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RESEARCH ARTICLE

Molecular approach to the characterization of lipase encoding genes from *Moraxella* sp. SBE01

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Abstract

Lipase from *Moraxella* sp. SBE01 is an expression of the gene encoding lipase. Detection and characterization of the *Moraxella* sp. SBE01 lipase coding gene is necessary for large-scale lipase production through genetic engineering. This study aimed to observe the molecular weight, amino acid sequence, length, and conserved amino acids in the DNA encoding the lipase gene, with the goal of identifying and characterizing the lipase-coding gene from *Moraxella* sp. *SBE01*. The primer design process was conducted to amplify the lipase gene from Moraxella sp. SBE01 using specialized software for sequence alignment and phylogenetic analysis. Amplification was carried out using PCR with the designed primer, forward primer (GTC ATG ATG TAC TTC CAY GGN GGN GG), reverse primer (GGT TGC CGC CGG CDS WRT CNC C). PCR was carried out under pre-denatured conditions at 95°C (3 minutes), followed by 30 cycles of denaturation at 95°C, annealing at 66°C (30 seconds), 70°C elongations (1 minute) and final elongation of 70°C (10 minutes). The PCR results were electrophoresed using 1% agarose gel with a 1 kb DNA marker. The PCR results were sequenced and analyzed for gene and amino acid sequences and the type of lipase expressed. Sequencing resulted in 387 bp of the nucleotide sequence. The gene and amino acid sequences from *Moraxella* sp. SBE01 had high homology with the gene and amino acid sequences from *Moraxella* sp. strain TA144. The lipase gene encodes a protein consisting of 129 amino acids and contains a conserved HGG (His-Gly-Gly) motif, which is characteristic of lipases in family IV, also known as the hormone-sensitive lipase (HSL) family. This conserved sequence suggests that the lipase shares structural and functional similarities with other enzymes in the HSL family, playing a key role in lipid metabolism.

1. INTRODUCTION

Lipase is an enzyme with a specific catalytic ability toward lipids or fats (1,2). This enzyme plays a crucial role in the hydrolysis of lipids, where it breaks down lipids into fatty acids and glycerol by cleaving the ester bonds that link these molecules (3–5). This hydrolysis process is essential for fat metabolism, allowing the body to access and utilize fatty acids as an energy source for various biological processes. Additionally, lipase is also known to participate in esterification reactions, where it facilitates the formation of ester bonds between fatty acids and

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alcohol, demonstrating the enzyme's dual role in biochemical reactions related to lipids (6–8). Therefore, lipase is not only involved in the breakdown of lipids but also to the reformation of lipid molecules through esterification reactions (9).

Lipase, with its remarkable biocatalytic properties, is an enzyme widely utilized in various sectors. It plays a crucial role in the production of numerous commercial products, such as in the detergent industry, where it enhances cleaning efficiency by breaking down fats and oils. In the cosmetic industry (10), lipase is employed in formulating products that require specific lipid transformations (11). Additionally, it is essential in the food and pharmaceutical industries (12), contributing to processes like flavor development, food preservation, and the production of bioactive compounds vital for health (13). Moreover, lipase significantly contributes to environmental management by breaking down pollutants into simpler, less harmful substances, making it a key tool in bioremediation efforts (7,14,15). Thus, lipase holds a vital role in modern industry and environmental sustainability.

A group of enzymes responsible for the hydrolysis of lipids has been extensively studied and isolated from various lipolytic bacteria. These bacteria, known for their ability to produce lipase, play a crucial role in natural lipid degradation processes (16). Lipolytic bacteria are a unique group of microorganisms that can utilize lipids as a primary nutrient source for their growth and metabolism. To access these nutrients, lipids must first be broken down by extracellular lipases produced by the bacteria, which catalyze the hydrolysis of fats into fatty acids and glycerol, making them more readily absorbable by the cells (17,18).

Lipases derived from lipolytic bacteria are particularly significant because of their versatile applications in various industries, including food, pharmaceuticals, biofuels, and bioremediation. These enzymes, naturally synthesized by the bacteria, hold immense potential due to their ability to function under a wide range of conditions, including extreme temperatures and pH levels (10). Several studies have identified a variety of lipolytic bacteria capable of producing lipase, highlighting the diversity of this group. Notable genera include *Pseudomonas*, *Vibrio*, *Acinetobacter*, *Proteus*, *Burkholderia*, *Chromobacterium*, *Serratia*, *Bacillus*, *Staphylococcus*, *Streptomyces*, *Moraxella*, and *Psychrobacter* (18–20). These bacteria have been isolated from different environments, each exhibiting unique lipase production capabilities that are tailored to their ecological niches.

One particularly interesting research by (21) focused on the lipolytic bacterium *Moraxella* sp. SBE01, which was isolated from spent bleaching earth, a waste product from the oil refining industry. This bacterium was found to produce lipase with notable efficiency and has been identified as a potential bioremediation agent for environments contaminated with oil. The ability of *Moraxella* sp. SBE01 to degrade lipids in such polluted environments underscores the importance of lipolytic bacteria not only in natural ecosystems but also in industrial applications aimed at environmental restoration and sustainability (22).

The lipase produced by *Moraxella* sp. SBE01 results from the expression of the lipase-coding gene. Genetic engineering can be employed to facilitate large-scale lipase production (23). The characters observed in this study include molecular weight, amino acid sequence, amino acid length and conserved amino acids in the DNA encoding the lipase gene. Therefore, the detection and characterization of the *Moraxella* sp. SBE01 lipase coding gene is necessary for large-scale lipase production through genetic engineering. The purpose of this study is to identify and describe the lipase-coding gene from *Moraxella* sp. SBE01.

2. MATERIALS AND METHODS

2.1. Primary Design

Nucleotide sequence data coding for lipase from various species within the *Moraxella* genera were obtained from the NCBI BLAST database (www.ncbi.nlm.nih.gov/BLAST/), a comprehensive resource for nucleotide and protein sequences. These sequences were carefully selected to encompass a broad range of lipase-coding genes to ensure a diverse dataset. Once acquired, the set of lipase-coding nucleotide sequences was subjected to alignment using MEGA6 (Molecular Evolutionary Genetics Analysis version 6), a software tool designed for sequence alignment and phylogenetic analysis. This alignment process was crucial to identify conserved regions and ensure the accuracy of the subsequent analyses. Following the alignment, the target nucleotide sequence identified in this process,was used to design specific primers using the Primer3 program https://www.primer3plus.com/index.html (24). The primer design produces forward primer (GTC ATG ATG TAC TTC CAY GGN GGN GG) and reverse primer (GGT TGC CGC CGG CDS WRT CNC C).

2.2. DNA isolation of *Moraxella* sp. SBE01

The DNA isolation process was conducted following the Zymo Research kit protocol (Quick-DNA Fungal/Bacterial Miniprep Kit, catalog number D6005) (17,25). The procedure involved several key stages: preparations of the bacterial cells, cell lysis, binding of the DNA, washing the DNA, and DNA elution. In the preparation stage, 50-100 mg of bacterial cells were placed into a 1.5 ml microtube. The cell lysis stage was carried out by adding 750 µLof Bashing Bead™ Buffer to bacterial cells. The mixture was vortexed at low speed for 1 minute and 3 minutes of cold shock. This process was repeated five times. For one minute, the mixture was centrifuged at 10.000 x g. The supernatant was collected.

DNA Binding stage was carried out by transferring the supernatant into the Zymo-Spin™ III-F Filter and Collection tube, which was then centrifuged at a speed of 8000 x g 1 minute. The filtrate obtained was added 1000 µL Genomic Lysis Buffer. In addition, 800 µL was transferred into Zymo-Spin TM IIC Columns and tubes. Centrifugation was carried out at a speed of 10.000 x g 1 minute (the process was repeated with the remaining mixture).

DNA Pre-Wash Buffer was added 100 µl into Zymo-Spin TM IIC Columns with a new tube, centrifuged at 10.000 x g for 1 minute. DNA Wash Buffer was added 500 µl g Zymo-Spin TM IIC Columns and centrifuged at 10.000 x g for 1 minute. The DNA elution stage was carried out by moving the Zymo-Spin TM IIC Column into a new 1.5 ml microtube and adding 100 µl of DNA Elution Buffer. Centrifuged at 10.000 x g for 30 seconds. The purity and concentration of DNA were assessed using nanodrop spectrophotometer Thermo Scientific™ NanoDrop™ 2000/2000C at a wavelength ratio of 260/280 nm. DNA purity and concentration are measured using the equation below (26).

$$DNA\ purity = \frac{Absorbance\ at\ 260\ nm}{Absorbance\ at\ 280\ nm} \quad (1)$$

$$DNA\ concentration\ (\mu g/ml) = Absorbance\ at\ 260\ nm\ x\ dilution\ factor\ x\ 50\ \mu g/mL \quad (2)$$

2.3. Amplification and Electrophoresis of the Lipase

Amplification of the lipase coding gene was executed using specifically designed forward and reverse primers. The forward primer sequence used was (GTC ATG ATG TAC TTC CAY GGN GGN GG), and the reverse primer sequence was (GGT TGC CGC CGG CDS WRT CNC C). These primers were meticulously designed to target conserved regions of the lipase gene, ensuring specificity and efficiency in the amplification process. The Polymerase Chain Reaction (PCR) was set up in a total reaction volume of 25 µL, which included 12.5 µL of GoTaq® Green Master Mix (Promega, GoTaq® Green Master Mix, catalog numbers M7122 and M7123). This master mix is a pre-mixed, ready-to-use solution containing DNA polymerase, dNTPs, $MgCl_2$, and reaction buffers optimized for PCR, providing convenience and reliability for high-fidelity DNA amplification. Additionally, the reaction mixture comprised 1 µL of forward primer, 1 µL of reverse primer, 1 µL of the DNA template, and 9.5 µL of nuclease-free water, ensuring a balanced and effective PCR reaction setup.

The amplification process commenced with an initial pre-denaturation step at 95°C for 3 minutes to denature the double-stranded DNA. This was followed by 30 cycles of the PCR process, each cycle consisting of three key stages: denaturation at 95°C for 30 seconds to separate the DNA strands, annealing at 66°C for 30 seconds to allow the primers to bind to the target sequences, and elongation at 70°C for 1 minute to enable the DNA polymerase to synthesize the new DNA strand. The PCR was concluded with a final elongation step at 70°C for 10 minutes, ensuring the complete extension of all amplified products. DNA amplification was conducted using Biolab Scientific Gradient Thermal Cycler BTHC-601.

The resulting PCR products were then subjected to gel electrophoresis using a 1% agarose gel. This concentration of agarose gel provides adequate resolution for the separation of DNA fragments based on size. A 1 kb DNA ladder (Geneaid, catalog number DL006) was included in the gel as a molecular weight marker to accurately determine the size of the amplified lipase gene fragments. After electrophoresis, the gel was stained with SYBR Safe DNA Gel Stain and visualized under ultraviolet (UV) light to confirm the successful amplification of the desired lipase gene fragments (17).

2.4. Sequencing and BLAST Analysis

Sequencing of PCR products was performed at the first BASE Malaysia. The GeneStudio tool was used to align the sequencing results, while the BLAST process was performed using the NCBI website

(www.ncbi.nlm.nih.gov/BLAST/). The amino acid sequence of the lipase gene was predicted from the DNA sequence using EMBOSS Transeq (www.ebi.ac.uk/Tools/st/emboss_transeq/) and BLASTp (www.ncbi.nlm.nih.gov/BLAST/), The amino acid sequences were predicted. The similarity of amino acid sequences to the database was compared through the alignment process. The amino acid sequences were aligned using www.ebi.ac.uk/Tools/msa/clustalo/ (27).

3. RESULTS AND DISCUSSION

3.1. DNA isolation of *Moraxella* sp. SBE01

The results of DNA isolation from *Moraxella* sp. SBE01 are presented in Figure 1. This image shows the results of DNA visualization using the 1% agarose electrophoresis method. The visualization results show that the *Moraxella* sp. SBE01 DNA band has a molecular weight of more than 10,000 bp. The glow of DNA bands in the ultraviolet light transilluminator looks clear, thick and bright. The DNA concentration can be inferred by comparing the thickness and luminescence of the genomic DNA band with the marker DNA. The luminescence of DNA bands in an ultraviolet light transilluminator determines the purity and concentration of DNA (28,29).

The success of downstream analysis is greatly influenced by the purity and concentration of DNA (30). Therefore, the purity and concentration of DNA were further confirmed using spectrophotometric methods. DNA purity and concentration were measured using a NanoDrop spectrophotometer with an absorbance ratio of A260/280. This ratio helps determine DNA purity and concentration, as nucleic acids exhibit maximum absorbance at 260 nm, while proteins absorb light at a f 280 nm. The absorbance ratio of A260/A280 for the extracted DNA is 1.90, a key indicator of the DNA's purity. This ratio is crucial as it reflects the quality of the DNA sample, particularly in terms of contamination by proteins or other impurities. A ratio value within the range of 1.8 to 2.0 is generally accepted as indicative of high-purity DNA, as it suggests minimal contamination and good sample integrity. The measured ratio of 1.90, therefore, falls within this optimal range, suggesting that the extracted DNA is of high purity and is not significantly contaminated by proteins (31,32).

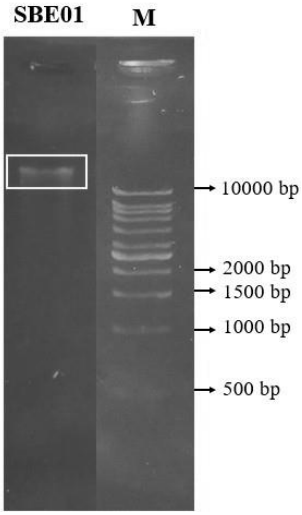

Figure 1. Electropherogram of the gene sequence from *Moraxella* sp. *SBE01* DNA. Note: M: Marker; SBE01: SBE01 isolate; The white box indicates that the molecular weight of the DNA band is greater than 10,000 bp.

This finding is consistent with the guidelines provided by (33), which emphasize that an ideal A260/A280 absorbance ratio for nucleic acids, included DNA, should fall within the range of 1.8 to 2.0. This ratio is a critical indicator used in molecular biology to assess the purity of DNA samples. Ratios within this optimal range are generally indicative of DNA that is relatively free from significant contamination, especially from proteins. Proteins are known to have a strong absorbance at 280 nm due to the presence of aromatic amino acids such as tryptophan, tyrosine, and phenylalanine. When proteins are present in a DNA sample, their absorbance at 280 nm can contribute significantly to the total absorbance at this wavelength, thereby reducing the A260/A280 ratio below the desired 1.8 threshold. A ratio lower than 1.8 would thus signal protein contamination in the DNA preparation, which could interfere with downstream applications such as PCR, sequencing, or cloning, where high purity is essential for reliable results. In such cases, additional purification steps might be necessary to remove the protein contaminants and enhance sample quality.

Conversely, if the A260/A280 ratio is significantly higher than 2.0, it might indicate the presence of RNA contamination. RNA, like DNA, absorbs strongly at 260 nm, but its contribution to the total absorbance can disproportionately elevate the ratio. An elevated ratio suggests that RNA molecules are present in the sample in substantial quantities, potentially affecting the accuracy and reliability of subsequent molecular analyses. RNA contamination is particularly concerning in applications requiring pure DNA, as it can compete with DNA in binding assays or distort quantification measurements (34).

3.2. Amplification and Characterization of the Lipase Coding Gene

Amplification of the lipase coding gene was performed using a specific primer PCR technique, forward primer (GTC ATG ATG TAC TTC CAY GGN GGN GG) and reverse primer (GGT TGC CGC CGG CDS WRT CNC C). The annealing temperature is optimized to obtain the optimum temperature for attaching the primer and DNA template. Previous studies (35) have indicated that the success of the DNA amplification process is influenced by several factors, one of which is the annealing temperature. The right annealing temperature will produce PCR products with good purity and concentration. Optimizing the annealing temperature begins with calculating the melting temperature (Tm) of the primer used. The temperature of 65°C is the Tm obtained from the calculation results. Based on these calculations, optimization was carried out twice at a temperature range of 58-67°C and 64-70°C. The annealing temperature was set by decreasing 3-5°C below the primer Tm and increasing 5°C above Tm temperature (36,37).

The initial optimization of the PCR conditions resulted in the observation of thin DNA bands at annealing temperatures of 66.3°C and 67°C, indicating some degree of successful amplification. In contrast, when the annealing temperatures were set between 58°C and 65°C, no visible amplicons were produced upon visualization, suggesting that these lower temperatures were not conducive to effective primer binding and subsequent DNA amplification.

In the subsequent round of optimization, the annealing temperature range was adjusted to span from 64°C to 70°C. Within this range, temperatures of 64-65°C and 68-70°C led to the formation of two distinct bands, approximately 250 bp and 400 bp in size, indicating the amplification of multiple DNA fragments. Interestingly, at 66°C, a single DNA band of approximately 400 bp was observed in the 4th well, suggesting that this temperature facilitated more specific amplification of the target sequence (Figure 2). According to study (38), successful PCR amplification is highly dependent on the annealing temperature. If the temperature is set too high, the primers may fail to bind to the DNA template, preventing the formation of PCR products. Conversely, an annealing temperature that is too low can result in non-specific binding of the primers to the DNA template, leading to the amplification of unintended sequences. The sequencing process was subsequently employed to determine the nucleotide sequence of the lipase-coding gene corresponding to the 400 bp band. This sequence is crucial for understanding the genetic basis of lipase production. As reported in previous studies (31,39), the optimal annealing temperature typically results in the production of a single, thick, and clear DNA band, which is indicative of specific and efficient amplification of the target gene.

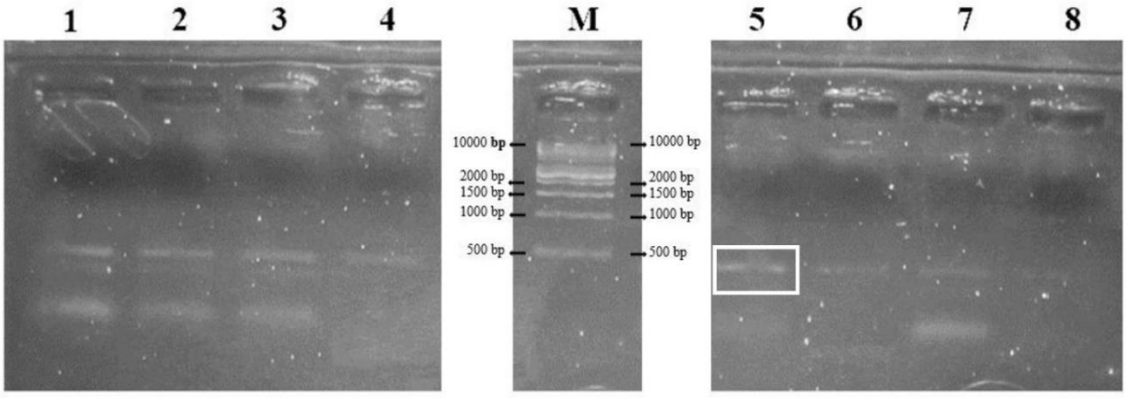

Figure 2. Amplification of the *Moraxella* sp. SBE01 lipase coding gene at different annealing temperatures: 1) 64°C; 2) 64.4.6°C; 3) 65.2°C; 4) 66°C; 5) 67.7°C; 6) 68.8°C; 7) 69.6°C; 8) 70°C; M) 1 kb DNA marker (500 bp, 1000 bp, 1500 bp, 2000 bp, 2500 bp, 3000 bp, 4000 bp, 5200 bp, 6200 bp, 7000 bp and 10,000 bp); The white box indicates that the molecular weight of the DNA band is greater than 400 bp.

Further, the amplification of the lipase coding gene of *Moraxella* sp. SBE01 was carried out at an annealing temperature of 66°C and yielding a molecular weight in the range of 400 bp (Figure 2). The sequencing process produced a nucleotide sequence of 387 bp. The identity of this nucleotide sequence was analyzed using BLASTx on the website. BLASTx is utilized to search protein databases using target nucleotide sequences. BLASTx analysis produced the highest percent similarity of 47.37% with the lipase gene sequence of *Moraxella* sp. strain TA144 (Table 1). The BLASTp method is also employed to ensure the homology of the protein coded by the target gene with the protein database at NCBI. The protein sequence was obtained by translating 387 bp nucleotides from the site https://www.ebi.ac.uk/Tools/st/emboss_transeq/. The analysis indicated that 387 bp nucleotides codes for 129 amino acids. The amino acid sequence was subsequently analyzed using BLASTp, yielding results consistent with the BLASTx analysis, where the protein from the *Moraxella* sp. Lip gene showed similarity to the lipase protein from *Moraxella* sp. strain TA144. The percentage of protein similarity between *Moraxella* sp SBE01 and *Moraxella* sp. TA144 is 47.37% (Table 1).

Table 1. BLASTx and BLASTp results of lipase and amino acid coding gene sequences from *Moraxella* sp. SBE01

Strain	Species	Similarity (%)	Accession number
Moraxella sp. SBE01	*Moraxella* sp. TA144	47.37	P24484.1
	Psychrobacter arenosus	45.23	WP_201498306.1
	Aquirhabdus sp.	38.11	HEY4712716.1
	Psychrobacter sp.	35.99	MDO769276.1
	Aquirhabdus parva	34.08	WP_162818176.1

The amino acid sequence of *Moraxella* sp. was subsequently aligned with the amino acid sequence of *Moraxella* sp. strain TA144 using the Clustal Omega tool, accessible via the European Bioinformatics Institute's website (https://www.ebi.ac.uk/Tools/msa/clustalo/). This alignment process is crucial for identifying conserved regions within protein sequences, which can provide insights into functional similarities between different strains. The alignment revealed that the conserved lipase produced by *Moraxella* sp. exhibits a distinct HGG (His-Gly-Gly) motif, as illustrated in Figure 3. This finding aligns with previous research conducted by Ramnath *et al.*, (19), which indicated that lipases from the *Moraxella* genus often possess conserved protein sequences characterized by the motifs GXSXG (Gly-Xaa-Ser-Xaa-Gly) and HGG (His-Gly-Gly). These motifs are critical for the enzymatic activity and structural stability of lipases. Specifically, lipases that display a conserved sequence with the HGG (His-Gly-Gly) pattern are classified within the family IV lipases, a group known for their specific substrate preferences and catalytic mechanisms (40).

Family IV lipases are known to possess a sequence that closely resemble the Hormone Sensitive Lipase (HSL) sequence found in mammals. Due to this similarity, lipases belonging to this family are often referred to as HSL-like lipases (12,30). This structural and functional parallel suggests that family IV lipases may share evolutionary and mechanistic traits with mammalian HSLs, making them particularly interesting for both biochemical studies and potential biotechnological applications.

Lipolytic bacteria capable of producing family IV lipases include a diverse array of species, such as *Pseudomonas* sp., *Alcaligenes eutrophus*, *Moraxella* sp., *Alicyclobacillus acidocaldarius*, *Escherichia coli*, *Paenibacillus calidifontis*, and *Archaeoglobus fulgidus*. These bacteria inhabit a variety of environments, from soil and water to extreme habitats like hot springs and hydrothermal vents, highlighting the adaptability and robustness of these enzymes in different ecological contexts (22).

The defining feature of family IV lipases is the presence of two conserved sequence motifs, GXSXG (Gly-Xaa-Ser-Xaa-Gly) and HGG (His-Gly-Gly). These motifs are critical for the catalytic function of the enzyme, with the GXSXG motif playing a key role in the formation of the active site, where the serine residue acts as a nucleophile during the hydrolysis of lipid substrates. The HGG motif is involved in stabilizing the enzyme's structure, particularly in the positioning of the histidine residue, which is essential for the enzyme's catalytic mechanism.

Family IV lipases exhibit activity over a broad temperature range, from 30°C to 90°C, demonstrating their versatility and potential for applications under various industrial conditions (21). This wide temperature tolerance is advantageous for processes that require stable enzyme activity at elevated temperatures, such as in bioreactors or in the production of biofuels.

The lipase enzyme produced by *Moraxella* sp. SBE01 has been specifically identified to exhibit optimal enzymatic activity at a temperature of 50°C, as detailed in a previous study (14). This finding is of particularly significant for industrial applications, as it demonstrates the enzyme's capability to maintain a high level of activity at a moderately elevated temperature. Such thermal stability is a desirable trait for enzymes used in industrial

processes, where reactions often need to occur at higher temperatures to increase efficiency and speed, reduce microbial contamination, and enhance the solubility of substrates. The high activity of the lipase from *Moraxella* sp. SBE01 at 50°C suggests that it can function effectively under conditions typically required in industrial settings, such as biodiesel production, the hydrolysis of fats and oils, and other lipid-related processes.

Figure 3. Alignment results of *Moraxella* sp. SBE01 amino acids with *Moraxella* sp. strain TA144 amino acids. The conserved lipase pattern is marked in black in the image above

The ability of *Moraxella* sp. SBE01 tp produce a lipase that is both thermally stable and highly active at this optimal temperature highlights the potential applications of family IV lipases in various biotechnological fields (41). These enzymes could be particularly useful in the degradation of fats and oils in wastewater treatment facilities, where high temperatures are often utilized to accelerate the breakdown of lipid contaminants. Additionally, the synthesis of biodiesel, a renewable energy source, involves the transesterification of fats and oils, a process that can benefit greatly from lipases that remain active and stable at higher temperatures. The use of such robust enzymes can lead to more efficient conversion rates and reduce the need for harsh chemical catalysts, making the process more environmentally friendly and cost-effective (42).

4. CONCLUSIONS

The lipase coding gene sequence of *Moraxella* sp. SBE01 is 387 bp long and encodes 129 amino acids. This sequence demonstrates homology with the lipase gene and corresponding amino acid sequence of *Moraxella* sp. strain TA144. The amino acids produced from this gene have the conserved pattern HGG (His-Gly-Gly) and are grouped into family IV (HSL). Lipases from family IV have activity over a wide temperature range, from mesophilic to thermophilic (30 - 90°C). Further research is needed to test the activity of lipase produced by *Moraxella* sp. SBE01 for various industries, pharmaceuticals, and medical biotechnologies.

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