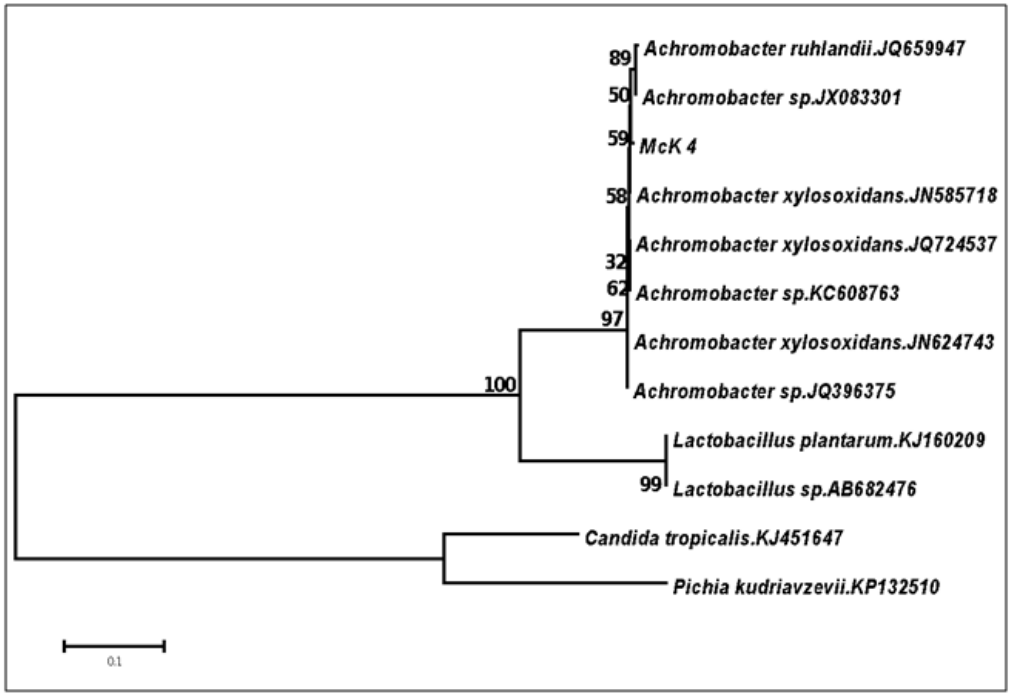


Fig. 3. Phylogenetic tree of the isolate (Mck-4)

3.2.1 Cell counts of seed culture

The viable cell count of $4.33\pm0.37x10^5$cell/mL was seen after staining with trypan blue to check for viability.

3.3 Optimization of Best Conditions for Production

3.3.1 Determination of pH of production medium

pH of final broth ranged from 7.2-9.0 in all production carried out indicating production of alkaline compounds by the organism.

3.3.2 Effect of incubation time on GI production by *Achromobacter xylosoxidans* Mck-4

The incubation time studied was from 12 to 96 hours with six-hour interval check. The organism started accumulating intracellular enzyme production after 12 hours and showed an increase in the enzyme accumulation till 48 hours. The enzyme accumulation at 12 hr was 1.02±0.27 U/mL and gave a peak increase at 48 hours with 3.65±0.34 U/mL but started decreasing sharply until 78 hours with 0.89±0.61 U/mL and remained stagnant till the 96 hours. The result indicates 48 hrs as the optimal time of production of the enzyme by *Achromobacter xylosoxidans* Mck-4. The enzyme is used in the primary metabolism of the organism's growth; therefore its production also coincided with the exponential growth phase.

3.3.3 Effect of pH on glucose isomerase production by *Achromobacter xylosoxidans*Mck-4

The optimal pH during the production of glucose isomerase by *Achromobacter xylosoxidans* Mck-4 was pH 7. Optimal production was observed at pH 7. Low GI activity was observed at pH 4 and 5. Protein content was 2.9±0.16 mg/mL at pH 6 and 4.35±0.31 mg/mL at pH 7. At pH 8 and 9, 3.45±0.38 U/mL of enzyme activity was observed and there was a sharp decrease in protein content at pH 10 and 11.

3.3.4 Effect of temperature on glucose isomerase production by *Achromobacter xylosoxidans* Mck-4

The production of glucose isomerase by *Achromobacter xylosoxidans* Mck-4 was found to be maximum at 30°C. Results from this study

indicate that the flasks incubated at the temperature lower and higher than 30°C had very low concentrations of enzyme. The glucose isomerase activity at 20°C was 0.92±0.24 U/mL, decreased to 0.52±0.11 U/mL at 25°C but was best at 30°C of 3.65±0.48 U/mL. Further increase in temperature decreased the glucose isomerase activity till the least increase of 50°C with little activity.

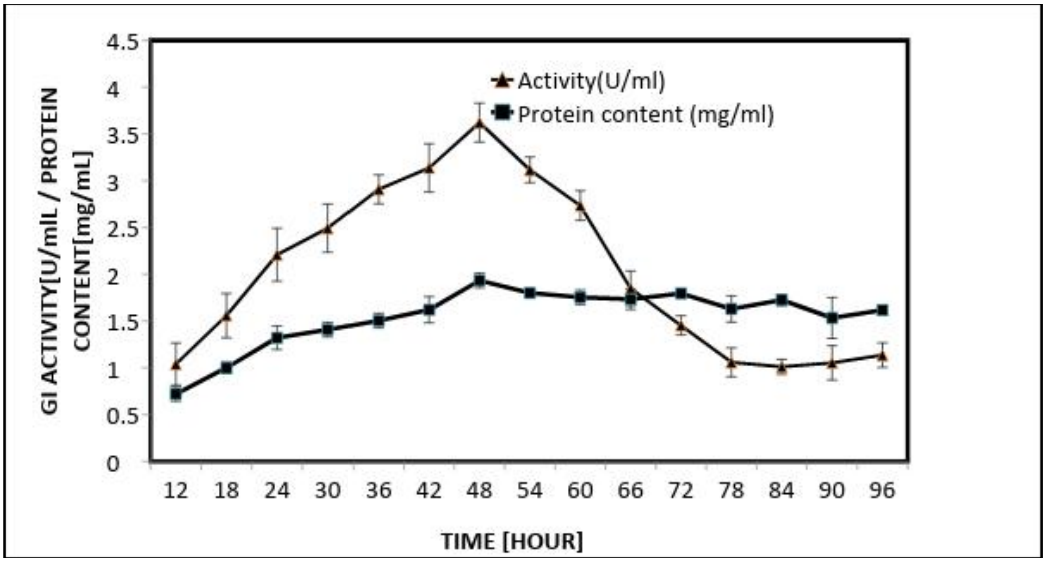


Fig. 4. Effect of incubation time on glucose isomerase production by *Achromobacter xylosoxidans* Mck-4

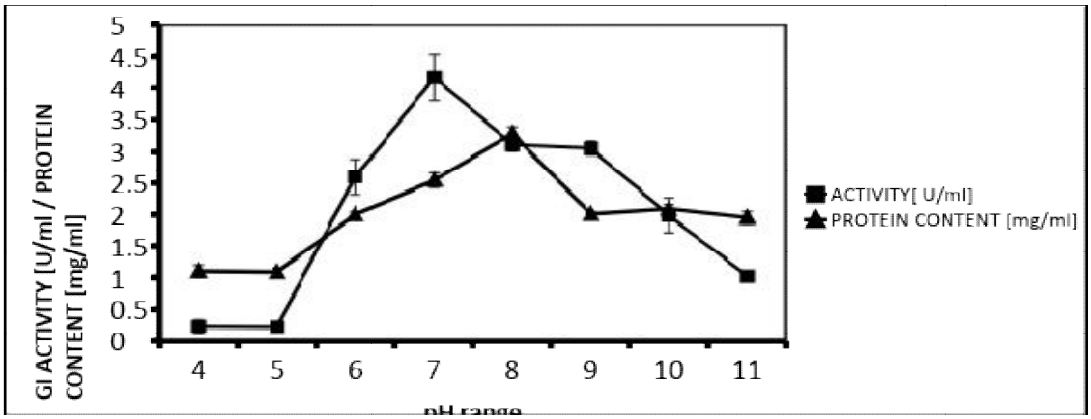


Fig. 5. Effect of pH on glucose isomerase production by *Achromobacter xylosoxidans* Mck-4

Table 2. Summary of glucose isomerase purification steps

Fraction	Activity [U/mL]	Total protein [mg/mL]	Specific activity [U/mg]	Fold purification	Percentage recovery
Crude Enzyme	3.1±0.18	2.3±0.14	1.3±0.10	1.0±0.00	100±0.00
70% NH_4S0_4 precipitation	7.8±0.07	1.8±0.32	4.3±0.17	3.2±0.16	41.6±1.2
Gel exclusion Chromatography on sephadex G-50.	8.6±0.05	1.3±0.24	6.4±0.52	4.7±0.13	30.9±1.6

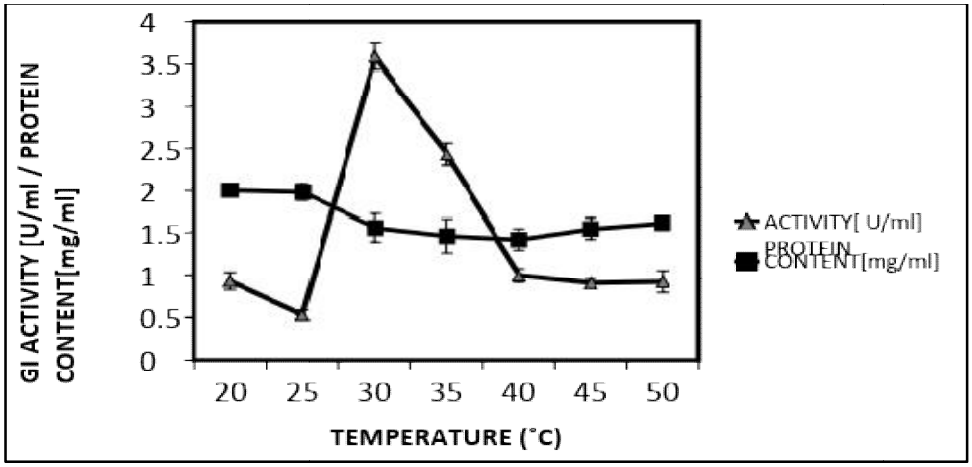


Fig. 6. Effect of temperature on GI production by *Achromobacter xylosoxidans* Mck-4

3.4 Partial Purification and Characterization of GI Produced by *Achromobacter xylosoxidans* Mck-4

3.4.1 Ammonium sulphate precipitation

The protein precipitated by ammonium sulphate was used to perform GI assay after dissolving it in 0.05 molar phosphate buffer. A considerable increase in the specific activity was observed. The specific activity was 1.853±0.32 U/mg of protein for the crude enzyme extract and 4.363±0.17 U/mg of protein of the precipitated enzyme. This step yielded 41.6±1.2 % recovery and 3.2±0.16 purification fold.

3.4.2 Gel filtration for removal of salt on Sephadex G-50

The desalting of concentrated protein containing enzyme was performed on Sephadex G-50. The fraction number G2, G3 and G4 contained the maximum amount of enzyme and they were pooled. G-2, G-3 and G-4 are further elutions from the gel exclusion chromatography. The specific activity determined for this step of purification was 6.4±0.52 U/mg of protein. The fold purification calculated was 4.7±0.13 times with 30.9±1.6 % recovery.

3.5 Results are Mean Values of Triplicate Readings with Standard Deviation

3.5.1 Molecular weight determination by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE)

The glucose isomerase enzyme is a tetramer of four polypeptide chains of 43 KDa. It was observed that the band of partially purified GI on SDS PAGE gel near the marker band of 43 KDa.

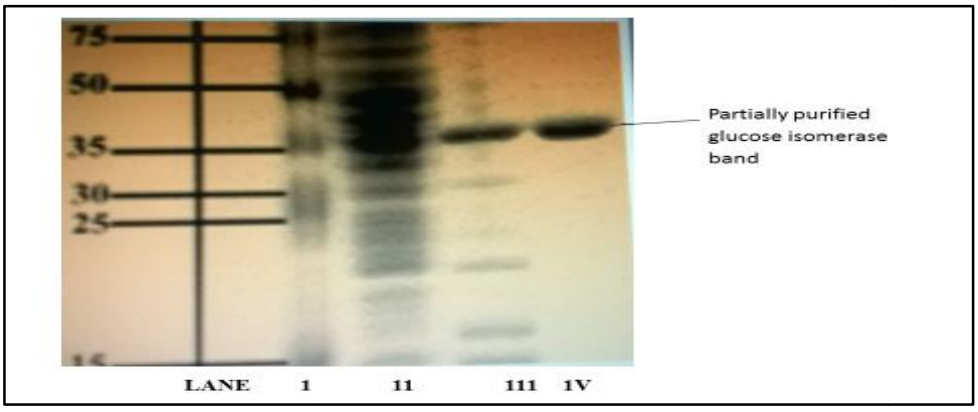


Fig. 7. SDS PAGE of partially purified GI from *Achromobacter xylosoxidans* Mck-4(Lane 11 and 111 shows a band near 43 KDa protein marker and Lane 1 shows protein marker bands)

3.6 Kinetic Characterization of Partially Purified GI

3.6.1 Effect of substrate concentration on gi activity produced by *Achromobacter xylosoxidans* Mck-4

The effect of substrate concentration on GI activity in our study increased progressively till 1.4 moles and there was no further increase observed. The Km calculated for glucose by Lineweaver-Burk plot is 0.89 moles and Vmax was 6.4±0.52 U/mg.

3.7 Effect of Temperature on Glucose Isomerase Activity Produced by *Achromobacter xylosoxidans* Mck-4

The maximum activity of enzyme produced by the isolate *Achromobacter xylosoxidans* Mck-4 was observed at 70°C with enzyme activity of 7.8±0.12 U/mL. The activity was good enough on and above 50°C with a broad range from 60 to 80°C.

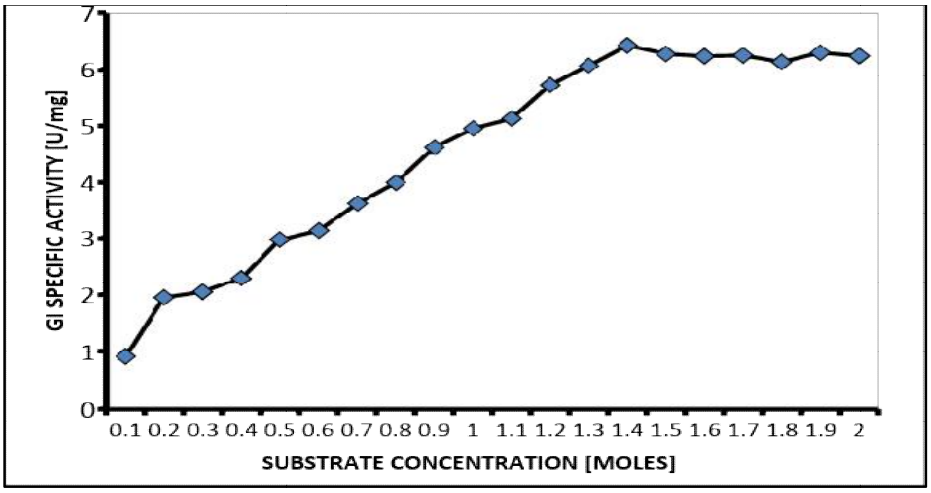


Fig. 8. Effect of substrate concentration on GI activity produced by *Achromobacter xylosoxidans* Mck-4

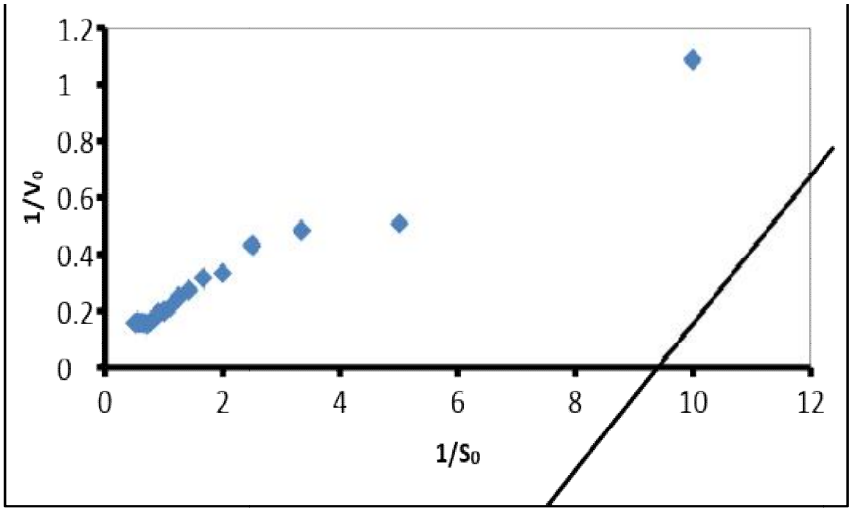


Fig. 9. Lineweaver-Burk plot for GI activity produced by *Achromobacter xylosoxidans* Mck-4

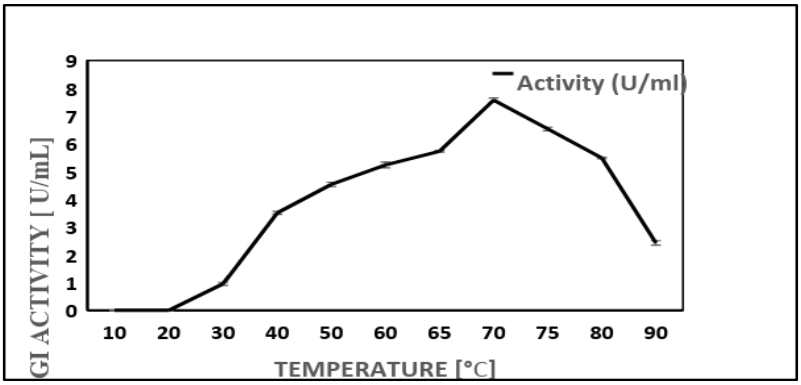


Fig. 10. Effect of temperature on GI activity produced by *Achromobacter xylosoxidans* Mck-4

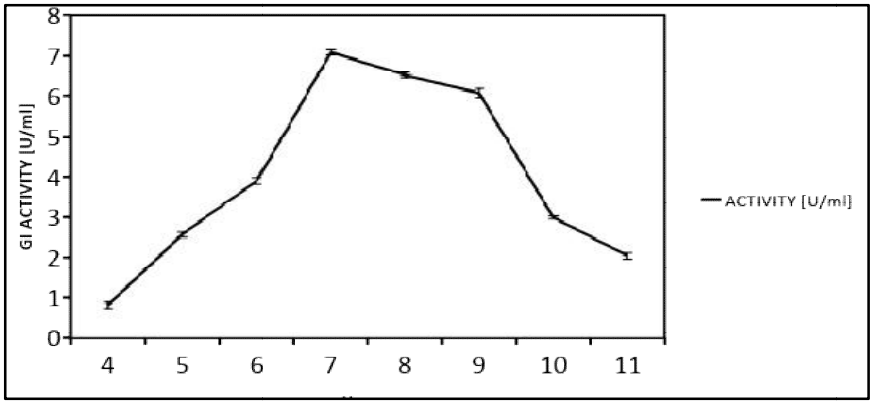


Fig. 11. Effect of pH on GI activity produced by *Achromobacter xylosoxidans* Mck-4

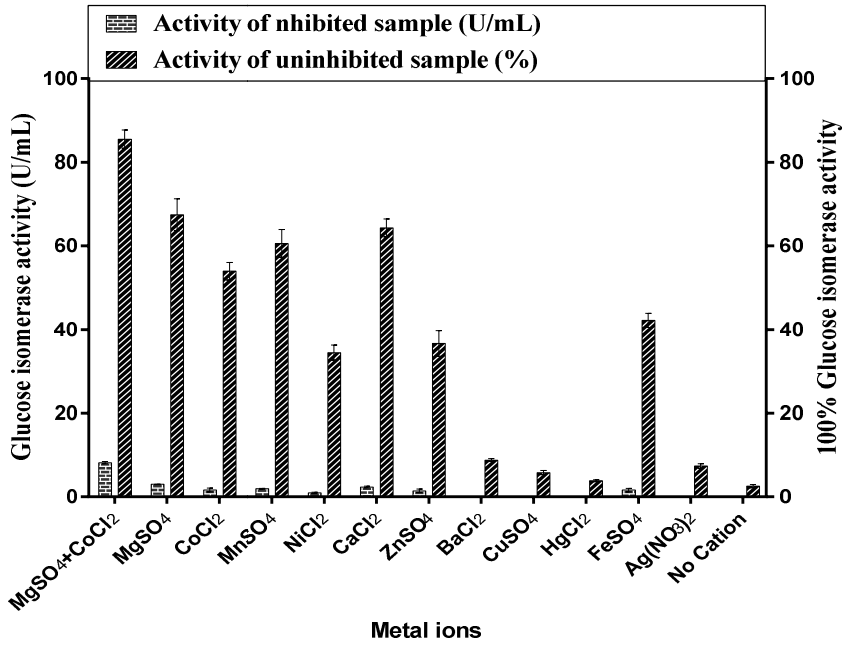


Fig. 12. Effect of metal ions on GI activity produced by *Achromobacter xylosoxidans* Mck-4

3.7.1 Effect of pH on GI activity produced by *Achromobacter xylosoxidans* Mck-4

The optimum pH range observed for GI produced by *Achromobacter xylosoxidans* Mck-4 was from 6 to 9. The highest activity was observed at pH 7 with enzyme activity of 7.1±0.23 U/mL. The results indicate a slightly alkalophilic preference for the enzyme activity with enzyme activity of 6.56±0.08 U/mL at pH 8 and 6.2±0.14 U/mL at pH 9 while lower or acidic pH of 4 and 5 showed low enzyme activity.

3.7.2 Effect of metal ions on GI activity produced by *Achromobacter xylosoxidans* Mck-4

Magnesium and cobalt are the known activators of the glucose isomerase and enhanced the activity of GI produced by *Achromobacter xylosoxidans* Mck-4 according to results from this study. Manganese is the next level of choice of some glucose isomerases which also proved to be an activator for the enzyme studied. Calcium was also found to activate the enzyme to a good extent but nickel, zinc and iron had a very little effect on glucose isomerase activity. On the other hand, barium, mercury and copper totally inhibited the enzyme activity.

4. DISCUSSION

The evolutionary history or phylogenetic tree was inferred using the Neighbor-Joining method [15]. *Achromobacter xylosoxidans* Mck-4 has a bootstrap score of 58 and 59 to *Achromobacter xylosoxidans* with accession numbers JN585718 and JQ724537 percentage match, meaning that the organisms are related but without a strong support. *Achromobacter xylosoxidans* Mck-4 is a pathogen and is not generally regarded as safe (no GRAS status) and therefore cannot be used in the commercial industrial production process for glucose isomerase with subsequent need to an application in high fructose corn syrup production or in other food production process. However, the gene for GI production should be isolated and cloned in GRAS status organism in further studies and industrial production application. In the biochemical characterization studies, *Achromobacter xylosoxidans* Mck-4 possessed the ability to produce amylase that breaks starch into maltose. The isolate gave a positive nitrate reduction test but showed a negative result in melanin production test. This test is done to observe the production of pigments from the organism and also the excretion of pigments into the media. These pigments vary greatly in nature, depending on the composition of different media, the condition of growth and age of culture. The isolate also showed a positive result in gelatin liquefaction when cultured on gelatin medium. The solid character of the medium depends upon the gelatin remaining in the gel state. Many microorganisms including *Achromobacter xylosoxidans* produce exoenzymes that are capable of hydrolyzing gelatin and liquefying the nutrient gelatin medium.

Achromobacter xylosoxidans Mck-4 started accumulating intracellular enzyme after 12 hrs showing an increase in the enzyme accumulation up to 48 hr. The highest production observed by *Achromobacter xylosoxidans* Mck-4 was in 48 hr. The enzyme is thought to be used in the primary metabolism of the organism's growth because its production coincided with the exponential growth phase. The time required for production has a direct influence on the cost of the production process. A fast-growing strain in an optimally rich medium is desirable and shall definitely give high yields as observed from the isolate Mck-4.

The progressive increase in the specific activity of enzyme through the partial purification with a 4.7 fold purification of enzyme with specific activity of 6.4±0.52 U/mg of protein and 30.9±1.6% enzyme recovery in the finally desalted sample, shows that the GI enzyme required purification as well as the effectiveness of the purification protocol used in this study to obtain partial pure enzyme extract and best maximal activity. A clear band of the partially purified enzyme near the protein marker of 43 KDa was observed, which indicates the molecular weight of this enzyme as the same from previous studies [1]. The glucose isomerase enzyme is a tetramer of four polypeptide chains of 43 KDa. The results of SDS PAGE are shown in Fig. 7. Gong et al. [16] also reported the presence of one protein band of glucose isomerase purified from *Actinoplanes missouriensis* on SDS-polyacrylamide gel. The molecular weight of this polypeptide band was measured to be 42 KDa. The molecular weight of glucose isomerase from *Bacillus coagulans* (58 KDa) is considerably larger than glucose isomerase from *Streptomyces sp.* [17]. Yassien et al. [18] determined molecular weight of *Streptomyces albanducus* to be 54 KDa by SDS-PAGE. Pandidurai et al. [8] stated 60 KDa as the molecular weight of *Enterobacter aglomerans* using SDS-PAGE. Borgi et al. [19] reported SDS-

PAGE analysis of recombinant glucose isomerase (SKGI) of *Streptomyces* sp. to be 180 KDa tetramer of four 43 KDa subunits. Different microbial glucose isomerases characterized previously vary in molecular weight from 80 KDa to 20 KDa and are composed of two or four identical subunits. The molecular weight of glucose isomerase produced by *Clostridium thermosulfurogenes* and *Thermoanaerobacter* strain B6A is 20 KDa with tetrameric subunit composition reported by Lee and Zeikus [20] was similar to the 19.5 KDa enzymes present in *Lactobacillus brevis* reported by Yamanaka [21]. Glucose isomerases characterized from *Streptomyces sp*. and *Arthrobacter* were observed to be 18.5 KDa as observed by Kasumi et al. [22] and Smith et al. [23]. GI from *Bacillus coagulans* and *Flavobacterium arborescens* also ranged between 15.7 KDa and 18.5 KDa with tetrameric subunit compositions. The size and shape of glucose isomerase crystals vary from different sources. Glucose isomerases from *E. coli,* alkalophilic *Bacillus*, *Actinoplanes missouriensis* and *Streptomyces olivochromogenes* are dimers, with a molecular weight of 80 - 120 KDa [20].

The effect of substrate concentration on GI activity in our study increased progressively till 1.4 moles after which there was no further increase observed. The Km calculated for glucose by Lineweaver-Burk plot is 0.89 moles and Vmax was 6.4±0.52 U/mg. The results are depicted in Fig. 9. This indicates that GI in this study possesses higher Km value for glucose with a significance of higher production volume and faster utilization of substrate for product yield. Glucose isomerase produced from *Actinoplanes missouriensis* exhibited Km value of 1.33 moles for glucose [15]. The Km reported by El-Shora et al. [24] from *Bacillus thuringiensis* was 8 mM for glucose. Dhungel et al. [4] reported 0.45 M as Km and 0.18 U/mg as Vmax for glucose by using psychrotolerant *Streptomyces* sp. Raykovska et al. [25] observed the Km and Vmax as 55.5 mM and 31.3 U/mg for *Streptomyces thermovulgaris*. Lee and Zeikus [20] reported Km of 140 and 120 mM for glucose by GI from *C. thermosulfurogenes* and *Thermoanaerobacter* respectively. Liu et al. [26] worked out Km for *Thermoanaerobacterium* strain JW/SL-YS 489 to be 130 mM and Vmax 6.0 U/mg. Fan et al. [27] observed Km and Vmax values to be 11 mM and 25 U/mg respectively for xylose. Glucose isomerase from *Thermotogamaritima* exhibited Km value of 118 mM for glucose [28]. The enzyme possesses higher Km value for glucose than fructose. Lama et al. [29] studied the glucose isomerase catalytic properties of *Bacillus thermoantarticus* and observed Km and Vmax values to be 167 mM and 6.3 U/mg respectively. Ogbo and Odibo [13] determined Km and Vmax for glucose isomerase produced by *Saccharococcuscaldo xylosilyticus* to be 334 mM and 0.92 U/mg of protein. Yanmis et al. [30] determined Km and Vmax for xylose isomerase produced by *Anoxybacillus gonensis* G2 to be 25±2 mM and 0.12±0.0002 U/mg.

The maximum activity of the enzyme produced by our isolate *Achromobacter xylosoxidans* Mck-4 was observed at 70°C. The activity was good enough on and above 50°C with a broad range from 60 to 80°C. This is attributed to the ability of this enzyme to be thermostable in the presence of cobalt and magnesium ions as there was no enzyme activity in the absence of these metal ions. This broad range optimum activity is beneficial for industrial purposes because maintenance of very critical and narrow temperature range is difficult at the commercial level. The results are depicted in Fig. 10. The optimal temperature reported for glucose isomerase from a thermophilic organism is usually above 80˚C. Brown et al. [28] observed 105-100°C as optimum temperature for GI from *Thermotoga maritime*. GI from *Thermoanaerobacter ethanolicus* was found to isomerize best at 85°C by Fan et al. [27] and the enzyme produced by *Thermotogane apolitana* was extremely thermostable with an optimum temperature at 95°C [31]. El-Shora et al. [24] found out that glucose isomerase from *Bacillus thuringiensis* isomerizing best at 50°C. Lee et al. [32] observed the optimal activity of GI from *Dunaliellater tiolecta* to be 60°C. Mu et al. [33] found optimal isomerization temperature of 80 ˚C for GI from *Acidothermus cellulolyticus*. Ogbo and Odibo [13] reported the highest activity of GI from *Saccharococcus caldoxylosilyticus* at 60˚C, Sriprapundh et al. [34] and Sanchez and Smiley [35] found GI from *Streptomyces* sp. isomerizing optimally at 85°C. *Streptomyces albus* isomerized optimally at 70-80°C. Kwon et al. [17] found GI from an alkalophilic *Bacillus sp*. isomerizing best at 60°C and Lama et al. [29] found GI from *Actinoplanes missouriensis* working best at 60°C. Raykovska et al. [25] studied *Streptomyces thermovulgaris* GI which has optimum activity between 60-85°C. Yassien et al. [18] found an optimum temperature of activity of GI for *Streptomyces albanducus* at 80°C. Yanmis et al. [30] determined an optimum temperature of activity for *Anoxybacillus*

gonensis GI at 85°C. Kim and Pyong-Su [36] found GI from *Streptomyces* sp. having temperature optima of 80°C.

Glucose isomerase is known to be a thermostable enzyme which has optimum activity at high temperature. Glucose isomerases are generally classified into two types of enzymes according to their thermal stability. Glucose isomerase from *Lactobacillus brevis* and *E. coli*, which are active at 37-50°C, belong to the thermolabile type of glucose isomerase [21]. Glucose isomerases from certain mesophilic microbial sources (e.g. *S. phaeochromogenes* and *A. missouriensis*), however, can display quite high thermal stability. Glucose isomerases produced from *C. thermosulfurogenes* and *Thermoanaerobacter, Thermotogamaritima*, *Thermotoganeapolitana* are highly thermostable and they belong to the latter type of glucose isomerase [20].

The optimum pH range observed for GI produced by *Achromobacter xylosoxidans* Mck-4 was from 6 to 9. The highest activity was observed at pH 7 but the results indicate a slightly alkalophilic preference for the enzyme activity which is in accordance with most of the earlier reports. The results are presented in Fig. 11. Fan et al. [27] observed pH 7 as optimum for *Thermoanaerobacter ethanolicus*. Ogbo and Odibo [13] found *Saccharococcus caldoxylosilyticus* exhibiting maximum activity at pH 6.4, Sriprapundh et al. [34] found an optimum activity for *Thermotogane apolitana* at pH 7, Lama et al. [29] *Bacillus thermoantarticus* pH 7. El-Shora et al. [24] found their glucose isomerase from *Bacillus thuringiensis* isomerizing best at pH of 7.0. Lee et al. [32] reported pH of 6.0 as optimum for *Dunaliellater tiolecta*. Mu et al. [33] observed optimal pH at 6.5 for *Acidothermus cellulolyticus*. Sukumar et al. [37] found GI optimal pH for isomerization for thermophilic *Bacillus* sp. to be 8.0**.** Lee and Zeikus [20] observed optimum pH for the glucose-isomerizing activity of *C. thermosulfurogenes* and *Thermoanaerobacter* at 7.0-7.5. Raykovska et al. [25] studied *Streptomyces thermovulgaris* which exhibited the highest activity at pH 7.0. Bazaraa and Hassan [38] observed theirs to be pH 7-7.8 as well as Paik and Dewey [31] for *Streptomyces sp*. Kwon et al. [17] found alkalophilic *Bacillus* sp. isomerizing best at pH 7.5, Sanchez and Smiley [35] also observed *Streptomyces albus* isomerizing best at pH 7 to 9. Gong et al. [16] reported *Actinoplanes missouriensis* optimal isomerization pH of 7.0. Kovalenko et al. [39] reported the shift in pH optima from neutral to acidic on developing a recombinant *E. coli* strain using xylose isomerase gene from *Arthrobacter nicotianae*.

Glucose isomerases from different microbial sources responded differently to the presence of divalent cations. El-Shora et al. [24] found their glucose isomerase from *Bacillus thuringiensis* requiring Mn^{2+} and Ca^{2+} as cofactors. Yanmis et al. [30] stated divalent cations Co^{2+}, Mg^{2+}, and Mn^{2+} were essential for xylose isomerase produced by *Anoxybacillus gonensis* whereas bivalent metal ions Ca^{2+}, Hg^{2+}, Ni^{2+}, Zn^{2+}, Fe^{2+} and Cu^{2+} showed inhibitory effects. Karaoglu et al. [40] reported glucose isomerase from *Anoxybacillus gonensis* G2 requiring the divalent cations of Co^{2+}, Mn^{2+} and Mg^{2+} for its maximal activity and thermostability. Fan et al. [27] studied *Thermoanaerobacter ethanolicus* and reported a great deal of enhancement in activity in the presence of magnesium and cobalt ions. Ogbo and Odibo [13] *Saccharococcus caldoxylosilyticus* requires Mn>Mg>Co. Copper was found to inhibit GI activity but calcium did not show such an inhibition**.** Lee and Zeikus [20] stated that the treatment of enzyme preparation with EDTA renders enzyme totally inactive.

5. CONCLUSION

*Achromobacter xylosoxidans*Mck-4 isolated in this study which showed remarkable ability to produce glucose isomerase using crude agro-residues is a pathogen and is not generally regarded as safe (no GRAS status) and therefore cannot be used in commercial industrial production process for glucose isomerase with subsequent need to apply in high fructose corn syrup production or in other food production process. However, the gene for GI production should be isolated and cloned in a GRAS status organism in further studies and used for the industrial production application. GI produced by *Achromobacter xylosoxidans* Mck-4 being maximally activated by the presence of both the cations magnesium and cobalt as well as Calcium is indicative of the possible use of this enzyme in simultaneous saccharification and isomerization reactions for high fructose corn syrup production because of the use of calcium ions as the main activator of amylase enzymes in saccharification reactions. All these parameters are well in the range of economic feasibility for industrial isomerization reactions for production of high fructose corn syrup (HFCS); therefore the

gene from our isolate should be isolated and cloned in GRAS status organism and enzyme production from the isolate can be scaled up and optimized for industrial production of HFCS in Nigeria using these agro-residues. This is the first report to our knowledge stating the production and characterization of glucose isomerase from *Achromobacter xylosoxidans* utilizing crude agro-residues in Nigeria and beyond.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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