Research Article

Genetic Identification of Methane-Oxidizing Bacteria with Soluble Methane Monooxygenase Genes and Enhancement of Bioremediation Using CRISPR Cas9

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Abstract | An innovative, economical, and ecofriendly approach is to use microorganisms to break down harmful ingredients and reduce soil pollution. This study aimed to identify novel *sMMO*-harboring methanotrophs, assess their potential for hydrocarbon bioremediation, and enhance their performance using CRISPR-Cas9. *Stutzerimonas balearica* (Accession number: LC777713.1 (; a novel strain of methanotrophic bacteria was isolated using methane as the sole carbon source. Soil samples were collected from Basra City southern Iraq. Based on morphological characteristics and detection of the *sMMO* gene, which encodes for the soluble methane monooxygenase enzyme, 11 bacterial isolates were identified and genotypically characterized. The chromosomal *sMMO* gene had single band of 369 bp in size. More than 99% of the reported legitimate genera were extremely closely connected to the BLASTN identity percentage and all the investigated sequences had an expected (E) value of 0. *Stutzerimonas balearica* showed positive results for naphthalene utilization when the colour of the colonies turned purple. The plasmid-treated CRISPR-Cas9 system was applied to *Stutzerimonas balearica*, and quantitative polymerase chain reaction (qPCR) analysis revealed a 25-fold increase in the expression of the *pMMO* gene. It was decided to investigate this gene and apply the CRISPR-Cas9 technology to increase the biodegradation effectiveness of the hydrocarbons [*i.e.*, n-alkane and polycyclic aromatic hydrocarbons (PAH)] in the laboratory. Within 7 d of incubation, the biodegradation rate of n-alkane by the modified *Stutzerimonas balearica* increased from 90.32% to 92.90%, while for PAH, the percentage increased from 83.16% to 94.07%. To the best of our knowledge, this study represents the first report in Iraq on the isolation of methanotrophic bacteria and, globally, the first application of CRISPR-Cas9 technology to enhance the biodegradation efficiency of methanotrophic bacteria for hydrocarbon compounds.

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Introduction

Methane-oxidizing bacteria are attracting more attention since they can use methane (CH_4) as their sole source of carbon and energy. As they can transform CH_4 into a range of useful bioproducts, including lipids, biopolymers, ectoine, and single-cell proteins, they play a crucial part in the carbon cycle.

Methanotrophs are gram-negative proteobacteria found in soil, marshes, sewage sludge, and waste treatment facilities. They are also widespread in natural gas fields and other arid environments (Guerrero-Cruz *et al.*, 2021; Dizon *et al.*, 2023). The existence of broad-spectrum methane monooxygenase enzymes (MMO), which carry out the oxidation of methane to methanol and represent the primary defining metabolic trait of methanotrophs, indicates that the bioremediation of a range of organic contaminants has been studied. A highly specialized class of aerobic bacteria known as methanotrophs are capable of oxidizing various organic contaminants, including alkanes, aromatics, and halogenated alkenes (Enbaia, 2019).

Methanotrophs can break down a variety of toxic chemical compounds, including chlorinated ethane through its enzymatic machinery (Shahid *et al.*, 2020). There are two different kinds of MMOs. A copper-containing, membrane-bound enzyme known as particulate MMO (pMMO), which is the most prevalent and is present in almost all methanotrophs. Some methanotrophs shut off the development of pMMO at low copper concentrations and instead produce a completely distinct, iron-containing soluble form (sMMO) (Banerjee *et al.*, 2019). Genetically engineered microorganisms (GEMs) are those that have been genetically modified (*i.e.* bacteria, fungi, and yeasts). Molecular techniques have been employed to modify these GEMs, and they can be used effectively for bioremediation (Liu *et al.*, 2019; Peeters *et al.*, 2019).

One method of a reprogrammable genome editing is CRISPR, modifying the internal DNA or RNA in a sequence-specific way. Gene editing is the process of precisely altering genes with the use of CRISPR-associated endonuclease Cas proteins. It has been effectively used in the areas of agriculture, medicine, treatment of infectious diseases, food industry, and bioproduction of energy (Nidhi *et al.*, 2021).

The CRISPR-Cas9 system is an innovative tool that enables precise and targeted genome editing in living cells. Genome editing requires a double-strand break in the target DNA, which the Cas9 nuclease induces when it attaches to the crRNA-tracrRNA duplex (Jinek *et al.*, 2012; Cong *et al.*, 2013). The objectives of this work were to find novel methanotrophs possessing the *sMMo* gene in the environment, utilize them to bioremediate hydrocarbon pollutants, and employ CRISPR-Cas9 gene editing to enhance their performance.

Materials and Methods

Soil sample collection

A total of 250 g of each oil-contaminated soil sample (5 samples) was aseptically collected in sterile plastic bags from the Mushrif oil station in Basra, southern Iraq (30.51714; 47.60423). The soil samples were taken at depths of 5-15 cm and kept at room temperature in the laboratory.

Isolation and purification of the methanotrophic bacteria

According to Dianou and Adachi (1999), methane-utilizing bacteria were isolated from the samples by placing 0.5 g of soil into 50 mL of nitrate mineral salt broth medium (NMS). The medium was placed in 100 mL bottles, which had rubber stoppers to seal the tops. Then, 20 mL of methane were injected into each bottle using a 0.22 µm filter syringe, providing about 18% methane in the head gas phase. Additionally, control bottles with no methane injection were created. The bottles were incubated under shaking condition in darkness at 30 ˚C and 180 rpm for 3 -4 weeks, while checks were undertaken every 3-d or one week. After incubation, 0.1 mL of each NMS broth culture was inoculated aseptically to NMS agar plate and spread using a sterile glass spreader. After incubation for 1-4 weeks, developing single colonies were streaked onto a petri plate containing NMS agar for identification, and incubated at 30 ˚C for 5-7 d. Following incubation, the colonies were Gram stained and examined using a microscope (Zeiss, Primo Star, Germany).

Molecular characterization of the methanotrophic bacteria

The Geneaid Presto™ Mini gDNA Kit (South Korea) was used to extract bacterial genomic DNA in accordance with the manufacturer's instructions. The purity and concentrations of the DNA were assessed using Nano-Drop (Optizen, South Korea). Amplification of the *sMMo* gene was carried out using polymerase chain reaction (PCR). The primers used for amplification of the *sMMo* gene were: mmoX1F(5́-CGGTC-CGCTGTGGAAGGGCATGAAGCGCGT-3́), and mmoX2R (5́-GGCTCGACCTTGAACTTG-GAGCCATACTCG-3́) (Miguez *et al.*, 1997).

The conditions for the *sMMo* gene amplification

reaction were established in accordance with the instructions attached to the Go Taq^{R} G2 Green Master Mix (Cat. No. M7822), 2X from the Promega kit. The following settings were programmed into the thermal cycler (Eppendorf, Germany) to carry out the PCR reaction: Initial denaturation at 95 ˚C for 2 min, 30 cycles each of 94 ˚C for 1 min, 59 ˚C for 1 min, and 72 ˚C for 1 min.

To identify *sMMo* gene bands, 2% agarose gel in 25 mL of 1XTBE solution was prepared, in addition to using a 100-bp DNA ladder (Promega, USA) and a UV transilluminator (ATTA, South Korea). The PCR products were stored at -20 ˚C for further analysis. Six samples, each containing 20 µL of *sMMo* PCR product were sent to Macrogen Company (South Korea) for sequencing. The query sequences for each sample were aligned to the BLASTN database (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch). Through the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov), the program was used to identify the bacterial species.

Methane monooxygenase activity assay

The used protocol for detecting methane monooxygenase activity relied on turning naphthalene into 1-naphthol, which was then colorimetrically monitored by adding the aromatic diazo compound o-Dianisidine to the reaction mixture. This technique was used to determine any soluble monooxygenase activity among microorganisms (Graham *et al.*, 1992). Broth cultures were grown in a 100 mL glass bottle at 30 ˚C in a copper-free NSM medium with 20% methane and agitated in a rotary incubator at 240 rpm. After 7 d, 50 µL of broth culture were spread on Mineral Salts agar (MSM) plates as mentioned before, and incubated in a jar at 30 ˚C with 25% methane gas for 7 d to allow the development of bacterial colonies.

Following incubation, naphthalene crystals weighing between 300 and 400 mg were placed on the plates lids, and the plates were incubated at 30 ˚C for 60 min to allow naphthalene to be converted to naphthol. 20 µL of freshly prepared o-ianisidine (5 mg/mL) were added directly to the bacterial colonies. The lid was replaced, and then incubated at 30 ˚C for 15 min. to permit color development.

Growth of methanotrophic bacteria in crude oil

One milliliter of a pure bacterial culture suspension was inoculated into a conical flask containing 100 mL of mineral salts medium (MSM) supplemented with 1% (v/v) crude oil. Bacterial growth was monitored by measuring the optical density at 600 nm (OD_{600}) using a spectrophotometer (Beckman Coulter DU530, UK). A negative control consisting of MSM supplemented with 1% crude oil without bacterial inoculation was included to allow comparison of daily growth measurements (Godini *et al.*, 2018).

Gene-editing experiment

Plasmid DNA extraction

Plasmid DNA was purified from the plasmid-transformed bacteria according to the manufacturer's instructions for Promega, using the PureYield™ Plasmid Miniprep System Kit (A1223, USA).

Gene editing plasmids

pCas9^{D10A} plasmid with 9,270 bp resistance to the ampicillin antibiotic containing Cas9 was ordered from Add gene (Tapscott *et al.*, 2019).

pgRNA plasmid

pgRNA plasmid with 2,584 bp resistance to the kanamycin antibiotic containing gRNA was ordered from Add gene (Tapscott *et al.*, 2019).

Antibiotic sensitivity assay

A stock solution (100 mg/ mL) was prepared by dissolving 1 g of an antibiotic powder in 10 mL of sterile distilled water. A 100 µL aliquot of bacterial suspension was uniformly spread using a sterile glass spreader onto Luria–Bertani (LB) agar plates to obtain a confluent lawn. Sterile 0.25-inch filter paper discs were aseptically placed on the surface of the inoculated plates, and 20 µL of the antibiotic solution were carefully applied to the center of each disc. The plates were allowed to stand for 30 min at room temperature to permit diffusion and drying of the antibiotic. Subsequently, the plates were incubated at 30 ˚C for 72 h. Antimicrobial susceptibility was determined by measuring the diameter (mm) of the inhibition zone surrounding each disc using a calibrated ruler (Desbois and Smith, 2015).

Preparation of the electro-competent cells

Primary cultures of *P. balearica* (5 mL) were grown at 37 ˚C. Subsequently, 500 µL of an overnight culture were used to inoculate 50 mL of nutrient broth in 250 mL Erlenmeyer flasks. The cultures were incubated at 37 ˚C with shaking at 250 rpm for 4–5 h until

the optical density at 600 nm reached 0.55–0.6. The cultures were chilled on ice for 30 min and transferred to 50 mL Falcon tubes, followed by centrifugation at 5,000 rpm for 10 min at 4°C. The resulting pellet was resuspended in 50 mL of ice-cold 10% (v/v) glycerol and washed three times under the same centrifugation conditions. After the final wash, the bacterial pellet was resuspended in 1000 µL of ice-cold 10% (v/v) glycerol. Aliquots (100 µL) of the cell suspension were dispensed, flash-frozen, and stored in liquid nitrogen (Belkhelfa, 2019).

Electroporation of the bacterial cells

Plasmid DNA was introduced into 50 µL of thawed bacterial cells on ice using electrocompetent cells, and the mixture was moved to a sterile, ice-cold Bio-Rad cuvette with a 0.1 cm gap. Using the electroporator, 1.8 kV was applied to the cuvette while it was in the pulsing chamber. The sample was resuspended in 1 mL of LB broth (without antibiotics) upon completion of the pulse. CRISPR modified bacterial strains were incubated for 1h at 37 ˚C with agitation. The bacteria were then centrifuged for 1 min at 4,000 rpm. The excess supernatant (900 µL) was thrown away, and each resulting pellet was placed individually on NA plates containing ampicillin after being re-suspended in 100 µL of broth medium. The bacterial plates were incubated at 37 ˚C for 24 h (Maki *et al*., 2024).

RNA extraction

In LB broth medium, both the bacteria and the recombinants were grown. The Genezol™ TriRNA Pure Kit from Geneaid (Taiwan) was used in line with the manufacturer's directions to extract the total RNA. A spectrophotometer nanodrop (Optizen, Korea) was used to quantify and quality of the RNA after three biological replications that had been employed to separate the the RNA from each isolate (Maki *et al*., 2024).

Quantitative polymerase chain reaction (qPCR) analysis

To create cDNA, 20 ng RNA were reverse-transcribed using the GoScript™ Reverse Transcription System (Promega, USA). Real-time PCR (RT-qPCR) analysis was carried out using the Go Taq® qPCR Master Mix from Promega (USA). A reference gene was employed, which encodes *16S rRNA*. RT-qPCR was performed in accordance with the manufacturer's instructions. Every reaction was performed three times. The $^{2-\Delta\Delta}$Ct method was used for relative quantification in RT-qPCR studies. The obtained Ct values were used as the starting point for calculating the relative transcriptional expression level of the candidate genes, which was then normalized against that of the *16S rRNA* gene. The extracted RNA samples were confirmed to be genomic DNA-free using an RT control sample, which was tested negative.

The *MMO* gene was amplified using the primer pairs PmoC374degenF (5′-AGCARGACGGYACNTGGC-3′) and PmoA344degenR (5′-ANGTCCAHCCCCAGAAGT-3′) (Ghashghavi *et al*. 2017). The *16S rRNA* gene was amplified using the primer pairs 16S-Fw (5′-TGAGATGTTGGGTTAAGTCCCGCA-3′) and 16S-Rv (5′-CGGTTTCGCTGCCCTTTGTATTGT-3′) (Zhang *et al*. 2016). The RT-qPCR program for the *pMMO* and *16S rRNA* primers was optimized as shown in Table 1. The reaction was submitted for a melting curve analysis to ensure primers specificity.

Table 1: *Real time-PCR program for expression of the pMMO and 16S rRNA genes.*

Gene	Initial denaturation	Denaturation	Annealing	Extension	Cycles
pMMO	96 °C 5 min.	96 °C 30 sec.	55 °C 60 sec.	72 °C 30 sec.	40
16S rRNA	94 °C 3 min.	94 °C 30 sec.	52 °C 30 sec.	72 °C 40 sec.	45

Bioremediation experiments

The ability of bacteria to degrade crude oil was examined. Conical flasks were inoculated with 1 mL bacterial suspension, 100 mL MSM broth medium, and 0.5% (v/v) crude oil. Flasks without bacteria served as controls. The bacteria were incubated for 7 d at 120 rpm at 30 ˚C (Maki *et al*., 2024). The residue crude oil was extracted and separated into aliphatic and aromatic fractions. The remaining crude oil was recovered using a separating funnel and a liquid-liquid extraction method. To ensure bacterial growth, 50 mL of carbon tetrachloride (CCl_4) solvent were added (Adebusoye *et al*., 2007). The residual oil was diluted with 25 mL of n-hexane after the aliphatic fraction was separated. The crude oil left over after removing the aromatic component was dissolved in 25 mL of benzene. The aromatic and aliphatic fractions were both examined using gas chromatography (GC) (Agilent, UK). The proportion of biodegradation involved was calculated using GC plots.

Results

Isolation and purification of the methanotrophic bacteria
After 30 d of incubation the bacteria were grown in NMS broth and streaked in NMS agar to enable their characteristics to be distinguished. All the developing colonies (6 isolates) were Gram-negative, rod-shaped, non-fluorescent, white, and medium in size.

Molecular characterization of the methanotrophic bacteria
Amplification of the *sMMO* gene by gene-specific primers resulted in single 369 bp band on agarose gel, representing the targeted area of the *sMMO* gene (Figure 1). The recovered six isolates considered as methanotrophic bacteria were sent for *sMMO* gene sequencing. The obtained results showed that the percentage of matching to the NCBI database as *Stutzerimonas balearica* (Accession no. LC777713.1) was greater than 99%. The results of sequence analysis were then used to search the NCBI database for sequences with similar characteristics using the Basic Local Alignment Search Tool (BLASTN).

Methane monooxygenase activity assay
Following the addition of naphthalene crystals and o-Dianisidine to the identified bacterial species, *Stutzerimonas balearica* only recorded a positive reaction, where the colour changed from white to purple.

Growth the bacteria on crude oil
Using crude oil as the sole source of carbon and energy for 7 d caused noticeable increases in bacterial cell density and concurrent reductions in the number of crude oil constituents detected by GC analysis. The recorded optical density of the *Stutzerimonas balearica* isolate on the culture medium was 0.17 (Figure 2).

Antibiotic sensitivity test
The subsequent stage of evaluating the ability of *Stutzerimonas balearica* to tolerate the antibiotics ampicillin and kanamycin, commonly used as selectable markers in genetic engineering, involved a preliminary antibiotic sensitivity assessment using the disc diffusion method on LB agar plates. After 3 d of incubation, the recorded minimum inhibitory concentrations (MICs) were 200 µg/ mL for ampicillin and 50 µg/ mL for kanamycin (Table 2).

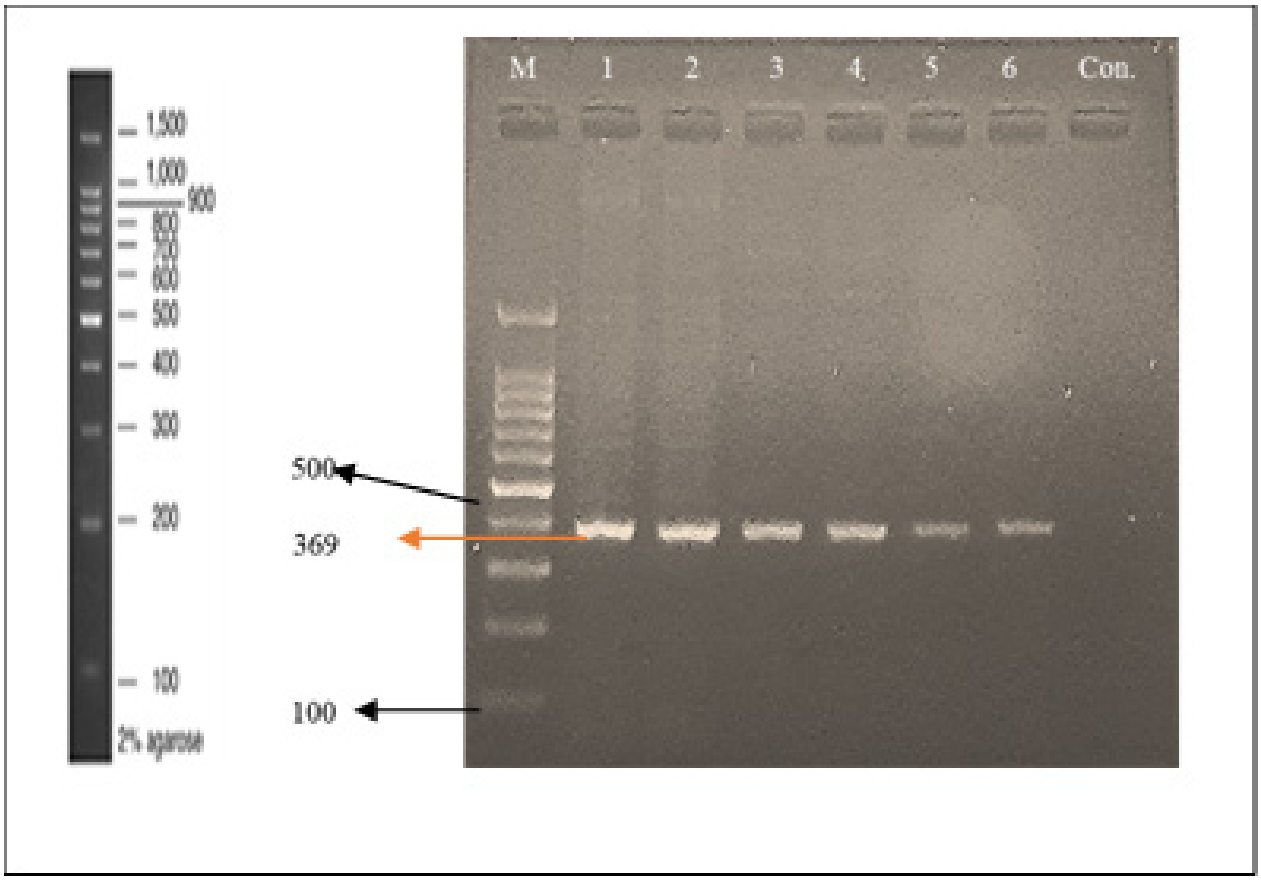

Figure 1: *Amplification of the sMMO gene (369bp) on 2% agarose gel electrophoresis at 60 volts. Lane M: 100 bp Marker, Lanes 1–6: sMMO gene bands of 6 isolates; Con.: negative control.*

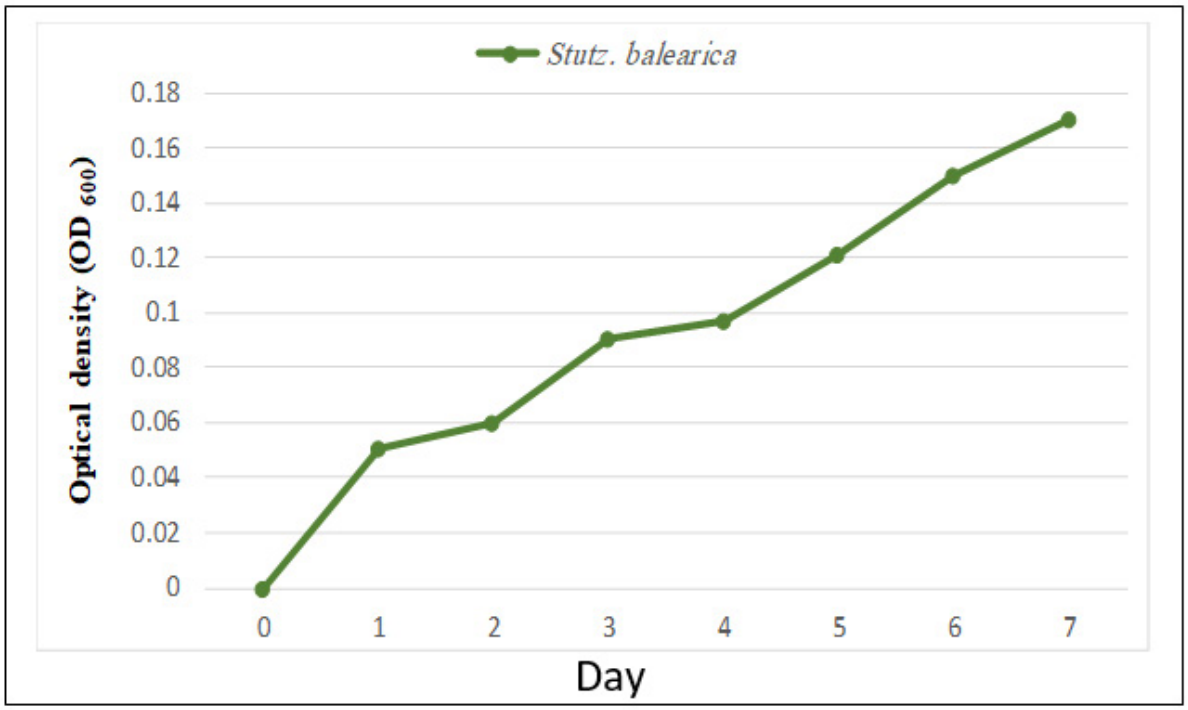

Figure 2: *The optical density of Stutzerimonas balearica reached 0.17 after 7d of incubation in MSM broth medium containing 1% crude oil.*

Table 2: *Antibiotic resistance assay pattern of the Stutzerimonas balearica on LB agar.*

Concentration µg/ L	Ampicillin	Kanamycin
	3 days	3 days
20	+	+
50	+	++
100	+	-
200	++	-
500	-	-

Where; (+): Sensitive, (-): Resistance, MIC: ++

Gene expression analysis of Cas9 in Stutzerimonas balearica

To investigate the expression of *pMMO* gene in *Stutzerimonas balearica* and its transformation, RNA was collected from cells treated with the $Cas9^{D10A}$ method and reverse transcribed to create the first strand of DNA for use as a template. *pMMO* expression after treatment with the CRISPR-Cas9 system was upregulated to 25-fold compared to the *Stutzerimonas balearica* before editing, which was normalized to 1. $^{\Delta\Delta}CT$ analysis was applied to conclude the *pMMO* gene expression. The qPCR and *PMMO* amplification primers were added to the cDNA templates. The expression levels of the genes were measured using SYBR green. The *16S rRNA* value was subtracted as a housekeeping gene.

Bioremediation experiments

Estimation of aliphatic portion biodegradation by Stutzerimonas balearica

The aliphatic components were examined using GC. The heavier compounds that were not eluted were observed by the hump. The sample of crude oil being tested exhibited carbon levels ranging from C_{12} to C_{37} after 7 d of incubation without bacterial inoculation as a control. Two types of *Stutzerimonas balearica* were used to degrade the aliphatic components. From the obtained results, we observed that the degradation by non-transformed bacteria reached 90.32%, meanwhile, the degradation by bacteria transformed with Cas9 was 92.9%.

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Estimation of aromatic portion biodegradation by Stutzerimonas balearica

The GC results indicated that about 83.16% of the aromatic components were degraded by non-transformed *Stutzerimonas balearica* after 7 d of incubation. The degradation rates of Benzo (A)-Anthrac, Fluoranthene, Phenanthrene, and Chrysene were 90.82%, 90.74%, 90.44%, and 89.86%, respectively. Meanwhile, Pyrene, Acenaphthyene, Benzo (B) Fluora, Benzo (A) Pyrene, Anthracene, and Fluorene were degraded at the rates of 83.48%, 81.34%, 78.03%, 76.78%, 76.77%, and 72.44%, respectively. In contrast, the 2-methylnaphtha, Acenaphthnen fractions, and Indeno (1, 2, 3-CD)

were completely degraded (100%).

Transformed *Stutzerimonas balearica* with Cas9 was highly efficient in degrading aromatic components, reaching 94.07%, based on the GC analysis. The Phenanthrene, Fluorene, Benzo (A) Anthrac, Benzo (B) and Benzo (A) were degraded at 94.19%, 94.37%, 95.62%, 95.61% and 94.55%, respectively. Conversely, Fluoranthene, Pyrene, and Benzo (K) were degraded at 80.83%, 88.80%, and 89.99%, respectively. Anthracene and Indeno (1, 2, 3-CD) were degraded at 73.37% and 73.64%. The 2-methylnaphtha, Acenaphthyene, and Acenaphthnen fractions were completely degraded (100%).

Discussion

In the current study, the gene *sMMO* was successfully detected and amplified in the genome of *Stutzerimonas balearica* (Figure 1). The *pMMO* is ubiquitous among all known obligate methanotrophs, whereas *sMMO* appears only in some methanotrophs such as facultative methanotrophs, and it expresses itself only in the presence of less Cu^{+2} (Nielsen *et al*., 1997). In fact, an unusual form of monooxygenase was detected in *Pseudomonas putida* four decades before. This enzyme was implicated in oxidizing 2-oxo-A3-4,5,5-trimethylcyclopentenylacetic acid (Ougham *et al*., 1983).

In this study, *Stutzerimonas balearica* was able to grow in NMS broth and used methane as the sole source of carbon and energy after 7 d of incubation. Qualitative measurement of sMMO activity *via* the naphthalene oxidation test revealed an encouraging result. This was accomplished by cultivating the bacteria on NMS agar with methane as the sole source of carbon. The production of 1-naphthol is an indicator of the activity of the sMMO on naphthaline and o-Dianisidine dye. These obtained findings are in agreement with those of Rani *et al*. (2021), who investigated a flooded paddy environment and found considerable methane used in 30 distinct bacterial taxa, including *Pseudomonas*, in addition to obtaining a positive naphthalene oxidation test. Dispersing the oil layers in the conical flasks at different levels and progressively raising the turbidity of the mineral medium during the incubation period, reflected the bacterial growth on crude oil as a carbon source, where *Stutzerimonas balearica* demonstrated a capacity to grow on crude oil.

This obtained result is consistent with a previous study conducted by Godini *et al*. (2018), where they found nine distinct types of bacteria in areas polluted with oil. It has been demonstrated that *Pseudomonas* spp. can live in their environment that has only 2% concentration of crude oil as their sole source of energy and carbon. Due to the emergence of bacterial resistance during this study demonstrated in Table 2, it was advised that an antibiotic sensitivity test be carried out on the plasmids to genetically modify the obtained bacteria under study. At a concentration of 50 µg/mL, *Stutzerimonas balearica* displayed susceptibility to kanamycin, in accordance with Pacheco *et al*. (2003) study, which revealed that *Pseudomonas sp*. was also susceptible to kanamycin.

Gene expression analysis before and after CRISPR-Cas9
Numerous attempts were conducted to clone *Stutzerimonas balearica* using *MMO gRNA,* where a 1-kb DNA repair template resulted in no effective transformation. Therefore, the authors focused on enhancing the genetic modification by adding supportive minerals such as copper. This allowed the bacteria to self-repair. Actually, copper is utilized to enhance and rewire gene expression networks by causing copper-responsive components to undergo a conformational shift to connect to DNA. A dose-dependent pattern resulted in an increase in gene expression that was hundreds of times greater than the control (Garcia-Perez *et al*., 2022).

Analysis of gene expression using the CRISPR-Cas9 system showed a 25-fold difference in the activity of the *pMMO* enzyme between the control *Stutzerimonas balearica* and the modified one. Meanwhile, Tapscott *et al*. (2019) noted in their initial findings that their evaluation of Cas9 or $Cas9^{D10A}$ expression in *Methylococcus capsulatus* demonstrated that nuclease expression didn't alter the bacterial growth in the absence of *gRNA* expression. Sundaresan *et al*. (2017) reported the addition of manganese ions (Mn^{+2}) to LB medium to improve the repair machinery in the modified bacterial strain. The normally dormant Cas9 enzyme became active when Mn^{+2} ions were added to the media. Cas12a and Cas9 may cleave DNA in the presence of Mn^{+2} ions, even in the absence of a guide RNA. Both Mn^{+2} and Co^{+2} ions have an extraordinary capacity to attach to the DNA strand between a guanine (Campbell and Jackson, 1980). Without a guide RNA, Mn^{+2} and Co^{+2} may help the DNA to cleave at the active site. Mn^{+2} and Co^{+2}

both enhance a productive orientation of the protein's metal, phosphate, and active site residues in DNA ligase D 3′-phosphoesterase (PE) enzyme crystal structures, whereas Zn^{+2} does not. A large number of DNA repair enzymes require Mn^{+2} but are inactive in the presence of Mg^{+2} (Zhu and Shuman, 2005; Das *et al*., 2012).

Currently, CRISPR-based gene editing with $Cas9^{D10A}$ nickase was employed successfully, corresponding to the similar works conducted by Xu *et al*. (2015); Song *et al*. (2017). This variant feature a RuvC1 nuclease domain has been deactivated so that it can cause single-stranded DNA (ssDNA) nicks to induce single-nick-assisted HDR (Jinek *et al*., 2012). Tapscott *et al*. (2019) reported that a $Cas9^{D10A}$-mediated nicking resulted in higher chromosomal recombination efficiency than Cas9-induced dsDNA breaks in *Methylococcus capsulatus*, leading to an increased number of transformants and improved editing efficiency at the *mmoX* locus. A previous study demonstrated that single-nick–assisted homology-directed repair (HDR) proceeds *via* a distinct repair mechanism with greater fidelity compared to dsDNA break–induced repair pathways (Metzger *et al*., 2011).

Estimation of biodegradation of aliphatic-oil component before and after CRISPR-Cas9

Stutzerimonas balearica was able to consume crude oil and reduce the amount of aliphatic material by 90.32%. These results are in accordance with a previous study reported by Yetti *et al*. (2018) indicating that *Stutzerimonas balearica* was a component of a bacterial group that degraded the total petroleum hydrocarbons (TPH) in crude oil. Similarly, Pandey *et al*. (2018) isolated *Stutzerimonas balearica* from oil-contaminated soil, which was able to metabolize n-alkanes and polycyclic aromatic hydrocarbons in diesel fuel. Hamad *et al*. (2021), demonstrated that microorganisms isolated from oil pollution sites are more effective in biodegrading hydrocarbon compounds than those isolated from unpolluted areas. Liu *et al*. (2022) revealed that the degradation percentage of in n-alkanes (C_{13}-C_{35}) by *P. aeruginosa* reached 87% to 100%. In addition, Fadhil and Al Baldawi (2020) reported that *P. stutzeri* could break down TPH at 68.6%, while displaying a strong capacity to digest C_{10}-C_{12}, C_{17}, C_{19}-C_{20}, and C_{24}-C_{26} over a course of 7 d.

In *Stutzerimonas balearica* modified using CRISPR-Cas9, the recorded percentage of aliphatic component residues degraded after 7 d of incubation with 0.5% crude oil was 92.90%. This study illustrates the power of the CRISPR system in improving biological treatment through boosting the rate of catalysis, in compatible with the increased gene expression of the bacteria modified using this system.

Estimation of biodegradation of oil aromatic components before and after CRISPR-Cas9

The *Stutzerimonas balearica* strain was effective in decomposing 83.16% of the aromatic component of the crude oil after being cultured for 7 d. Similarly, according to a study conducted by Medić *et al*. (2020), *P. aeruginosa* was able to decompose polycyclic aromatic hydrocarbons (PAHs) (Fluorene, Phenanthrene, and Pyrene) with efficiencies of 96%, 50%, and 41%, respectively, at starting concentrations of 20 mg/L and over a span of 7 d. In addition, Smułek *et al*. (2020) discovered that *P. mendocina* and *Brevundimonas olei* were capable of degrading more than 60% of the total concentration of PAHs during the course of a test lasted for 28 d. According to the findings of the current work, prolonged exposure to aromatic chemicals caused the bacteria to start using PAHs as a source of both carbon and energy.

Kumari *et al*. (2018) demonstrated that several PAHs, including naphthalene, phenanthrene, enzo(b) fluoranthene, and fluorene were biodegraded by *Microbacterium esteraromaticum*, *Stenotrophomonas maltophilia*, and *P. aeruginosa* at rates greater than 50% in 45 d. Patowary *et al*. (2018), revealed that in just 3 months, *P. aeruginosa* was able to break down more than 60% of the petroleum oil used in their experiment. Currently *Stutzerimonas balearica* modified using the CRISPR-Cas9 system had 94.07% of its aromatic component residues destroyed after being cultured with 0.5% crude oil for 7 d. An increased breakdown of the aliphatic and aromatic compounds was observed, in consistence with gene expression of the CRISPR-treated bacteria.

Conclusions and Recommendations

Stutzerimonas balearica was the predominant genus isolated from crude oil–contaminated soil in this study. This bacterium demonstrated a strong potential for application in the bioremediation of hydrocarbon-polluted environments, as it can utilize crude oil as its sole source of carbon and energy. In light of these

results, further studies are recommended to identify additional bacterial species capable of methane utilization in contaminated soils, contributing to the development of more effective and sustainable bioremediation strategies.

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Novelty Statement

To our knowledge, this study reports for the first time globally the isolation of *Stutzerimonas balearica* with the ability to grow in the presence of methane, attributable to the presence of the soluble methane monooxygenase (*sMMO*) gene. Furthermore, the biodegradation capacity of crude oil by this bacterium was enhanced through application of the CRISPR-Cas9 system.

Author's Contribution

AAM, AMRA-T and ZWA: Conceptualization, data curation, investigation, supervision, validation, roles, writing original draft, writing review and editing.

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Ethical approval
Non-applicable.

Generative AI and AI-assisted technology statement
The authors declare that no generative AI and AI assisted technology was used in the creation of this manuscript.

Conflict of interestS
The authors declare that they haven't any conflicts of interest regarding publication of this manuscript.

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