

Optimization of Catalase Production from CRISPR-Cas9-Mediated Editing of the *LacZ* Gene in *Escherichia coli* Using Various Carbon Sources (*Manihot esculenta* Crantz, *Oryza sativa* L., *Solanum tuberosum* L., and *Glycin max* (L.) Merr.)

Minari, J.B; Ekundayo, D.D; Anams, C. P; Kehinde, S. T; Ojo, D.O; Dada, I.S

Genome Editing Research Group, Department of Cell Biology and Genetics, Faculty of Life Science, University of Lagos, Nigeria

Abstract

Catalase is an important antioxidant enzyme widely applied in food processing, textiles, and biomedical industries, yet it is largely imported into Nigeria at considerable cost. This study investigated the use of CRISPR-Cas9-edited *Escherichia coli* HB101-pBRKan and locally available agricultural by-products as low-cost carbon sources for catalase production. Cassava peel, potato peel, rice bran, and soybean meal were processed into powdered substrates and incorporated into a basal medium containing KH_2PO_4 (2.13 g/L), MgSO_4 (0.5 g/L), and NaH_2PO_4 (1.5 g/L). Fermentations were carried out at varying temperatures (4, 37, and 50 °C) and pH values (4.5, 6.5, and 9.0). Growth was monitored for seven days using $\text{OD}_{540 \text{ nm}}$, and the spectrophotometer was used to assess the catalase activity through hydrogen peroxide decomposition. The *lacZ* gene was targeted with CRISPR-Cas9 to examine its influence on catalase production. Colony colour on X-gal plates reflected editing outcomes: blue colonies appeared where *lacZ* remained intact, white colonies indicated successful editing, and plates lacking arabinose showed no growth due to insufficient Cas9 induction. Multiplex PCR confirmed gene disruption, producing an approximately 650 bp amplicon in edited strains and 1100 bp fragment in unedited controls. Temperature influenced enzyme output markedly. The edited strain showed improved tolerance to higher temperatures, achieving its highest catalase activity at 50 °C (1.6 U/mL), whereas the unedited strain reached maximal activity at 37 °C (1.0 U/mL). Clear differences were observed in the substrates: cassava peel supported the highest catalase yield ($\text{OD}_{540} = 1.591$ at pH 6.5 and 50 °C), while potato peel substrates demonstrated optimal activity ($\text{OD}_{540} = 0.595$ at pH 6.5 and 37 °C). In contrast, soybean meal and rice bran favoured the unedited strain in both growth and enzyme production. Overall, this study shows that CRISPR-Cas9-Edited *E. coli* can produce higher levels of catalase under the right environmental conditions. It also identifies cassava peel waste as a strong, practical option for local enzyme production. Together, these findings point to a realistic path for turning agricultural waste into value and using gene-edited microbes to cut Nigeria's reliance on imported catalase.

Keywords: CRISPR-Cas9 gene editing, *lacZ* gene, catalase, substrates fermentation, *E. coli*.

Introduction

The catalase enzyme, (E.C. 1.11.1.6), is an important antioxidant enzyme. The enzyme is present in nearly all aerobic organisms. To avoid cell damage due to oxidation reactions, it is important for the degradation of hydrogen peroxide into water and oxygen (Rasheed, 2024). The enzyme is very conserved among different

species, including bacteria, plant life, and animals, and is differentiated based on their structure, function, and evolution (Pan *et al.*, 2022). Although the enzyme can be used in industries to bleach textiles, remove starch from fabric, and make other important materials, access to this enzyme is very limited and expensive because it is being imported into Nigeria (Kumar *et al.*, 2021). Importation of this

important enzyme, therefore presents a very big problem, especially where industries such as textile industries require this enzyme in large quantities for most of their operations, such as the degradation of lignin. Therefore, it is important to look for other alternative sources of this enzyme, which will make it cheap and available to textile industries in developing countries such as Nigeria. Getting this important enzyme will therefore require considering other materials derived from local resources, which are not being used. Based on this idea, this research will therefore focus on locally producing this important enzyme using agricultural by-products such as cassava peel waste, potato peel, rice bran, and soya meal in Nigeria using Clustered Regularly Interspaced Palindromic Repeats and CRISPR-associated proteins (CRISPR-Cas9)-edited *E. coli*, which will alter the *lacZ* gene in *E. coli*. The above idea will be very important since cassava peel, peel of potatoes, rice bran, and soya meal can be regarded as important agricultural by-products in Nigeria, which are vastly underutilized. Their conversion into another very important product such as this enzyme will therefore have a very positive impact on ensuring proper waste management takes place in this country.

The *lacZ* gene codes for an enzyme called β -galactosidase, which hydrolyzes lactose into glucose and galactose. *E. coli*'s *lacZ* gene has considerable significance in molecular biology since it dates back to studies conducted by Jacob and Monod, which highlighted the regulation of genes and focused on β -galactosidase production from *lacZ* in the presence of an inducer (Beal *et al.*, 2023). Beyond its metabolic role, *lacZ* is one of the most widely used reporter genes in molecular biology (Lambre *et al.*, 2023), including in CRISPR-Cas9 systems where disruption of the gene can be visually confirmed through blue–white screening (Yang *et al.*, 2024). Altering *lacZ* can shift carbohydrate utilisation pathways, potentially influencing oxidative stress responses. Since catalase helps neutralise reactive oxygen species, such metabolic adjustments may affect its production (Karkare *et al.*, 2021).

CRISPR-Cas9 is a gene editing tool that has revolutionized research in a wide array of disciplines, including but not limited to medicine, agriculture, and biotechnology (Mengstie *et al.*, 2021). While CRISPR-Cas9 is primarily derived

from a bacterial defence system, it has since been harnessed to make precise cuts in DNA in model organisms, where this technology can allow scientists to insert, remove, or perhaps repair genes in a cell (Li *et al.*, 2023). CRISPR-Cas9 is being used to this day to knock out or knock in genes or edit existing ones in *E. coli* bacteria (Tong *et al.*, 2023). *Escherichia coli* can be considered a model prokaryote with different strains that include both pathogenic and non-pathogenic variants. For this study, a non-pathogenic variant of *E. coli* (HB101-pBRKan) was used because of its natural ability to synthesise catalase for protection against reactive oxygen species such as hydrogen peroxide (Fasnacht & Polacek, 2021).

The agricultural by-products considered in this work have different nutritional qualities suitable for microbial fermentation. Cassava peel, obtained from *Manihot esculenta* Crantz, contains many carbohydrates such as cellulose and hemicellulose present in its cell structure, which favors microbial growth and enzyme production (Mohidin *et al.*, 2023). Peels of potatoes are mainly high in starch and non-starch carbohydrates, which make them ideal for microbial enzyme production in bioprocesses (Javed *et al.*, 2019). Rice bran has a combination of carbohydrates, proteins, vitamins, and minerals with nutritional potential in increasing microbial metabolic functions during fermentation (Devi *et al.*, 2021). Soybean meal is a good nutritional source of proteins, which serves as a nutrient-balanced by-product in the production of soybean oil, making it ideal for increasing microbial activity and enzyme production in different bioprocesses (Miao *et al.*, 2023).

The use of these under-exploited resources in producing catalase can therefore offer a chance for improving local biotechnology, practising a circular economy, and ensuring industries have access to a cheaper enzyme. The work will examine the possibility of using CRISPR-Cas9 edited *E. coli*, which can grow using chosen agro-waste biomass, to increase production of the enzyme catalase.

Materials and Methods

Ethical Approval / Biosafety Considerations: The protocol for this study was submitted to the University of Lagos Research Ethics Committee (UNILAGREC), Cell Biology and Genetics Branch, and approval with the **Reference Number UNILAGREC/26/01/021**, was obtained. UNILAGREC serves as the Ethical and Biosafety Committee of the University of Lagos. All experiments were carried out under the supervision of the UNILAGREC members at the Department and in accordance with the National Biosafety Guidelines and Institutional Regulations.

Materials: CRISPR-Cas9 gene-editing reagents and the associated instructional kit were obtained from Bio-Rad Laboratories (USA). All chemicals were of analytical grade. Cassava peel, potato peel, rice bran, and soybean meal were sourced from local markets in Ekiti, Lagos and Ogun States, Nigeria. Other media components were purchased commercially.

Gene Editing

Preparation of LB Agar Plates: Lysogeny Broth (LB) agar plates supplemented with kanamycin, IPTG, and X-gal (collectively referred to as KIX plates) were prepared following the Bio-Rad CRISPR kit protocol, with minor adaptations. Three types of plates were required: KIX plates, containing kanamycin, IPTG, and X-gal; KIX/ARA plates, containing KIX additives plus arabinose; and KIX/SPT plates, containing KIX additives supplemented with spectinomycin.

To make additive solutions, arabinose and spectinomycin were rehydrated with sterile distilled water. KIX mix (kanamycin, IPTG, X-gal) was dissolved in distilled water according to the kit protocol.

A volume of distilled water sufficient for each formula was added to sterile Erlenmeyer flasks, followed by a powdered LB agar mix. After complete suspension of the KIX mix, the agar mixtures were heated in a microwave in short intervals until fully melted. KIX/ARA plates were prepared by adding rehydrated arabinose to the molten KIX agar before dispensing. KIX/SPT plates were prepared by cooling the molten agar to approximately 50 °C before adding spectinomycin to prevent heat degradation of the antibiotic.

Plates were poured to a depth of approximately 10 mL, allowed to solidify, and left to dry at room temperature for 48 hours in the dark to improve transformation efficiency. Dried plates were wrapped in aluminium foil to protect the light-sensitive additives, returned to their plastic sleeves, and stored inverted at 4 °C until use, typically for no longer than two weeks.

Rehydration of E. coli: Lyophilised *E. coli* HB101-pBRKan cells were rehydrated in sterile LB broth prepared according to standard microbiological procedures. Approximately 250 µL of room-temperature LB broth was added to the vial, and the cells were gently re-suspended and incubated at 37 °C for 8–24 hours to obtain an overnight culture. Fresh LB broth was stored at 4 °C and reheated to room temperature as required. For all transformation reactions and fermentation experiments, 1% (v/v) of the overnight culture was used as the inoculum. Experiments were performed in triplicate to ensure reproducibility.

Preparation of Starter Plates: Starter cultures were prepared by streaking the rehydrated strain onto KIX and KIX/ARA plates using the quadrant streaking method. Plates were incubated inverted at 37 °C for 24 hours in the dark to allow colony development. In addition, colonies were allowed to grow, when necessary, at room temperatures (20-25°C).

Preparation of Transformation Reagents: The transformation reagents used in this protocol include transformation solution (TS), LB broth, donor plasmid (pLZDonor), and donor guide plasmid (pLZDonorGuide). The tubes used in preparing these reagents were microcentrifuge tubes, which were sterilized. The tubes were labeled appropriately. Transformation solution and LB broth were placed in separate tubes, but 25 µL of either pLZDonor or pLZDonorGuide were placed in separate tubes for plasmids. In addition, all reagents were stored at 4°C before being used. The tubes containing plasmids were centrifuged briefly to allow all content to be at the bottom.

CRISPR-Cas9 Gene Editing Procedure: The gene-editing workflow followed the manufacturer-recommended CRISPR-Cas9 protocol with minor adjustments. Four microcentrifuge tubes (A–D) were placed on ice and pre-chilled transformation solution was added. Bacterial colonies were collected from KIX or KIX/ARA plates and

suspended in the transformation solution in each tube.

Tubes A and C received the donor plasmid (pLZDonor), while tubes B and D received the donor-guide plasmid (pLZDonorGuide). After gentle mixing, samples were incubated on ice for at least 10 minutes followed by a brief heat-shock step at 60 °C for 50 seconds. Tubes were immediately returned to ice to stabilise the cells.

LB broth (250 µL) was added to each tube to support recovery, and samples were incubated at room temperature for 20 minutes or left overnight. Aliquots (100 µL) of each transformation mix were spread onto separate KIX/SPT plates labelled A–D and incubated at 37 °C for 24 hours.

Following incubation, plates were examined for the appearance of blue and white colonies corresponding to unsuccessful and successful CRISPR-Cas9 editing, respectively. When colour contrast was weak, plates were refrigerated for 1–5 days to improve visual differentiation.

Guide RNA and Donor template DNA design: A single guide RNA (sgRNA) targeting the *lacZ* locus was designed using the CHOPCHOP online platform (<http://chopchop.cbu.uib.no>), selecting targets with high predicted editing efficiency and minimal off-target activity. The sgRNA sequence was chosen to be complementary to the 20-nucleotide protospacer adjacent to the PAM site within the *lacZ* gene. The corresponding donor template DNA was designed to include short homology arms flanking a 10-nucleotide insertion sequence to facilitate homology-directed repair during CRISPR-Cas9 editing.

The sgRNA and donor template were designed around a defined target site within the *lacZ* locus. The genomic region selected for editing corresponded to the sequence 5'-

tacaccaacgtgacatccattacgggtcaatccgccgtttgttcc
cacggagaatccg-3'.

In this region, a 20-nucleotide protospacer (5'-caacgtgacatccatta-3') was identified and used to guide Cas9 to the intended cleavage site. The resulting sgRNA incorporated this protospacer sequence in its RNA form as 5'-caacgugaccuaucgauua-3'.

For homology-directed repair, a donor DNA template was constructed with an inserted sequence (tgcgccatc) flanked by two homology arms. The upstream region of homology (5') was identified as (ctatccattacggt), and the downstream region of homology (3') as (tacgggtcaatccgcc). The entire system worked in tandem to allow for a precise cut in the targeted region after Cas9 nuclease activity. Everything was provided in accordance with specifications from a CRISPR-Cas9 kit.

Genomic DNA Extraction

Genomic DNA was obtained from colonies grown on KIX/SPT plates using InstaGene Matrix reagents (BioRad). A single colony was resuspended in a volume of 250 µl InstaGene reagent, incubated at 56 °C for 15 minutes to lyse the cells, and incubated at 95 °C for 10 minutes to inactivate nucleases. Centrifugation at 12,000 × g for 2 minutes gave a supernatant containing pure genomic DNA, which can be used immediately as a template for polymerase chain reactions.

Polymerase Chain Reaction (PCR) Analysis

PCR reactions were performed to verify the disruption of the *lacZ* locus via CRISPR-Cas9. Reactions were set up with a 25 µL volume using a template of genomic DNA, a pair of primer specific to *lacZ*, dNTPs, Taq DNA polymerase, and a buffer solution. The thermal profiles were initiated with a denature step of 94°C for 5 minutes, followed by a cycle of 94 °C for 30 seconds, 62 °C for 30 seconds, and 72 °C for 1 minute for a total of 35 cycles, with a final extension of 5 minutes at 72 °C.

To distinguish between the unedited gene and donor template, multiplex PCR reactions were performed. A total of three primer combinations were used: Unedited *lacZ*, which gives an expected product size of approximately 1100 bp; Edited allele with a donor inserts, which gives an expected product size of approximately 650 bp; and Internal control locus, which gives a product size of approximately 350 bp.

In all matters pertaining to PCR design and representation, standards established by MIQE were adhered to in order to reinforce methodology accuracy (Bustin *et al.*, 2009).

Gel Electrophoresis

The agarose gel electrophoresis of PCR product consisted of 1% agarose in TAE buffer. The samples were mixed with loading buffer and electrophoresed at 100 V for a period of 30 minutes. Ethidium bromide staining under UV light allowed visualization of DNA bands. The agarose gel contained a molecular weight marker along with samples of genomic DNA from unedited starter plates, DNA from transformation Plate C, duplicate samples from Plate D, positive control, and negative control.

Variations in banding patterns, deletion of the 1100 bp band or emergence of a 650 bp edited product band, were used to confirm successful CRISPR-Cas9 editing of the *lacZ* gene.

Substrate Preparation

Cassava peels were obtained from Oja Oba Market in Ado-Ekiti (Ekiti State, Nigeria). The peels were cleaned, sun-dried, processed into flour, and sterilized. Moreover, since agro-waste samples obtained from local markets are composed of heterogeneous varieties (cultivars), cassava varieties were not specified. Generally, in fermentation research, rather than identifying varieties, composition informs the suitability of a material to be used in fermentation studies (Javed *et al.*, 2019; Devi *et al.*, 2021). The cassava-based medium was prepared using 30 g/L cassava peel powder dissolved in a mineral solution containing KH_2PO_4 (2.13 g/L), MgSO_4 (0.5 g/L), and NaH_2PO_4 (1.5 g/L).

The potato peels were derived from sweet potatoes bought from a food seller in Oshodi, Lagos State. They were cleaned, dried for three days, minced into a powdered form, and preserved in a container before use. Like cassava peels, identification of varieties is not possible because they come from different local vendors. The medium for growing potatoes can be obtained by mixing 0.03g of sweet potato peels with 1 L of mineral solution, which consists of KH_2PO_4 , MgSO_4 , and NaH_2PO_4 , based on a technique described by (Minari & Agho, 2018).

Rice bran samples were obtained from small scale rice milling industries in Ogun State. The bran was washed thoroughly with distilled water, sun-dried, and stored in airtight containers. Genotype information could not be verified because regional mills process multiple rice varieties. Rice-bran medium was prepared using 0.03 g of bran

in 1 L of the mineral salt solution described by (Minari *et al.*, 2022).

Soybean meal was purchased from local markets in Lagos State. As commercially sold soybean meal typically contains mixed cultivars, genotype identification was not possible. For this substrate, 50 g/L soybean meal was dissolved in deionised water and the pH was adjusted to 4.5, 6.5, or 9.0 using 1 M HCl or NaOH. All media were sterilised at 121 °C for 15 minutes before inoculation.

Submerged Fermentation

Thirty millilitres of each prepared substrate medium (cassava peel, potato peel, rice bran, and soybean meal) were dispensed into 11 sterile tubes: five assigned to the edited *E. coli* strain, five to the unedited strain, and one to an uninoculated control. The fermentation tests were carried out in different environmental conditions, such as temperatures of 4 °C, 37 °C, and 50 °C, and pH levels of 4.5, 6.5, and 9.0. The control tubes were set to a pH of 7.5 and a temperature of 37 °C.

Media were sterilized at 100°C using a pressure pot for 1 hour, allowed to cool, and adjusted to their respective conditions before being inoculated. Fermentation took seven days. Bacterial growth was measured every day by using OD 540 nm to check biomass and consumption of substrates based on the procedure described by (Minari *et al.*, 2022).

Isolation of Crude Enzyme

After fermentation, centrifuging of cultures at 10,000 rpm for a period of 10 minutes was used to obtain bacterial pellets. Lysing of bacterial cells can be achieved using a freeze-thaw cycle, where pellets were either frozen at a temperature of 20 °C for a period of 30 minutes or thawed at room temperature for a period of 15 minutes. The freeze-thaw cycle took place three times to ensure proper cell lysis. The lysed samples were resuspended in 1X phosphate-buffered saline and centrifuged for another 10 minutes at 10,000 rpm. A supernatant with crude catalase activity was collected for subsequent assays (Lappe *et al.*, 2023).

Measurement of Catalase Activity

The activity of the catalase enzyme was measured based on the decay of hydrogen

peroxide (H_2O_2) concentrations using a UV-Visible spectrophotometer. The reactions were a mixture of crude extracts of the enzyme, 50 mM PBS buffer solution at pH 7.0, and 30% H_2O_2 solutions. Absorbance decreases at 240 nm were measured with time, which stood for the decay of H_2O_2 concentrations. The activity of each enzyme was measured in units per mL (U/mL) and indicated the volume of the enzyme solution required to decompose 1 mL of H_2O_2 per minute. Activities were measured using a calculated molar extinction coefficient for H_2O_2 at a wavelength of 240 nm at $43.6 \text{ M}^{-1} \text{ cm}^{-1}$, which considers total volumes in a reaction and lengths of light paths in cuvettes (Hadwan *et al.*, 2018).

Characterization of Catalase

The thermal stability of the enzyme was determined by incubating crude samples at 4 °C,

30 °C, and 50 °C for 30 minutes, after which remaining catalase activity was determined. The effect of pH on catalase activity was determined using a series of phosphate buffer solutions with a pH range of 4.0 to 9.0. Activity was measured based on the decomposition rate of hydrogen peroxide at 240 nm, and the temperatures and pH at which maximum activity occurred were determined (Zeng *et al.*, 2010; Jia *et al.*, 2017).

Statistical Analysis

Catalase activities from CRISPR-Cas9–edited and unedited *E. coli* strains were compared using one-way analysis of variance (ANOVA). Statistical analyses were performed in GraphPad Prism, and figures were generated using Microsoft Excel. A significance threshold of $p < 0.05$ was applied.

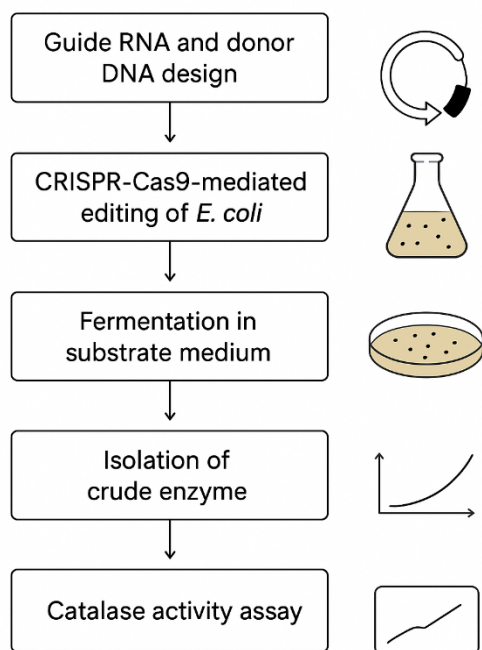


Figure 1: Overview of the experimental design and workflow

Results

The CRISPR-Cas9 editing experiment was performed on four treatment plates. Every one of them corresponds to a different combination of Cas9, sgRNA, and arabinose induction. After incubation for a period of 24 hours at 37°C,

different colony appearances were observed (Fig. 2A-D).

In Plate A (Figure 2A), *E. coli* bacteria with Cas9 but without the sgRNA or arabinose grew to form blue colonies. This indicated a functional *lacZ* gene and therefore a functional β -galactosidase. Plate B in Figure 2B did not have any colonies

because the bacteria were not induced with arabinose.

On Plate C (Figure 2C), where cells received Cas9 and arabinose but no sgRNA, blue colonies were again observed, consistent with the absence of

targeted cleavage at the *lacZ* locus. In contrast, Plate D (Figure 2D), containing Cas9, sgRNA, and arabinose, produced white colonies. These colourless colonies indicated loss of β -galactosidase function and confirmed successful CRISPR-Cas9-mediated disruption of *lacZ*.

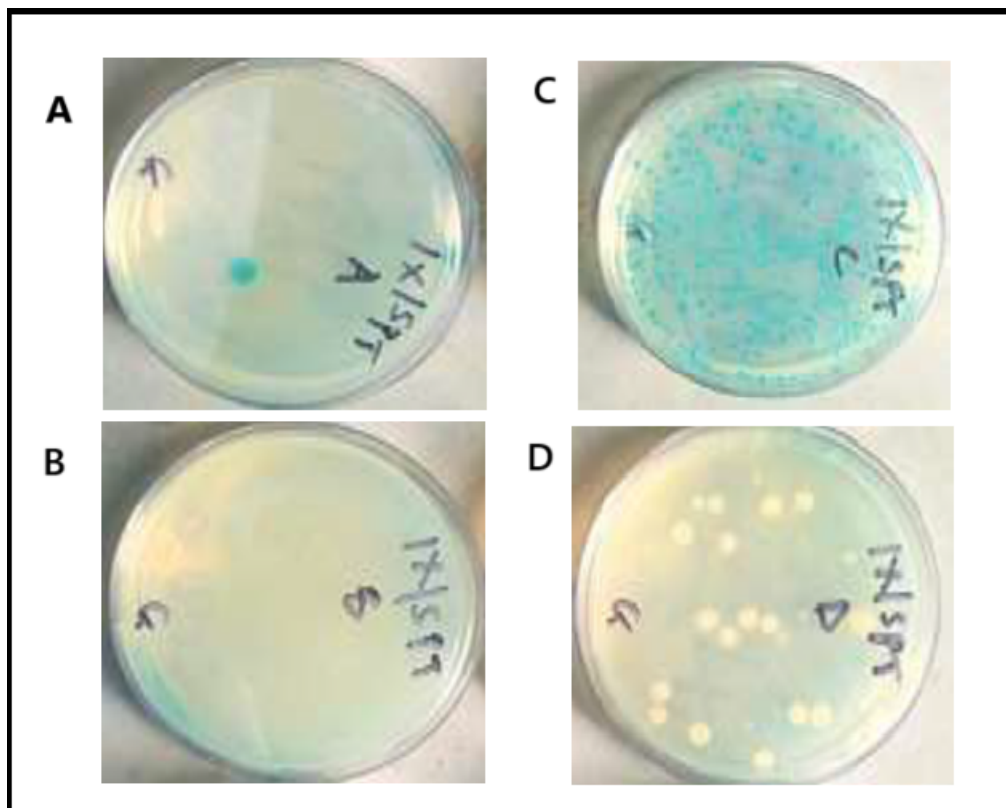


Figure 2: CRISPR-Cas9 gene-editing outcomes of the *lacZ* gene in *E. coli* HB101-pBRKan. (A) *E. coli* exposed to Cas9 only (no sgRNA, no arabinose) showing blue colonies, indicating intact *lacZ* and β -galactosidase activity. (B) *E. coli* transformed with Cas9 and sgRNA but without arabinose, showing no colony growth, confirming the requirement of arabinose for Cas9 expression. (C) *E. coli* exposed to Cas9 and arabinose but without sgRNA, producing blue colonies, indicating no targeted cleavage of *lacZ*. (D) *E. coli* containing Cas9, sgRNA, and arabinose showing white colonies, indicating successful CRISPR-Cas9-mediated editing and disruption of *lacZ*.

Re-Culturing of Edited and Unedited Strains

To generate fresh cultures for fermentation experiments, colonies from two plates were re-streaked. Blue colonies from the unedited control plate (Figure 2A) were re-cultured to produce re-cultured unedited *E. coli* plate (figure 3A).

Similarly, white colonies from the successfully edited plate (Figure 2D) were re-cultured to obtain re-cultured edited *E. coli* plate (figure 3B). As expected, the re-cultured unedited *E. coli* plate exhibited blue colonies, while re-cultured edited *E. coli* plate consistently showed white colonies, validating the stability of the edited phenotype.

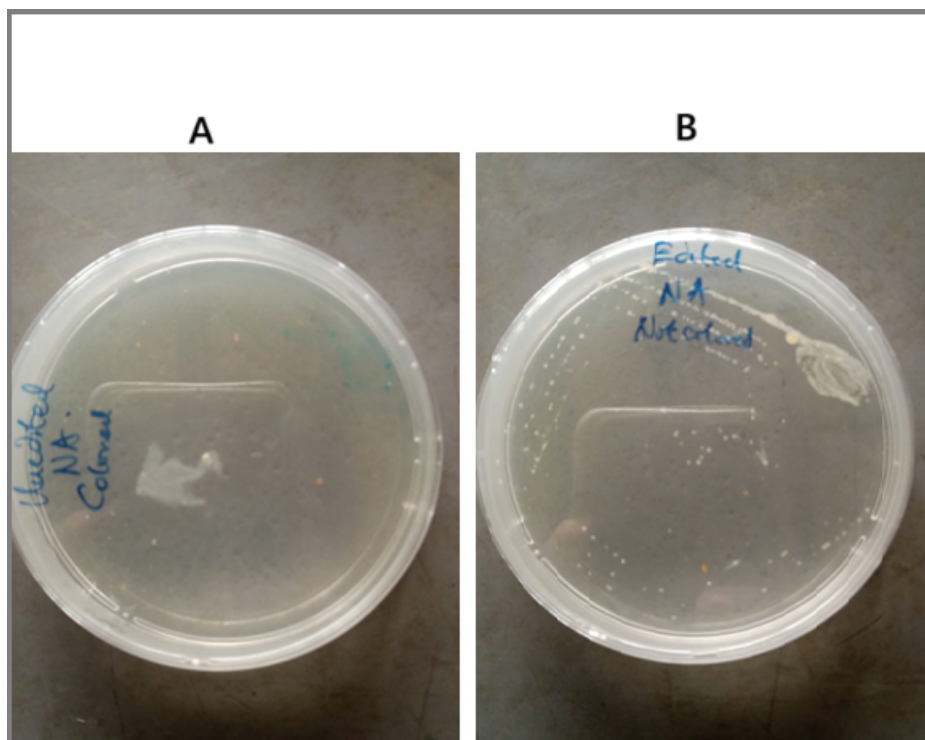


Figure 3: Re-cultured colonies of edited and unedited *E. coli* used for submerged fermentation. (A) Re-cultured unedited *E. coli* plate showing blue colonies used as the unedited strain for fermentation experiments. (B) Re-cultured CRISPR-Cas9-edited *E. coli* plate showing white colonies used as the edited strain for fermentation experiments.

Multiplex PCR Verification of lacZ Editing

Figure 4 presents the agarose gel electrophoresis profile of multiplex PCR products from edited and unedited *E. coli* samples. All lanes containing biological samples showed a ~350 bp band amplified by the internal control primer set, which targets a chromosomal region unrelated to *lacZ* and verifies successful DNA extraction and PCR amplification.

Lane 1 contains the molecular weight marker used to estimate fragment sizes. Lane 2 (starter plate DNA) displayed a strong ~1,100 bp amplicon corresponding to the intact *lacZ* gene of unedited *E. coli*, serving as the reference for comparison. Lane 3 (Plate C DNA), derived from cells transformed with Cas9 and arabinose but

lacking sgRNA, also showed an unmodified ~1,100 bp band, confirming that the absence of guide RNA prevents targeted cleavage.

Lanes 4 to 6 are the DNA from Plates D1 to D3. This DNA was edited with Cas9, sgRNA and arabinose. Each of these lanes has a band that is around 650 base pairs. This is what is expected because it means the donor template was inserted correctly and the native *lacZ* sequence was disrupted. The DNA in Lane 7 is the positive control. It has three bands: one is around 1,100 base pairs, one is around 650 base pairs and one is 350 base pairs. This shows that all of the primer sets are working correctly. Lane 8 is the negative control. There are no bands in this lane. This is good because it means there is no contamination and the reagents are good.

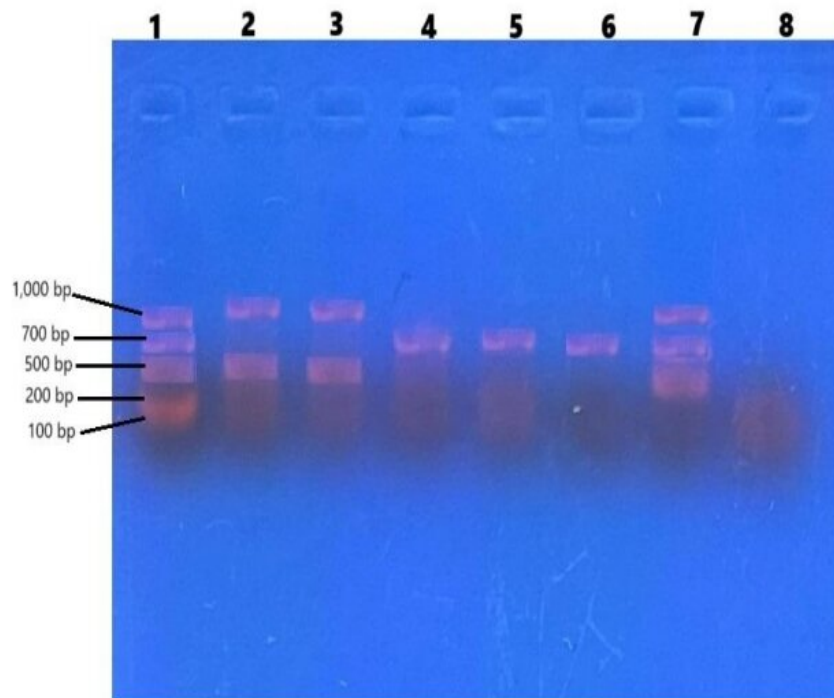


Figure 4: Gel electrophoresis of multiplex PCR products and controls samples

KEY: **Lane 1:** Molecular weight marker (size reference); **Lane 2:** Starter plate DNA; **Lane 3:** Plate C DNA; **Lane 4:** Plate D1 DNA; **Lane 5:** Plate D2 DNA; **Lane 6:** Plate D3 DNA; **Lane 7:** Positive control; **Lane 8:** Negative control.

Catalase Activity Under Different Temperature and pH Conditions

The catalase production was different in experimental conditions. The highest activity of catalase was found at a pH of 6.5 and a temperature of 50 °C in both strains of *E. coli*, which was 1.591 U/mL. When the pH was 6.5 and the temperature was 4 °C, the edited strain of *E. coli* did not produce any observable bubbles even though it had a high optical density of 0.845. In general, the edited *E. coli* had bubble formation and less biomass than the unedited strain of *E. coli*. However, the catalase enzyme in the edited *E. coli* was more stable and active at increased temperatures, which is a big difference and this result is statistically significant with a p value of less than 0.05 ($p < 0.05$).

*Growth Profiles of Edited and Unedited *E. coli**

The optical density measurements that were taken over six days showed that the growth responses of the strains depended a lot on the pH and temperature. When the temperature was at 37 °C, the growth was the highest at pH 6.5 for

both strains, as you can see in Figures 5 and 6. The optical density measurements of the strain were higher than the edited strain under these conditions; the unedited strain had an optical density of 2.05 ± 0.03 , while the edited strain had an optical density of 1.82 ± 0.02 . The growth of the strains went down fast at pH 4.5 and pH 9.0. This shows that acidic and alkaline environments had a bad effect on the growth of both the optical density measurements of the strains. The optical density measurements of the strains were affected by the pH and temperature. The optical density measurements showed that the strains did not grow well in alkaline environments.

Temperature Effects on Catalase Activity

As shown in Figure 7, catalase activity in unedited *E. coli* increased significantly with temperature (one-way ANOVA, $p < 0.01$), going up from 0.60 ± 0.01 U/mL at 4 °C to 1.00 ± 0.03 U/mL at 37 °C and further at 50 °C. The edited strain produced significantly higher catalase activity than the unedited strain at 4 °C (0.80 ± 0.02 U/mL; $p < 0.01$). Activity decreased slightly at 37

°C (0.60 ± 0.02 U/mL; $p > 0.05$), then increased to 1.60 ± 0.04 U/mL at 50 °C, significantly surpassing the unedited strain ($p < 0.001$).

Effect of pH on Catalase Activity

Figure 8 showed that the catalase activity of the catalase reached its peak at pH 6.5 in both strains. The catalase activity of the unedited strain was a lot lower at pH 4.5 ($p < 0.05$). This was a difference. The catalase activity of the catalase strain also went down a lot at pH 9.0 ($p < 0.01$). In contrast, the edited catalase strain had catalase activity that stayed the same across the whole pH spectrum. The edited catalase

strain did a lot better than the catalase strain when it was acidic and when it was alkaline ($p < 0.05$).

Overall Comparison of Strain Performance

The results demonstrate that CRISPR-edited *E. coli* possesses enhanced catalase thermostability and greater tolerance to pH extremes. The unedited strain, however, exhibited faster growth and higher biomass accumulation under optimal conditions (37 °C, pH 6.5). The complementary strengths of both strains underline the functional trade-offs associated with genetic modification.

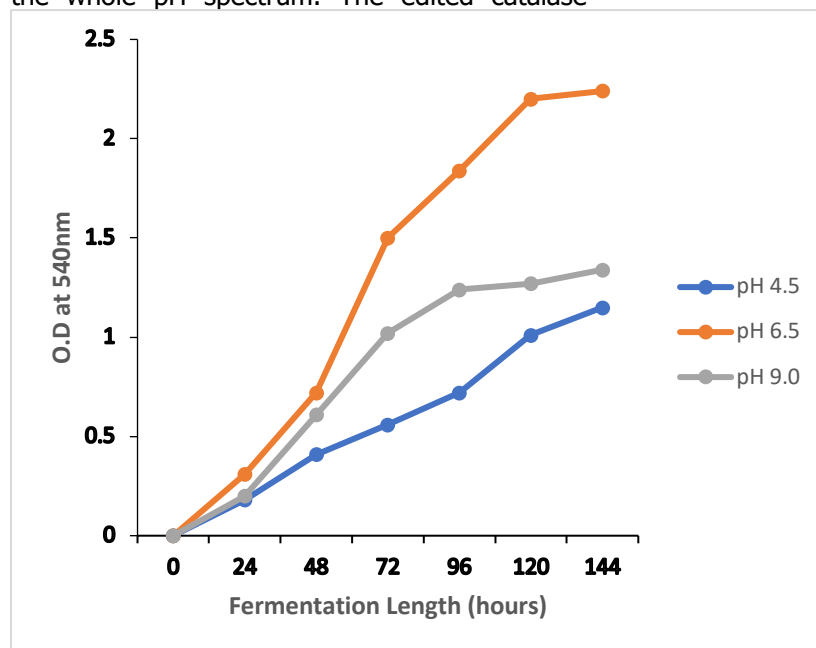


Figure 5: Growth trends of unedited *E. coli* strains at 37 °C across pH values 4.5, 6.5, and 9.0.

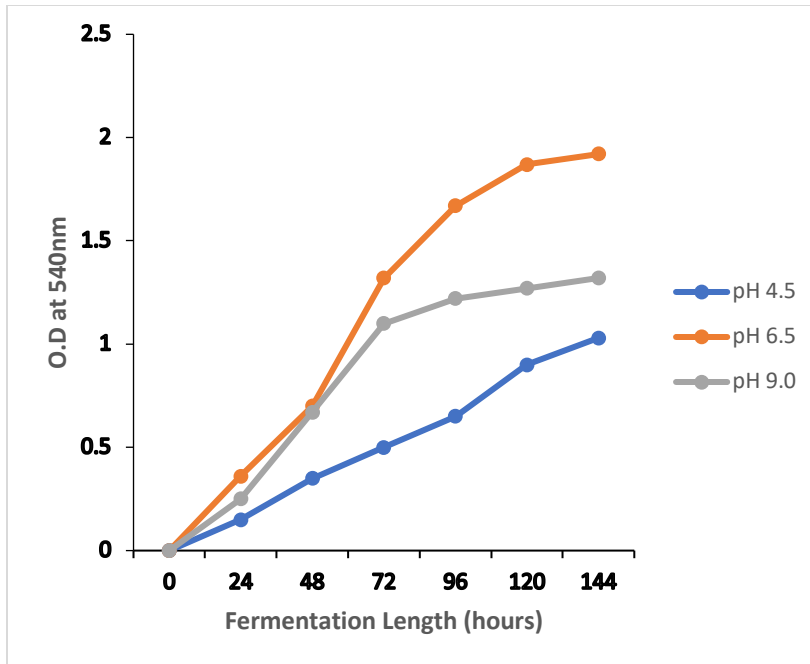


Figure 6: Growth trends of edited *E. coli* strains at 37 °C across pH values 4.5, 6.5, and 9.0.

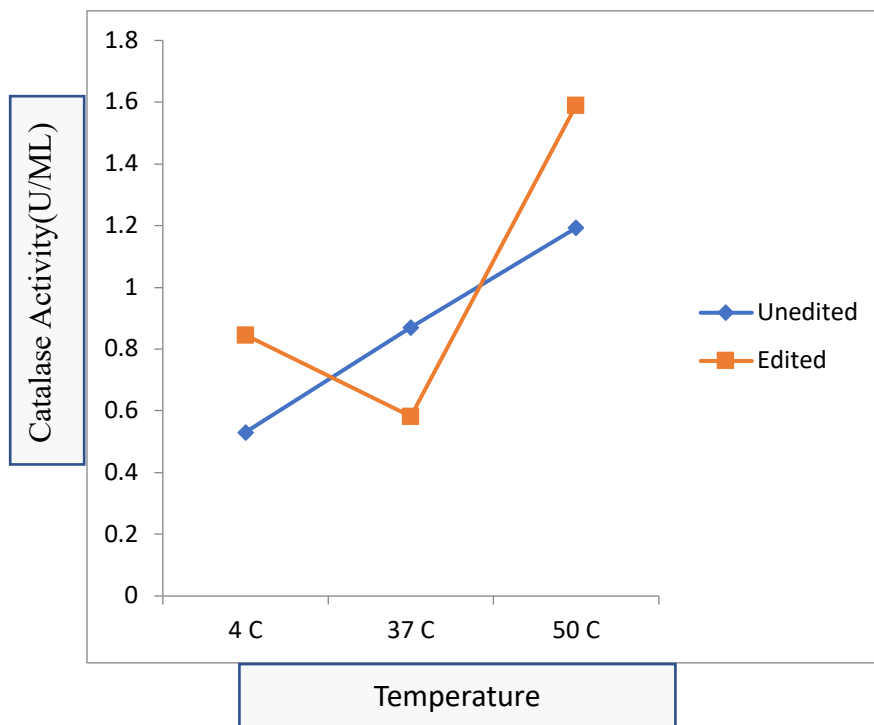


Figure 7: Temperature effect on catalase from CRISPR-edited and unedited *E. coli* substrates.

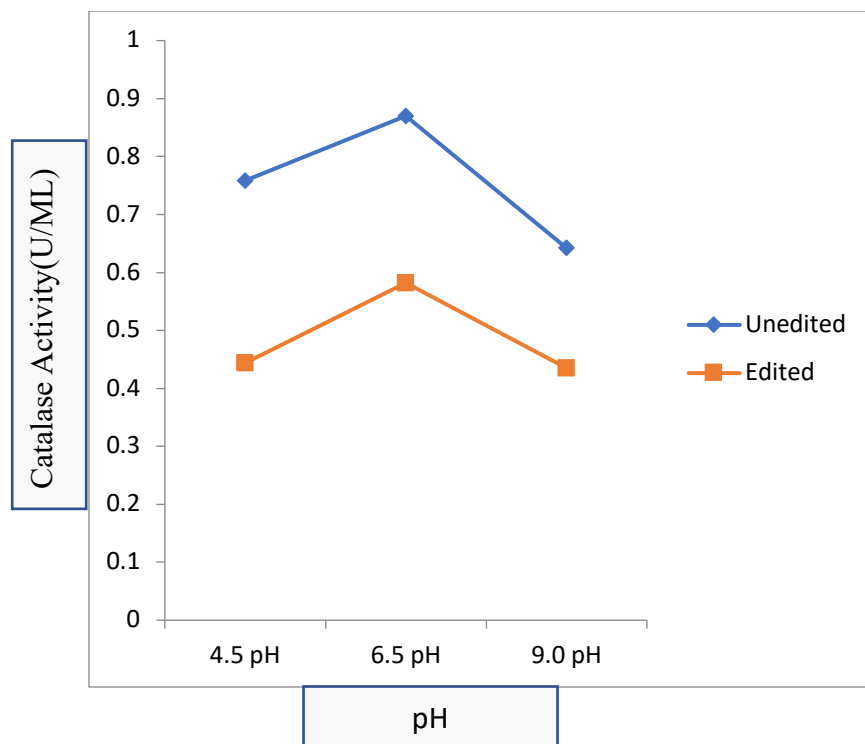


Figure 8: pH effect on catalase from CRISPR-edited and unedited *E. coli* substrates.

Discussion

The white colonies that showed up on the X-gal plates in the edited strain meant that the *lacZ* gene was successfully disrupted. On the other hand, the plates that did not have sgRNA or arabinose still had blue colonies. This shows that Cas9 needs both sgRNA and its inducer to cut the DNA in the place (to achieve targeted DNA cleavage). Multiplex PCR further supported the editing outcome: The unedited strains produced the expected piece of DNA that was 1,100 base pairs long, from the *lacZ* gene. The edited strains also produced a piece of DNA that was around 650 base pairs long, which is what is expected to be seen when the donor template is integrated. These results are based on the rules for editing with CRISPR-Cas9, in which sgRNA guides Cas9 to the target locus, enabling homology-directed repair (Li *et al.*, 2023).

The edited *E. coli* showed markedly increased catalase activity at 50 °C when compared with that of the unedited strain, suggesting improved enzymatic thermostability. By comparison, unedited cells exhibited peak activity at 37 °C, the temperature of their normal physiological optimum. The enhanced thermostability could correspond to metabolic adaptation coupled to

the knockout of *lacZ*, in which metabolite alterations linked with carbon usage might modulate redox homeostasis promoting over-expression or stabilization of catalase. The growth of the edited strain was decreased at 37 °C, but its improved performance for decolourization at 50 °C indicates industrial feasibility for high-temperature applications (e.g. textile bleaching). These results agree with previous studies where gene disruption brought about an upregulation of antioxidant responses in bacteria (Fasnacht & Polacek, 2021) and modifications can improve enzyme stability under heat stress (Tong *et al.*, 2023). The observed temperature and pH trends also align with prior work on bacterial catalase systems (Zeng *et al.*, 2010; Jia *et al.*, 2017).

The highest catalase production was recorded on cassava peel among the carbon sources screened. This could be due to the higher cellulose and hemicellulose produced, which serve as an ideal carbon matrix for microbial fermentation (Mohidin *et al.*, 2023). The optimum pH for the catalase activity in all substrates was 6.5, which was in accordance with the neutral condition preference of the enzyme. The catalase activity was reduced at alkaline pH,

the suggest that the stability of the enzyme has partially decreased. Worthy of note was the fact that even at reduced growth, the edited strain maintained strong catalase production. This shows improved metabolic efficiency rather than increased cell numbers.

One limitation of this study was the reduced growth rate of the edited strain relative to the unedited counterpart. Loss of *lacZ* may impair carbohydrate metabolism and decrease energy available for cell division. While catalase production was not negatively affected, large-scale fermentation may require strategies that balance growth and productivity, such as regulating *lacZ* expression rather than fully knocking it out, or supplementing pathways affected by the mutation. Future experiments should also evaluate strain performance in bioreactors and assess long-term genetic stability.

The use of cassava peel is especially important in the Nigerian context, where more than 20 million tons of cassava waste are generated each year. Turning this waste into a substrate for enzyme production supports the circular economy and provides a low-cost alternative to imported catalase, with the potential to significantly cut industrial production costs (Kumar *et al.*, 2021). Although cassava peel yielded the best results, soybean meal and other substrates demonstrated moderate potential and could be improved through optimization of fermentation conditions.

Although CRISPR-Cas9 allows highly precise genome editing, the possibility of off-target cuts cannot be completely ruled out. In this study, sgRNAs were carefully designed using the CHOPCHOP platform to reduce sequence mismatches. However, further analyses such as whole-genome sequencing or targeted deep sequencing would be needed to fully confirm that the observed effects result only from disruption of the *lacZ* gene (Zischewski *et al.*, 2017; Tycko *et al.*, 2018).

Conclusion

Targeted disruption of the *lacZ* gene in *E. coli* by CRISPR-Cas9 led to a clear improvement in catalase activity at higher temperatures and broadened the effectiveness of the enzyme across different pH conditions. When combined with cheap agricultural waste materials,

especially cassava peel, this approach provides a practical and sustainable pathway for local catalase production. The findings show a strong potential to reduce dependence on imported enzymes while supporting industrial growth and promoting effective waste reuse in Nigeria.

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