PRINT ISSN 1119-8362
Electronic ISSN 2659-1499

Full-text Available Online at
https://www.ajol.info/index.php/jasem
https://www.bioline.org.br/ja

J. Appl. Sci. Environ. Manage.
Vol. 28 (10) 2981-2989 October, 2024

Isolation and Characterization of Amylase Produced by CRISPR-Cas 9 Edited *LacZ* Gene and Unedited *Escherichia coli* using Potato Peel (*Ipomea batata*) as Enzyme Source

***MINARI, JB; NWOSU, GE; DADA, IS; ABDULAZEEZ, DO**

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

*.*Corresponding Author Email: jminari@unilag.edu.ng*
**ORCID: https://orcid.org/0000-0001-8346-9936*
**Tel: +234 803 248 8513*

Co-Authors Email: dadaidowusamuel1@gmail.com; nwosugift32@yahoo.com; abdulazeezdhik@aol.com

ABSTRACT: Genome editing, particularly using CRISPR-Cas9, is a powerful tool for manipulating genomes, including *Escherichia coli*. This study aimed to genetically engineer the *lacZ* gene in *E. coli* using CRISPR-Cas9 to assess its role in amylase production during submerged fermentation of sweet potato peels (Ipomoea batatas). Edited and wild-type *E. coli* were cultured at 37ºC, under pH 6.2, 7.0, and 8.4 conditions, and the resulting amylase was purified using ammonium sulfate. Amylase production was screened using starch as a glucose source, with enzyme characterization performed at varying temperatures and pH levels. CRISPR-Cas9 edited *E. coli* without the guide RNA (gRNA) and arabinose showed blue colonies, while those with gRNA, Cas9, but without arabinose showed no colonies. Edited *E. coli* with Cas9, and arabinose, but without gRNA also produced blue colonies. Colonies displayed a white phenotype when subjected to Cas9, gRNA, and arabinose. Gel electrophoresis revealed that *E. coli* exposed to Cas9 and arabinose had two bands at 650 bp, while blue colonies exposed to Cas9 without gRNA and arabinose showed bands at 1,100 bp. The positive control exhibited three distinct bands, whereas the negative control had none. Amylolytic screening shows similar clear zones of wild-type *E. coli* and CRISPR-edited *E. coli*. pH 8.4 provides the most favorable conditions for wild-type *E. coli* growth and pH 7.0 for the CRISPR-edited *E. coli* growth during 15 days of fermentation. Temperature and pH assays indicated that both wild-type and CRISPR-edited *E. coli* showed similar maximum amylase activity at 45ºC and pH 7, with no significant difference in enzyme production. These results suggest that the *lacZ* gene does not significantly affect amylase production in *E. coli*.

DOI: https://dx.doi.org/10.4314/jasem.v28i10.5

Cite this Article as: MINARI, J. B; NWOSU, G. E; DADA, I. S; ABDULAZEEZ, D. O. (2024). Isolation and Characterization of Amylase Produced by CRISPR-Cas 9 Edited *LacZ* Gene and Unedited *Escherichia coli* using Potato Peel (*Ipomea batata*) as Enzyme Source. *J. Appl. Sci. Environ. Manage.* 28 (10) 2981-2989

Dates: Received: 07 July 2024; Revised: 15 August 2024; Accepted: 19 August 2024 Published: 05 October 2024

Keywords: CRISPR Cas9 gene editing, *lacZ* gene, *E. coli*, Potato peel fermentation, Amylase

The ideal metabolic catalyst are enzymes, which provide various endogenous biochemical reactions via a clearly defined pathway. (Singh *et al*., 2019). Since enzymes are present across all naturally occurring species, including plants, animals, and microscopic microorganisms, they can be used for industrial reasons. Furthermore, under controlled circumstances, a variety of microbial enzymes are recognized catalysts for the production of various compounds from a wide range of substrates. The two most important enzymes for industry out of all of them are amylases and proteases. According to Mondal *et al*., (2022), amylase, which breaks down starch or glycogen, makes up more than 30% of all enzymes generated worldwide. Microbes, animals, and plants are just a few of the sources from which they can arise.

*.*Corresponding Author Email: jminari@unilag.edu.ng*
**ORCID: https://orcid.org/0000-0001-8346-9936*
**Tel: +234 803 248 8513*

Microbes are easily manipulated to produce enzymes with desired properties, which is the main benefit of employing amylases derived from microorganisms (Golgeri *et al*., 2024). Several techniques have been used to increase enzyme production, and techniques such as protein engineering, gene shuffling, and directed evolution have enabled the development of enzymes (Sharma *et al*., 2021). Genetic research now includes studies of enzymes among its many uses, thanks to the identification of CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) and CRISPR-associated (Cas) proteins (Uddin *et al.* 2020).

According to Sandomenico *et al*., (2020) and Osgerby and Overton (2023), the gram-negative bacterium *Escherichia coli* is the preferred expression host for producing 30% to 40% of recombinant enzymes (also known as recombinant protein production, or RPP) in the industrial setting. This bacterium can be grown on relatively inexpensive media, replicates quickly, and exhibits elevated intracellular product titers. It is typically employed for insertion of the desired gene since these advantages frequently exceed several purification processes and the absence of glycosylation sequence on the recombinant result (Kielkopf *et al*., 2021). We have moved past the studying phase of genetic data to the post-genomic period, as rewriting as well as a novel creation of the genome are becoming realities, thanks to the quick growth of biotechnology and life sciences. The development of many life science fields has been greatly advanced by the simplicity and effectiveness of CRISPR-based gene editing tools, which have become mainstream through constant exploration and iteration (Wei and Li 2023) and are the reason behind our isolation and characterization of amylase generated from CRISPR Cas9 edited *lacZ* gene in *Escherichia coli* using potato peel as enzyme source.

MATERIALS AND METHODS

Materials: Out of the Blue CRISPR Kit with catalog number12012608EDU was purchased from Bio-Rad Research Company, USA. Containing; lyophilized *E. coli* HB101-pBRKan, Transformation solution, 80ng/µlpLZDonor, 80ng/µl pLZDonorGuide, LB broth capsule, Streptomycin, Arabinose, KIX Mix (containing the antibiotic kanamycin. K) Instagene mix (IG), Master Mix Plus Primers (MMP), sterilized conical flasks,500ml and 1L, sterilized plates, plastic inoculating loop, four capped 2ml tubes, four micro centrifuge tubes, 250ml bottle, distilled water, arabinose, weighing balance, incubator, vortex machine, water bath, ice bath, micropipette and tips, (100-1000µl), (20-200µl), (2-20µl), 2ml micro test tubes, ice bath, tube racks.

Potato peels were collected from a local potato chips company at Akure, Ondo State, Nigeria. All other reagents used were obtained commercially and of analytical grade.

Genome Editing Analysis: LB Agar Plate Preparation: Two beakers 500ml and 1L flask were labeled as KIX and KIX/SPT respectively, 3ml of distilled water was added to the arabinose vial for reconstitution and the mixture was vortexed for 10mins till it was fully dissolved. 500 µl of distilled water was added to the streptomycin vial and the mixture was vortexed, shaking it after each vortex until it was fully dissolved.700 ml of distilled water was added to the flask labeled KIX/SPT and left to stand. 3.0 ml of distilled water was added to the KIX Mix vial (which contains kanamycin, IPTG and X-gal) and the mixture was shaken for 5 secs. The vial containing the KIX Mix was thoroughly rinsed until the mixture became clear. The KIX Mix mixture was then poured into the flask labeled KIX/SPT containing 700ml distilled water and the solution was swirled until the white powder was evenly suspended. 200ml of the solution was then poured into the 500ml KIX flask. 7g of LB agar powder was added to the KIX flask and the remaining to the KIX/SPT flask. Both flasks were microwaved and brought to boiling point 3 times in the microwave. The flask was allowed to cool a bit but careful not to allow the mixture to gel up, while the KIX/SPT flask was kept at 50ºC in a water bath 8 plates were pre-labeled IX and another 8 IX/ARA 40 other plates were labeled IX/SPT. The 8 plates labeled IX were quickly filled with the molten KIX LB agar up to one-third of the sterile Petri dish. 1.0 ml of rehydrated arabinose was added to the remaining molten KIX LB agar.

The mixture was swirled to reconstitute and poured up to one-third full into the eight Petri dishes labeled IX/ARA. Once the KIX/SPT flask could be held comfortably, 500µl of rehydrated streptomycin was added and the mixture was swirled. The 40 plates labeled IX/SPT were filled to one-third full with the molten agar. All the plates were allowed to solidify for about 30mins, then allowed to dry for 48hrs in a dark sterile room before it was stored upside down and refrigerated at 4ºC for use.

LB Broth Preparation: 50ml distilled water was poured into a sterile 250ml bottle. LB broth capsule was added to the 50ml distilled water and placed in the microwave with the cap of the bottle loosely tightened before bringing it to boiling point 3 times. The broth was allowed to cool to room temperature before the cap was tightened and stored in the refrigerator at 4ºC.

Rehydration of E. coli: 250µl of LB broth at room temperature was measured and added to a vial of lyophilized *E. coli*, the vial was gently shaken to resuspend the bacteria. The remaining LB broth was then microwaved to boiling point once with cap tightened loosely to prevent contamination.

Streaking of Starter Plates: The eight IX and IX/ARA plates were streaked with the rehydrated *E. coli* using a sterile plastic inoculating loop. The quadrant method of streaking was used to spread the bacteria properly on the plate. A fresh inoculating loop was used for each plate to avoid contamination. The starter plates were then incubated upside down in a dark incubator for 24 hours.

Dispensing of Solutions: Four 2ml tubes were labelled; TS, LB, pD and pDG. The LB, pD, and pDG were brought to room temperature by rubbing against the palms, careful not to shake up the contents of the vials. The pLZDonor and pLZDonorGuide vials were pulse spun to collect liquid at the bottom of the tubes.1.2ml of transformation solution (TS) was measured and added to the tube labeled TS. 1.2ml of sterile LB broth was measured and added to the tube labeled LB. 25µl of pLZDonor was measured and added into the tubes labeled pD. All dispensed solutions were capped and stored in a refrigerator at 4ºC for the genome editing.

Editing the lacZ Gene with the sgRNA and the Donor Template DNA: Four 2ml microcentrifuge tubes were labeled A, B, C, and D, and incubated on ice. IX (IPTG/X-gal) were added into tubes A and B while IX ARA(IPTG/X-gal/ARA) were added into tubes C and D. 250µml of T.S (Transformation solution) were added into tubes A, B, C and D, 10µml of plasmid donor (Pd) were added into tubes A and C while 10µml of plasmid donor guide (Pdg) were added to B and D. All four tubes were incubated on ice for 10 mins and heat shocked for 50sec at 60ºC, tubes were immediately transferred to an ice bath and incubated for 2mins. The four tubes were returned to the rack and 250µml of LB was added to them. Tubes were incubated at room temperature for 30 minutes. Four plates were labeled A, B, C, and D. 100µml of tube A was transferred to plate A and spread using a sterile spreader, 100µml of tube B was transferred to plate B and spread using a sterile spreader, 100µml of tube C was transferred to plate C and spread using a sterile spreader, 100µml of tube D was transferred to plate D and spread using a sterile spreader, the plates were covered properly and stacked. Plates were then incubated at 27ºC for 72 hours and observed for significant changes.

Genomic DNA Extraction: Five capped tubes were labeled S, C, D1, D2 and D3. The insta-gene matrix (IG) was inverted to mix up beads and 250µl was measured and pipetted into each tube. A sterile pipette tip was used to pick a single colony from the starter plate, and was transferred into tubes. A fresh sterile tip was used to pick up another single blue colony from plate C and transferred to tube C, the tip was swirled until no bacteria remained on the tip. Another fresh tip was used to pick a single white colony from plate D. The Colony was transferred to tube D1 and swirled until no bacteria was left on the tip. Another colony from plate D was picked and transferred to tube D2, and the last colony was picked from plate D and transferred to tube D3. The capped tubes are closed properly and vortexed to mix properly. The tubes were then incubated in a dry bath for 15 minutes at 56ºC. the tubes were allowed to cool slightly before it was vortexed again. The capped tubes were then incubated in a dry bath at 95ºC for 8 mins. Tubes were allowed to cool slightly before vertexing to mix again. The tubes were put in the centrifuge and spun at 6000 rev/min for 5 minutes.

PCR Sample Preparation: Seven PCR tubes were labeled S, C, D1, D2, D3, -, and +. 10µl of master mix plus primer (MMP) was added to each tube. 10µl of supernatant from each of the screwed-capped tubes was added to their complementary PCR tubes, using a fresh tip each time. The mixture was mixed thoroughly using the pipette. 10µl of the positive PCR control DNA was added to the positive (+) tube, and 10µl of the negative control DNA was added to the negative tube (-). The tubes were capped, placed in the thermocycler, and amplified.

Gel Electrophoresis: To mix what was contained in the tubes, PCR samples were vortexed briefly. For each sample, 5µl of loading dye (LD) was pipetted using a brand-new pipet tip, and the solution was gently mixed with the pipet. When preparing the 1% TAE agarose gel, it was positioned with the wells nearest to the black electrode and put into the electrophoresis chamber. To completely cover the gel, 400 ml of TAE electrophoresis buffer was added to the electrophoresis chamber. A fresh pipette tip was used for every sample as 15µl was poured into each of the eight wells. After replacing the electrophoresis chamber cover, the power supply was linked red to black and red to red. 30 minutes at 100 volts were spent running the gel. The gel was gently taken out and placed under the UV lamp for visualization after 30 minutes.

sgRNA and Donor Template DNA Design
DNA target

5’tacac**caacg tgacctatcc cattacggt**c aatccgccgt ttgttcccac ggagaatccg 3’
3’atgtggttgc actggatagg gtaatgccag ttaggcggca aacaagggtg cctcttaggc 5’
20-nucleotide protospacer:
3’gttgc actggatagg gtaat 5’
5’caacg tgacctatcc catta 3’
3’ guugc acuggauagg guaau 5’
5’caacg ugaccuaucc cauua 3’

The designed sgRNA
5’caacg ugaccuaucc cauua3’5’UUUUAGAGCUAGAAAUAGC AAGUUAAAAUAAGGCUAGUCCGUUAUCAAC UUGAAAAAGUGGCACCGAGUCGGU GCUUUUUU3’.

Donor template DNA
Inserted sequence: tgcgcccatc
3’ |homology arm |
5’tacac**caacg tgacctatcc cattacggt**c aatccgccgt ttgttcccac ggagaatccg 3’
5’ |homology arm|
3’atgtggttgc actggatagg gtaatgccag ttaggcggca aacaagggtg cctcttaggc 5’
5’ homology arm: ctatcc cattacggt
3’ homology arm: tacggtc aatccgcc
Note: The PAM sequence is colored in blue, and the target sequence is colored in red.

Donor template
Deleted base
ctatcc cattacggt tgcgcccat**c** tacggtc aatccgcc
5’homology arm inserted gene 3’homology arm.

Enzyme Synthesis
E. coli Inoculum Preparation: The test strains were kept as stock cultures for five days at room temperature and then stored at 4°C for routine subculturing. The cultures were actively growing for two days. The composition of the used media was as follows (in g/L): KCl2.4H2O 0.5, MgSO4.7H2O 0.5, Peptone 6.0, and Starch 1.0 were the inoculation media used in all of the tests. After being injected into sterile inoculation media, the pure culture was cultured for the entire night at 37°C on a rotary shaker. For the growth investigation and enzyme production, the fresh culture cultured overnight served as an inoculum.

Substrate Preparation: Potatoes peels served as the substrate for the enzyme production. Sweet potatoes were gotten and peeled to get the back; the back was then oven dried for 1hr till they were crispy. The potato peels were then crushed with a mortar and pestle to break them to small size before they were blended to a smooth powder form. 20g of potato flour was measured and poured into 1000 ml of distilled water.

Amylase Extraction/ Isolation: Utilizing the submerged fermentation method, the bacterial strains of both wild-type and modified isolates were examined for their capacity to produce amylase. Depending on the number of isolates, 100 mL of the potato peel media were distributed into a 250 mL conical flask in triplicate with three different pH conditions (pH 6.2, 7.0, 8.4). The media was then autoclaved at 121^0C for 15 minutes to sterilize it. Following sterilization, the flasks were given some time to cool before 2 mL of the isolates were added to the potato peel medium in each of the 250 ml flasks. Then, the flasks were positioned in a shaker incubator that was set to run at 30 °C and 120 rpm for 15 days, and optical density was taken every three days. Fermentation broths that had been inoculated were kept at 37ºC for a full day. The crude enzyme source was extracted from the supernatant of the fermentation broths after they had been centrifuged for 15 minutes at 5000 rpm in a chilled centrifuge after the incubation period.

Amylase Assay: Amylase activity of edited and wild type was assayed according to (Saha *et al*., 2023). Utilizing the subsequent formula, the enzyme activity was calculated:

$$Enzyme\ Activity\ \left(\frac{IU}{mL}\right) = \frac{Amount\ of\ sugar\ reduced \times 1000 \times\ dilution\ ratio}{Amount\ of\ glucose\ in\ moles \times time \times volume\ of\ the\ enzyme}$$

A single enzyme activity unit (U) is the quantity of enzyme that, in a conventional assay, releases one micromole of glucose from a substrate in one minute.

Enzyme Purification
Ammonium Sulfate Fractionation: To achieve the ideal ammonium sulfate ratio, varying saturation ratios (20, 40, 60, and 80%) were added. Salt was gradually added to each 20 ml of crude amylase enzyme in an ice bath while stirring, and the mixture was centrifuged for 25 minutes at 6000 rpm. The precipitate was then taken and dissolved in 5 ml of distilled water, and the protein content and enzyme activity were assessed for each distinct fraction (Dennison 2002).

Partial Purification of Amylase: Following submerged fermentation, the protein solution was transferred into a magnetic-bar-equipped beaker and heated to 4°C. A milliliter of the protein solution was taken, and 0.6g of ammonium sulfate was added. The protein solution was then agitated, and before adding

the next portion, a small amount of ammonium sulfate was added and allowed to dissolve. At last, the beaker was left to stand for the entire night (Agho *et al.*, 2022).

Characterization of Amylase enzyme: The pure amylase was characterized by determining several parameters, including the ideal pH and ideal temperature.

Amylase optimum pH and temperature determination: By changing the substrate's pH in 0.1 M phosphate buffer to different values (4.5, 5.0, 6.0, 7.0, and 8.0), the ideal pH was found. The ideal pH was maintained at the required temperature. The best temperature for the enzyme was found by adjusting the ideal pH range for the incubation temperature, which is 25, 35, 45, 55, and 65 degrees Celsius. the activity of enzymes was calculated using the Mandels technique (Witazora *et al*., 2021).

Statistical Analysis Used: Statistical analyzers used include; mean, variances, standard deviations bar charts, and plots.

RESULT AND DISCUSSION

CRISPR Cas9 gene editing of the *lacZ* gene in *E. coli* was carried out in four different petri dishes. The observations of the petri dish after 48 hours of incubation at 37°C are shown in Fig 1. The results from the genome editing revealed a mixture of *lacZ* genes with and without editing. Plate A contains a bacterial source without arabinose with an active plasmid donor without a guide RNA and an active repair mechanism resulting in the blue color of colonies growth, it yielded the growth of blue colony bacteria. Plate B comprised an active plasmid donor, a guide RNA system, and a bacterial source devoid of arabinose as well as an inactive repair mechanism. The donor template DNA contains a stop codon that inhibits the development of functional b-galactosidase, which is why Plate C also produced growth of blue colonies while Plate B produced no bacterial cell growth at all. A bacterial source with arabinose, an active plasmid donor guide, a guide RNA system, and an active repair mechanism are found on Plate D. Plate D revealed a white bacterial growth; the bacteria's white growth indicates that their DNA has been disrupted and repaired. Plate 2 shows PCR samples that were run on an agarose gel by electrophoresis with different band pattern obtained from the CRISPR-Cas9 experiment. Each PCR sample yielded specific amplicons by the primer sets, which provided the information about the DNA insertions. The bands on each lane represents the amplicons yielded by each PCR sample. The molecular ruler on zzthe extreme left produced amplicons with weight; 1000bp, 700bp, 500bp, 200bp and 100bp. Wild-type *lacZ* gene(unchanged) produced an amplicon with 1,100bp as seen in the positive (+), S and C, while the edited *lacZ* gene produced an amplicon with 650bp as seen in D1, D2 and D3.

Plate 1: A = Petri dish showing CRISPR Cas9 unedited *E. coli* with Cas9 enzyme but without single guide RNA (sgRNA) and repair machinery (arabinose (ARA)) showing blue colonies; **B =** Petri dish showing CRISPR Cas9 edited with Cas9 enzyme and sgRNA but not subjected to repair machinery (no arabinose (ARA)) with no colony of *E. coli;* **C =** Petri dish showing CRISPR Cas9 unedited *E. coli* with Cas9 enzyme and active repair machinery (arabinose (ARA)) but without single guide RNA (sgRNA) exhibiting blue colonies; **D =** Petri dish showing CRISPR Cas9 edited *E. coli* with Cas9 enzyme and sgRNA subjected to active repair machinery showing white colonies.

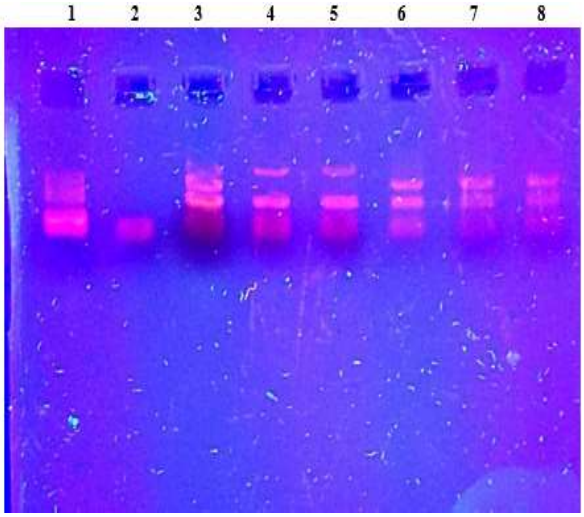

Plate 2: Amplicons of the CRSIPR Cas9 edited *E. coli* and unedited *E. coli* PCR products

Key: 1 = Molecular weight ruler (MWR); **2 =** Negative PCR Control (-); **3 =** Positive PCR Control (+); **4 =** PCR Sample S; **5 =** PCR Sample C; **6 =** PCR Sample D1; **7 =** PCR Sample D2; **8 =** PCR Sample D3

Key: D3=Edited *E. coli* 3; **D2**=Edited *E. coli* 2; **D1**=Edited *E. coli* 1; **S** = Starter plate *E. coli*, **C**= wild-type *E. coli;* **(+)** = Positive control**, (-)** = Negative control

Enzyme Amylase Analysis: E. coli. Plate 3a shows the wild-type *E. coli* petri dish and plate 3b shows the CRISPR Cas9 edited *E. coli* petri dish. Plate 4a shows amylolytic activities of wild-type/unedited *E. coli* petri dish after being subjected to iodine had a clear zone surrounding the colonies; 4b shows amylolytic activities of CRISPR Cas9 edited *E. coli* petri dish after the medium was subjected to iodine also having similar clear zones surrounding the colonies in the amylase medium as in 4a. After submerged fermentation of potato peel with CRISPR Cas9 edited *E. coli* and unedited *E. coli*, the crude enzyme was extracted. Plate 5 shows the purified amylase enzyme extracted from CRISPR Cas9 edited *E. coli* and unedited *E. coli* after ammonium sulfate was used to partially purify the amylase enzyme extracted from both CRISPR Cas9 edited and wild-type E

Plate 3a: Wild-type *E. coli* (right) on amylase medium

Plate 3b: CRISPR Cas9 edited *E. coli* (right) on amylase medium

The study compared enzyme production between wild-type *E. coli* and CRISPR-Cas9-edited *E. coli* under different pH conditions. Fig. 1 presents the growth of wild-type *E. coli* in a potato peel medium over 15 days at pH levels 6.2, 7.0, and 8.4, showing that growth was most significant at pH 8.4.

Plate 4a: Amylolytic activity of wild-type *E. coli* (left)

Plate 4b: Amylolytic activity of CRISPR Cas9 edited *E. coli* (right)

Plate 5: Crude enzyme extracted from different pH conditions.

Fig. 2 shows that CRISPR-Cas9 edited *E. coli* exhibited a significant increase in optical density (O.D.) at pH 7.0, particularly after day 3.

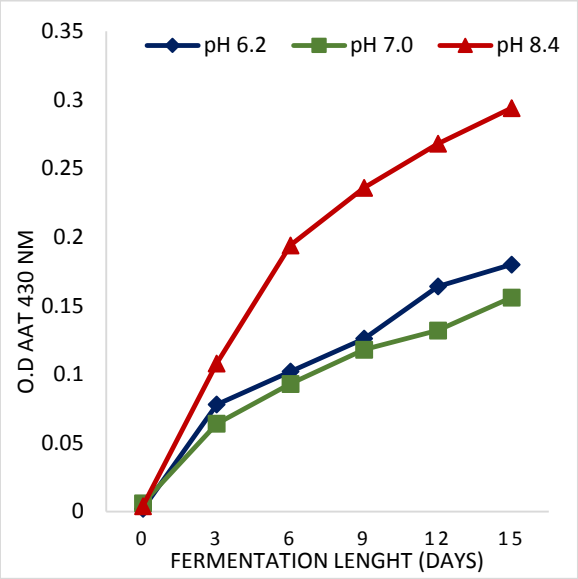

Fig. 1: Effect of pH on CRISPR Cas9 unedited *Escherichia coli* in potato peels medium. (Each plotted value is a mean of two determinations ± SD)

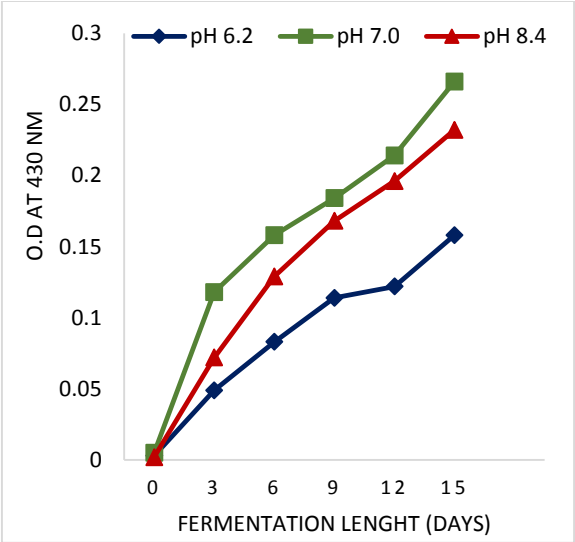

Fig. 2: Effect of pH on CRISPR Cas9 edited *Escherichia coli* in potato peels medium. (Each plotted value is a mean of two determinations ± SD)

The effect of temperature on amylase production by wild-type *E. coli* is represented in Fig. 3, which showed that amylase activity gradually increased with temperature, peaking at 45°C ($p<0.05$) with an activity of 0.544 U/ml. Above this temperature, amylase activity decreased to 0.284 U/ml at 55°C and 0.077 U/ml at 65°C ($p<0.05$). The temperature activity curve for amylase produced by CRISPR-Cas9 edited *E. coli* also peaked at 45°C with the same activity of 0.544 U/ml, followed by a steady decrease to 0.284 U/ml at 55°C and 0.077 U/ml at 65°C. The effect of pH on amylase activity is shown in Fig. 4, with a steady increase ($p<0.05$) from pH 4 to a maximum activity of 0.821 U/ml at pH 7. Then, a significant decrease ($p>0.05$) was observed with further increases in pH, dropping to 0.483 U/ml at pH 8. Fig. 4 also shows the rise in amylase activity in CRISPR-Cas9 edited *E. coli* with an increase in pH ($p<0.05$) until it reached a maximum activity of 0.821 U/ml at pH 7, followed by a sharp decrease in activity to 0.483 U/ml at pH 8 ($p>0.05$).

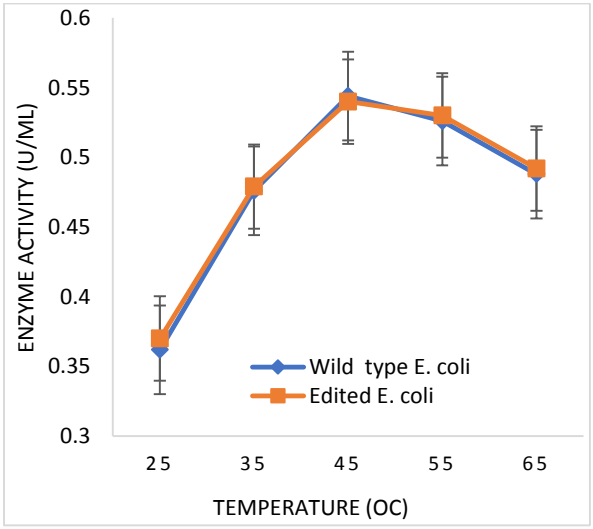

Fig. 3: Effect of temperature on purified amylase enzyme obtained from potato peels with wild-type *E. coli* an CRISPR-editeded *E. coli* (Each plotted value is a mean of two determinations ± SD).

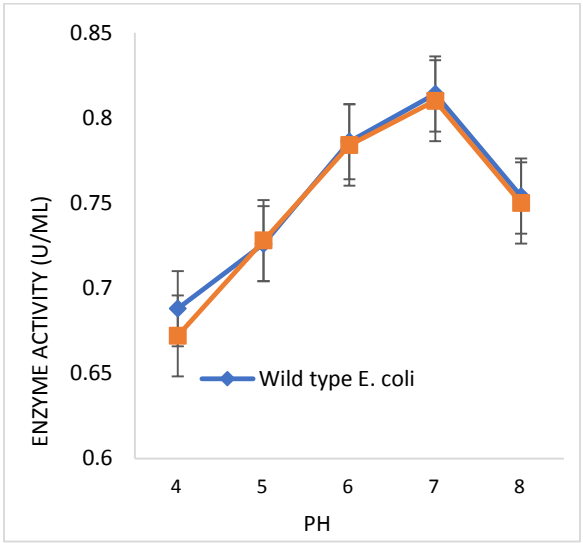

Fig. 4: Effect of pH on purified amylase enzyme obtained from potato peels with wild-type *E. coli* and CRISPR-edited *E. coli*. (Each plotted value is a mean of two determinations ± SD)

The emergence of the CRISPR-Cas9 system has been revolutionary, allowing precise modification of the *lacZ* gene in *Escherichia coli* using donor template DNA and sgRNA through the HDR mechanism (Deligianni and Kiamos, 2021). The successful editing of the *lacZ* gene demonstrated the effectiveness of the sgRNA and donor template DNA. In the genome editing experiments, small bacterial colonies formed on each plate, resulting from the editing of the *lacZ* gene in *E. coli* HB101-pBRKan, using either pLZDonor or pLZDonor Guide plasmids to activate HDR and insert DNA. Blue colonies indicated functional beta-galactosidase activity, which hydrolyzes X-gal (5-bromo-4-chloroindol-3-yl-b-D-galactopyranoside) and is expressed when the *lacZ* gene is functional and induced by IPTG (Isopropyl beta-D-1-thiogalactopyranoside). The absence of blue color in the presence of both X-gal and IPTG suggested that the *lacZ* gene was not functional. Most samples produced the expected results, with the sgRNA and donor template DNA leading to the anticipated colony colors, except one plate where bacteria grown without arabinose did not survive.

This study highlights that the CRISPR-Cas9 system requires engineered crRNA (or sgRNA) and donor template DNA for gene editing. Modified *lacZ* genes produced white colonies, while unmodified genes resulted in blue colonies. Amplicon amplification provided further evidence of DNA alteration. In multiplex PCR, disruptions in primer binding sites or the absence of donor template DNA inhibited target sequence amplification. Each PCR sample produced specific amplicons with the primer sets. A 1,100 bp amplicon indicated a failure to modify the Cas9 cut site, while a 650 bp amplicon showed successful repair using donor DNA. PCR samples with a 350 bp amplicon contained chromosomal DNA and passed the PCR test. Gel electrophoresis visualized the PCR products as DNA bands, with a fluorescent dye band indicating successful PCR.

The desired sequence appeared as a single DNA band, while multiple bands suggested additional sequences. The blue-white screening technique confirmed successful genome editing. PCR results validated the successful knockout of the *lacZ* gene in samples D1, D2, and D3, as they produced amplicons without the 1,100 bp gene representing *lacZ*. These findings align with the discovery of Naeem, (2022). Amylase production and characterization from *E. coli* supported the findings of Hassan and Jebor (2018), which stated that *E. coli* isolates are suitable for amylase production in technological applications. The potato medium used in this study effectively supported the growth of the organism, aligning with Kargapolova *et al*., 2020, who noted that all microbiological media must meet the organism's needs for carbon, nitrogen, minerals, growth factors, and water, without containing inhibitory substances. pH 8.4 provides the most favorable conditions for wild-type *E. coli* growth during fermentation, as it shows the highest increase in O.D. (0.29) over 15 days and pH 7.0 shows the highest increase in O.D., This agreed with Dong *et al*., 2021 who reported this indication may be the most favorable pH for CRISPR *E. coli* growth under these conditions. Optimization studies on amylase enzyme production by *E. coli* in fermented potato medium revealed that various factors significantly impacted amylase production. This finding agrees with Fentahun and Kumari (2017), who emphasized that optimizing culture conditions, and chemical and physical parameters like pH, temperature, salt concentration, and incubation time is crucial for microbial amylase production. In this study, amylase produced from wild-type *E. coli* was compared to that from Cas9-edited *E. coli* regarding temperature and pH. Temperature studies (Fig. 3) showed that wild-type *Escherichia coli* and Cas9-edited *Escherichia coli* in potato fermentation medium exhibited maximum enzyme activity of 0.544 ± 0.005 U/mL/min and 0.543 ± 0.002 U/mL/min, respectively, at 45°C in both assays. This result suggests that the *lacZ* gene does not affect amylase activity. pH studies (Fig. 4) showed that both wild-type and Cas9-edited *Escherichia coli* exhibited a steady increase in amylase activity, reaching maximum enzyme activity of 0.821 ± 0.005 U/mL/min and 0.820 ± 0.005 U/mL/min, respectively, at a pH of 7 in both assays, indicating that the *lacZ* gene does not significantly influence amylase production. These findings suggest that the *lacZ* gene is not a critical factor in amylase production, as both wild-type and CRISPR Cas9-edited *E. coli* displayed similar enzyme activity regarding pH, temperature, and enzyme yield.

Conclusion: Overall, the study's results indicate that the temperature and pH of the wild-type and CRISPR Cas9 edited *E. coli* samples yielded similar results for their amylase activity. Therefore, it can be concluded from the aforementioned research that the *LacZ* gene has no discernible effect on the production of amylase, and that both the wild-type and CRISPR Cas9 isolates of *E. coli* can serve as a solid basis for the production of amylase that is used in industry and technology.

Declaration of Conflict of Interest: The authors declare that there is no conflict of interest.

Data Availability Statement: The authors declare that data for this research are available upon request from the corresponding author

REFERENCES

Agho EE; Minari JB; Emelumadu EN; Adeniyi OA; Olajig FR. (2022). Characterization of laccase from the fungi *Fusarium* isolated from potato peels using carbon and nitrogen sources. *Adv. Technol.* 2(2), 183-197. https://doi.org/10.31357/ait.v2i2.5400

Deligianni E; Kiamos IS. (2021). Gene editing in Plasmodium berghei made easy: Development of a CRISPR/Cas9 protocol using linear donor template and ribozymes for sgRNA generation. *Mol. Biochem. Parasitol.* 246, 111415. https://doi.org/10.1016/j.molbiopara.2021.111415

Dong Y; Zhang H; Wang X; Ma J; Lei P; Xu H; Li S. (2021). Enhancing ectoine production by recombinant *Escherichia coli* through step-wise fermentation optimization strategy based on kinetic analysis. *Bioprocess. Biosyst. Eng*. 44, 1557-1566. DOI:10.1007/s00449-021-02541-7

Fentahun M; Kumari PV. (2017). Isolation and screening of amylase producing thermophilic spore-forming *Bacilli* from starch-rich soil and characterization of their amylase activity. *Afr. J. Microbiol. Res.* 11(21), 851-859. DOI:10.5897/AJMR2017.8543

Golgeri MDB; Mulla SI; Bagewadi ZK; Tyagi S; Hu A; Sharma S; Nadda AK. (2024). A systematic review on potential microbial carbohydrases: current and future perspectives. *Crit. Rev. Food. Sci. Nutr.* 64(2), 438-455. https://doi.org/10.1080/10408398.2022.2106545

Hassan BA; Jebor MA. (2018). Amylase production, purification and characterization from *Escherichia coli*. *J. Pharm. Sci. Res.* 10(7), 1691-1696.

Kargapolova KY; Burygin GL; Tkachenko OV; Evseeva NV; Pukhalskiy YV; Belimov AA. (2020). Effectiveness of inoculation of in vitro-grown potato microplants with rhizosphere bacteria of the genus *Azospirillum*. *Plant Cell Tissue Organ Cult.* 141, 351-359. https://doi.org/10.1007/s11240-020-01791-9

Kielkopf CL; Bauer W; Urbatsch IL. (2021). Expressing cloned genes for protein production, purification, and analysis. *Cold Spring Harb .Protoc.* 2021(2), pdb-top102129. DOI: 10.1101/pdb.top102129

Mondal S; Mondal K; Halder SK; Thakur N; Mondal KC. (2022). Microbial Amylase: Old but still at the forefront of all major industrial enzymes. *Biocatal. Agric. Biotechnol.* 45, 102509. https://doi.org/10.1016/j.bcab.2022.102509

Naeem MA. (2022). CRISPR/Cas9 gene editing in bacteria: Leading to hematopoietic stem cell editing in Pakistan. *J.Haematol.Stem* Cell Res., 2(1), 4-7.

Osgerby A; Overton TW. (2023). Approaches for high-throughput quantification of periplasmic recombinant proteins. *New Biotechnol.* 77, 149-160. https://doi.org/10.1016/j.nbt.2023.09.003

Saha SP; Ghosh S; Mazumdar D; Ghosh S; Ghosh D; Sarkar MM; Roy S. (2023). Valorization of banana peel into α-amylase using one factor at a time (OFAT) assisted artificial neural network (ANN) and its partial purification, characterization, and kinetics study. *Food Biosci*. 53, 102533. https://doi.org/10.1016/j.fbio.2023.102533

Sandomenico A; Sivaccumar JP; Ruvo M. (2020). Evolution of *Escherichia coli* expression system in producing antibody recombinant fragments. *Int. J. Mol. Sci*. 21(17), 6324. https://doi.org/10.3390/ijms21176324

Sharma A; Gupta G; Ahmad T; Mansoor S; Kaur B. (2021). Enzyme engineering: current trends and future perspectives. *Food Rev. Int.* 37(2), 121-154. http://dx.doi.org/10.1080/87559129.2019.1695835

Singh RS; Singh T; Pandey A. (2019). Microbial enzymes—an overview. *Adv. Enzyme. Technol*, 1-40. DOI:10.1016/B978-0-444-64114-4.00001-7

Wei J; Li Y. (2023). CRISPR-based gene editing technology and its application in microbial engineering. *Eng. Microbiol.* 3(4), 100101. https://doi.org/10.1016/j.engmic.2023.100101

Witazora Y; Suhartati T; Satria H; Hadi S. (2021). Production, purification and characterization of the α-amylase from local bacteria isolate *Bacillus subtilis* ITBCCB148. *In J. Phys. Conf. Ser*. 1751 (1). 012096. IOP Publishing. DOI 10.1088/1742-6596/1751/1/012096