GREENING INDIA'S FUEL: *BACILLUS VELEZENSIS* DAA1, A GAME-CHANGER IN SUSTAINABLE LIPID SYNTHESIS FOR BIOENERGY

Sugandha Asthana[1], Aarti Sharma[2], Neelam R Yadav[3] and Deepshikha Pande Katare[1]*

Address(es):
[1] Lab 320-Proteomics & Genomics Lab, Amity Institute of Biotechnology, Amity University, Uttar Pradesh, Sector-125, Noida, India-201301.
[2] Neurodiagnostic Inc., Rockville (MD), USA.
[3] Department of Molecular Biology, Biotechnology and Bioinformatics, CCS Haryana Agricultural University Hisar-125004, Haryana.

Corresponding author: dpkatare@amity.edu

ARTICLE INFO

ABSTRACT

India presently depends on imports of crude oil from overseas sources. However, the increasing demand for fossil fuels and the depletion of these finite resources are contributing to a rise in the costs associated with these imports. There has been a pressing demand for the development of cost-effective, renewable, and environmentally sustainable solutions. Biofuels, which are obtained from a wide range of biomass sources such as plants, animals, and microbes, present a potentially viable answer. Microorganisms exhibiting the ability to accumulate lipids in excess of 20% in the form of triacylglycerols and wax esters are commonly denoted as "oleaginous". The primary objective of this work was to isolate and screen bacterial strains with high lipid accumulation potential from soil samples collected along riverbanks. A total of fourteen strains were isolated and subjected to screening in order to assess their lipid production capabilities. Among these strains, *Bacillus velezensis* strain DAA1 exhibited the highest level of lipid synthesis. Response Surface Methodology was used to optimize carbon and salt concentrations. The experimental findings indicated that the utilization of carbon at a concentration of 12.5 g/L with salt concentration of 17.07 g/L, resulted in a noteworthy lipid content of 79%. The major fatty acid found through Gas Chromatography-Mass Spectrometry (GC-MS) for lipid characterization was determined to be 12-methyltetradecanoic acid, constituting 41% of the overall lipid composition. This work highlights the potential of *Bacillus velezensis* DAA1 as a promising alternative for lipid production, with possible implications for biofuel generation in the industry.

Keywords: Oleaginous, *Bacillus,* Alternate fuels, Response Surface Methodology, Lipids

INTRODUCTION

With the increase in industrialization, there has been a significant increase in energy demand. According to Petroleum Planning and Analysis Cell (PPAC), India's crude oil imports from March 2021 to December 2022 amounted to a total value of 119.2 billion USD, which included 4.398 million USD worth of fuel oils. Prioritizing the enhancement of local fuel oil production and the reduction of imports are key objectives. The increasing popularity of non-renewable fossil fuel alternatives is driven by the need to mitigate pollution and greenhouse gas emissions. According to on prevailing energy consumption trends, it can be inferred that our existing energy reserves will be sufficient to supply the anticipated demand until the year 2030 (**Gautam *et al.,* 2022**). Among the available alternatives, biofuels have emerged as a promising and sustainable substitute for traditional fossil fuels in the foreseeable future. Biofuels, predominantly derived from biomass, offer a sustainable and environmentally friendly energy alternative characterized by little ecological repercussions and limited adverse consequences (**Talha Akhtar, 2023**). Notably, they exhibit no contribution to the greenhouse effect and result in reduced carbon emissions (**Demirbas, 2007**). Biodiesel, is typically produced via the transesterification reaction involving biomass-derived acylglycerols, a catalyst, and a short-chain alcohol (e.g., methanol or ethanol) (**Neeti *et al.,* 2023**).Primary biofuel resources use unprocessed organic matter as fuel, while secondary biofuel resources convert a wide variety of biomasses, such as edible and non-edible plant materials and microbial agents (**Leong *et al.,* 2018**). In India, around 32% of the country's primary energy is derived from biomass sources, a form of energy that is utilized by nearly 70% of the population. Based on the data provided by the Ministry of New and Renewable Energy (MNRE), India possesses an annual biomass reserve of 750 million metric tons. The research findings indicated a requirement for an additional 230 million metric tons of biomass. Various microorganisms, such as bacteria, yeasts, filamentous fungi, and unicellular algae, possess the ability to biosynthesize lipids under appropriate environmental conditions (**Maza *et al.,* 2020**). Oleaginous microorganisms are characterized by their capacity to amass lipids over 20% of their dry cell biomass (**Ratledge, 1994**). The microorganisms undergo a process of converting sucrose, carbon dioxide, and organic acids into a single cell oil (SCO). This SCO holds potential as a viable source for the manufacturing of biodiesel, serving as an alternative to traditional methods (**Agrawal & Kumar, 2023**). Under nitrogen

limitation, it has been found that specific bacteria have the capacity to accumulate lipids up to 60% of their Dry Cell Weight (DCW) (**Prabhu *et al.,* 2019**). The bacterial cytoplasm is characterized by the presence of lipid bodies, which mostly consist of Triacylglycerols (TAGs), Free Fatty Acids (FFAs), and sterol esters. The lipid components play a crucial role as precursors in the production of biofuels (**Yao *et al.,* 2012**). Triacylglycerols (TAGs) are stored in significant quantities in some bacteria, including Mycobacterium, Rhodococcus, Bacillus, Nocardia, and Streptomyces (**Wältermann *et al.,* 2005**). Due to their high energy content, TAGs and fatty acids extracted from microorganisms can potentially be used to produce biofuels (**Lestari *et al.,* 2009**). The elongation of fatty acid (FA) chains acts as a mechanism for generating fuels with distinctive properties. For example, fatty acids (FAs) with carbon chain lengths ranging from C10 to C14 can be employed as components of jet biofuels, whereas FAs with carbon chain lengths spanning from C16 to C18 are appropriate for the production of biofuels (**Sarsekeyeva *et al.,* 2014**).

The use of Response Surface Methodology (RSM) enables the comprehension of interactions among variables and the calculation of the prospective quantity of subsequent responses. The approach is characterized by its efficiency and cost-effectiveness, as it necessitates a reduced number of tests and consequently fewer resources in total. (**Ghosh *et al.,* 2014**). The present methodology is presently extensively employed for the purpose of optimizing medium constituents and environmental circumstances in order to enhance lipid production. This is achieved by determining the best growth parameters that establish the most favorable relationship between numerous independent factors and a variable, with the aim of maximizing lipid synthesis (**Nair *et al.,* 2018**).

In this study, our primary objective was to isolate oleaginous bacterial strains from soil samples gathered along a riverside. The strains were thoroughly evaluated for their ability to produce triacylglycerols (TAGs), utilizing Nile-Red as a fluorescent dye to indicate the presence of TAGs. Out of the total of fourteen isolated isolates, six demonstrated fluorescence, which serves as an indicator of lipid production. The lipids were subsequently isolated from these strains utilizing the Bligh and Dyer method. Significantly, a particular strain, denoted as DAA1, exhibited the maximum lipid production and was then chosen for comprehensive analysis. Following that, we utilized the central composite design in Design Expert-13 software to optimize the ratios of carbon, nitrogen, and salt for the purpose of enhancing lipid production. The utilization of gas chromatography was

employed as a means to characterize the lipids that were generated. The strain was identified as *Bacillus velezensis* was determined using phylogenetic analysis, and the associated sequence has been archived in the NCBI database. The lipid production of DAA1 was found to be noteworthy, constituting almost 75% of its cellular dry weight. The predominant fatty acids identified were 12-methyltetradecanoic acid (41%), palmitic acid (21%), and stearic acid (16%). This data indicate that this particular strain exhibits considerable potential as a viable substitute for the manufacture of biodiesel.

MATERIALS AND METHODS

Sample collection and Isolation of bacterial strain

For this study, soil samples were collected from four different sites along the Ithikkara riverside (8.8525° N, 76.7907° E) in Kerala, India. Top soil was collected from 5-6cm underneath the surface of the riverside and placed in a sterilized plastic bag. Bacterial strains were isolated from the soil collected by performing serial dilution. Dilutions of 10^{-5}, 10^{-6} and 10^{-7} were spread on nutrient agar plates to obtain individual colonies. Visually distinct colonies were further purified by streak plate method **(Bradbury, 1970)**.

Screening of lipid producing bacterial strains

Qualitative screening was performed by incorporating Nile Red in low C:N ratio media containing 20g/L glucose, 1g/L yeast extract, 1/gL peptone and 1g/L sodium chloride. Nile red was dissolved in DMSO (1mg/ml) and added in media by filter sterilization to make the final concentration 1µg/ml. The isolated bacterial strains were spotted onto the agar plates and incubated at 37°C for 120h. The plates were visualized under UV light and the strains emitting fluorescence were considered potential lipid accumulating bacteria and further screened.

Small-scale lipid extraction

The extraction of lipids was conducted using the Bligh and Dyer method **(Bligh & Dyer, 1959)** with certain modifications. The media underwent centrifugation at a speed of 6000 revolutions per minute (rpm), resulting in the formation of a pellet. This pellet was air dried till constant weight was observe and subjected to a treatment of methanol and chloroform in a ratio of 2:1. The solution underwent sonication for a duration of 2 minutes utilizing an ultrasonic sonicator (Sonics, Vibra-Cell). The solution was maintained at room temperature for 30 minutes and thereafter centrifuged at a speed of 6000 rpm for 10 minutes. The solution was allowed to separate in a separating funnel containing a mixture of methanol, chloroform, and water in a ratio of 1:2:1. The organic phase, which contains lipids, was subsequently retrieved and air dried. The lipid yield percentage was determined by employing the formula, as reported by **Qadeer et al., 2018**.

$$\frac{\text{Weight of lipid (g)}}{\text{Cell dry weight (g)}} \times 100$$

Where,
Weight of lipid= Weight of tube after chloroform evaporation – weight of empty tube
Cell dry weight = Weight of tube after pelleting and air drying – weight of empty tube

Growth Kinetics analysis

The strain, DAA1, was grown in optimized C:N ratio media and was incubated at 37°C. To understand the growth pattern of these strains, optical density (OD) reading was taken using spectrophotometer (Spectra, Eppendorf) at 600nm after every 24 hours for 5 days. The data was plotted using GraphPad Prism 5.0.

Process optimization by Response Surface Methodology using central composite design (CCD)

RSM was used to optimize the most significant variables. In this study, two factors, Carbon and salt ratio, were considered. They were studied at five levels: 1, 0, +1 and +α. A set of 13 experiments were carried out based on the design built by the statistical software Design-Expert (v13.0.0, Stat-Ease, Minneapolis, USA).

Identification

Morphological, physiological and biochemical characteristics were used to identify potential oleaginous bacteria. Physiological properties were determined according to Bergey's manual of systematic bacteriology. Standard protocols were used biochemical characterization including TSI, Indole, citrate, motility and peroxide. For identification of the strain, DAA1 was grown in nutrient broth for 24 hours at 37ºC and DNA was isolated using CTAB method **(Andreou, 2013)**. 16S rRNA sequencing using ITS primer (F 5' AGAGTTTGATCCTGGCTCAG 3' and R 5' ACGGCTACCTTGTTACGACTT 3') was used for further confirmation.

PCR was performed using the standard protocol of Thermo Scientific Phusion High-Fidelity kit under the following conditions: 95ºC for 3 min, 95ºC for 30 sec, 56ºC for 30 sec, 72ºC for 1 min and final extension of 5 min 72ºC for 30 cycles. The purified PCR product was for sequenced. The resulting sequence was analyzed using NCBI and a phylogenetic tree was made using MEGA 11. The sequence was submitted to GenBank.

Characterization of lipid using Gas Chromatography- Mass Spectrometry

To make Fatty Acid Methyl Esters (FAMEs) from extracted lipid, 2ml of 2.5% H_2SO_4 in methanol was added. This was heated at 85ºC for one hour and cooled at room temperature. To this, 3 ml of deionized water and 400ul of hexane was added. This was vortexed for 20−30 s and then centrifuged to facilitate phase separation **(Guo et al., 2015)**. The upper organic phase (150–200 µL) was collected and transferred. The sample was analyzed for GC-MS using Shimadzu's GCMS-QP2010 series, India. The initial temperature of the oven was programmed at 140ºC for 5 min followed by final temperature of 280ºC where it was held for 10 minutes. The sample was run for 50 minutes.

RESULTS

Isolation and screening of bacterial strains from soil samples

In this study, soil samples were collected from four distinct points along the river. To isolate morphologically diverse bacterial strains, serial dilution and spread plating was performed. A set of fourteen distinct strains was chosen and subsequently purified by means of the streak plate method in order to produce single colonies. Result is shown in Figure 1. Specific bacterial strains, when grown in limited nitrogen environment and excess carbon, they amass lipid compounds, including triacylglycerides (TAGs) and wax esters, in the form of intracellular granules. The lipid inclusions within the cells can be observed with the use of Nile Red, a lipophilic dye, for staining purposes. According to **Greenspan et al., 1985**, Nile Red exhibits fluorescence in a lipophilic environment. In order to ascertain the presence of lipid-producing bacteria, all isolated strains were spotted onto Nile Red plates. The lipophilic inclusions are stained by Nile Red, resulting in the emission of fluorescence when observed under UV light as shown in Figure 2. The observed fluorescence indicates the existence of lipid inclusions inside the cytoplasm, implying the capacity for storing and subsequently using fatty acids.
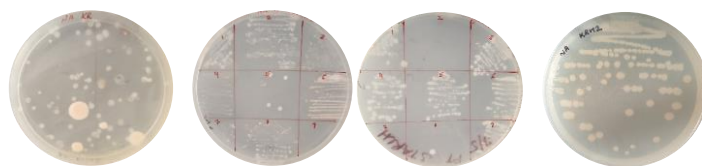

Figure 1 (a) Showing strains isolated from soil samples (b) Fourteen strains were selected and streaked on nutrient agar plates (c) showing the morphological characteristics of selected strain DAA1 (initially termed as KRH2)
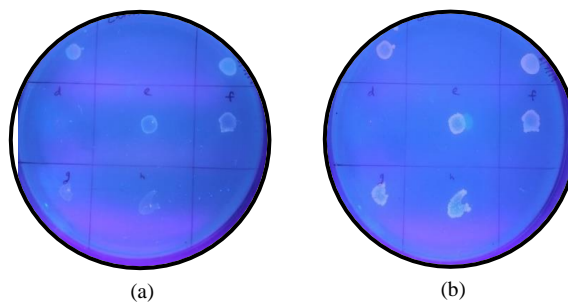

(a) (b)

Figure 2 Showing nine strains on (a) Day 1 and (b) Day 5. Six strains showing fluorescence were selected for further studies.

Small scale lipid production

The most extensively researched bacterial species capable of lipid production is *Rhodococcus* sp. Alvarez *et al.* were the first to isolate this strain from soil samples **(Alvarez et al., 1997)**. *R.opacus* PD630 is capable of accumulating as much as 87% of the lipids of its cell dried weight **(Alvarez et al., 2013)**. To ascertain the lipid percentage in isolated strains from our study, primary lipid extraction was conducted using the Bligh and Dyer method (Tab 1). The results indicated that strain DAA1, which is highlighted in Figure 2, exhibited the highest lipid production at 47.5% of its cell dry weight. As a result, this strain was chosen for subsequent identification and process optimization.

Table 1 Initial lipid produced from each positive strain

Strain	Dry biomass (g)	Lipid percentage (%)
Strain A	0.594	5.5
Strain C	0.084	4.8
Strain E	0.067	47.5
Strain F	0.300	1.3
Strain G	0.527	2.08
Strain H	0.256	2.34

Identification

The *Bacillus velezensis* strain, designated as DAA 1, was assigned the accession number ON680948. It was initially documented by Ruiz-Garcia (**Ruiz-García et al., 2005**) as this particular strain, which exhibited a close genetic relationship with *Bacillus subtilis* and *Bacillus amyloliquefaciens*. The agar plate exhibited a pearly white coloration, accompanied by colonies that possess an irregular shape, umbonate elevation, and undulate margins. The surface was arid and smooth. The observed biochemical characteristics are detailed in Tab 2. A phylogenetic tree was constructed utilizing Mega 11.0 and 100 bootstrap replications in accordance with the neighbor-joining method as seen in Figure 3. The strain is closely related to another strain of *B.velezensis* RH21, which is closely related to *B.subtilis* SkS27, according to the tree.

Table 2 Biochemical characteristics of *Bacillus velezensis*

Test	Result
Gram staining	Gram positive rods
Capsule staining	Positive
Endospore staining	Negative
Acid-fast staining	Negative
Motility	Negative
Indole test	Negative
MR test	Positive
VP test	Negative
Catalase test	Negative
TSI test	Lactose, glucose, sucrose fermentor, no gas production

Figure 3 Showing the phylogenetic tree designed using Mega 11.0 using neighbour joining method. It shows the strain DAA1 is closely related to OM996115.1 *Bacillus velezensis*

Growth Kinetic analysis

A succession of distinct growth phases transpires throughout microbial growth in a broth: log, lag, stationary and death phases (**Ruiz-García et al., 2005**). The stationary phase is a significant feature of oleaginous bacteria, as it is distinguished by a discontinuity in the growth kinetics curve (**Breidt et al., 1994**). This stage occurs prior to the death phase and is characterised by nutrient depletion in the medium and an increased death-to-growth ratio. Under the experimental conditions depicted in Figure 4, strain DAA1 attains the stationary phase on Day 5, following which the decline phase initiates. It is anticipated that both maximal lipid production and storage will transpire during this transitional phase. As a result, Day 5 has been designated as the most favourable time point for lipid extraction in subsequent experiments.
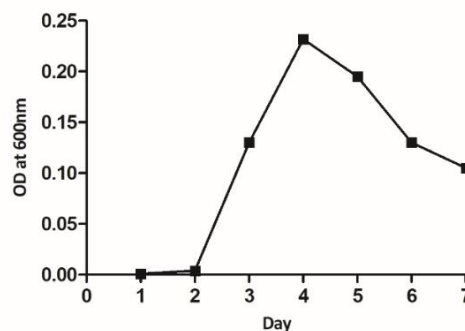

Figure 4 Shows the growth pattern of DAA 1 over 7 days. This strain reaches stationary phase between days 4 and 5 and maximum lipid production should take place.

Response Surface Methodology using CCD

Central Composite Design (CCD) of RSM was used in a series of 13 experiments to determine the ideal carbon and salt concentration for the maximum lipid production. Result is shown in Figure 5. The CCD matrix and responses for various combinations are shown in Tab 3. A regression equation was determined by the tool, which reflects the interaction between response and variables and was as follows:

$$Y = +40.43 + 24.63*A + 21.78*B$$

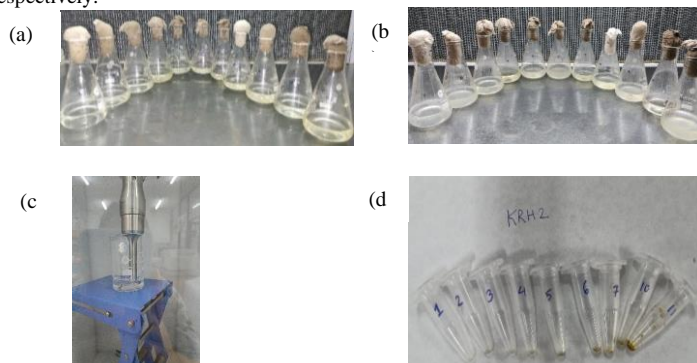Where Y is the predicted response and A and B are Carbon and Salt concentration, respectively.

Figure 5 Showing different experiment run as designed by Design Expert Software v 7.0.0 (a) inoculated with strain DAA 1 at day 0 (b) growth observed at Day 5 (c) sample being ultrasonicated for 2 minutes (d) lipid extracted after phase separation and chloroform evaporation

The statistical analysis conducted on our experiment identified a significant result. The p-value of 0.0224 and the F-value of 6.34 obtained from the ANOVA test (Tab 4) provided evidence for the statistical significance of the model. Significantly, the probability that this F-value is attributable to random noise is a mere 2.95%. Moreover, Prob>F values less than 0.05 indicate that model terms A and B are significant. The "Lack of Fit F-value," which was calculated to be 2.23, indicated that the lack of fit is not statistically significant in comparison to inherent error, with a 22.83% probability of noise interference. The "Adeq Precision" metric, which represents the signal-to-noise ratio, provided confirmation of signal adequacy with a value of 6.449. As a result, this model serves as a reliable instrument for methodical investigation of the design space.

The interactions between outcomes and variations in salt and carbon concentrations are effectively illustrated by the 3D response surface plots seen in Figure 6. When individual factors are taken into account, lipid production is correlated with lower concentrations of both carbon and sodium. In the second cycle, a maximum lipid production of 87.5% was recorded using 23.1 g/L of carbon source and 10 g/L of salt source. However, biomass production was comparatively minimal at this concentration. As a result, run 3, comprising 12.5 g/L of carbon source and 17.07 g/L of salt source, was chosen for subsequent experiments due to its attainment of the second-highest lipid concentration at 79.13%. As a primary carbon source, high-salt waste streams from industries such as meat processing, epoxy resin, and corn starch have the potential to reduce lipid production expenses. This not only facilitates the utilization of industrial waste but also contributes to the reduction of overall costs in biofuel production.

Table 3 Shows the design chart with responses of lipid production after 13 experiments were carried out in triplicates. Run 11 and 13 were not included in the analysis as no growth was observed in this ratio.

Run	Space Type	Factor 1 A: Carbon Concentration (g/L)	Factor 2 B- Salt Concentration (g/L)	Response 1: Dry Biomass (g)	Response 3: Lipid percentage (%)
1	Axial	12.5	2.93	0.18	3.89
2	Axial	23.11	10	0.008	87.5
3	Axial	12.5	17.07	0.115	79.13
4	Axial	1.89	10	0.278	4.32
5	Centre	12.5	10	0.22	5
6	Centre	12.5	10	0.016	43.75
7	Centre	12.5	10	0.016	25
8	Centre	12.5	10	0.22	5
9	Centre	12.5	10	0.223	4.93
10	Factorial	5	5	0.068	13.24
11	Factorial	20	15	0	0
12	Factorial	5	15	0.024	37.5
13	Factorial	20	5	0	0

Table 4 Shows the F value and p value of carbon and salt concentration

Source	Sum of Squares	F value	p-value
Model	5535.14	5.65	0.0295 (significant)
A-Carbon Concentration	2689.56	5.49	0.00471
B- Salt Concentration	2845.58	5.81	0.0425
Lack of Fit	2703.77	2.23	0.2283 (not significant)

Design-Expert® Software

Lipid Percentage
87.5
0

X1 = A: Carbon Concentration
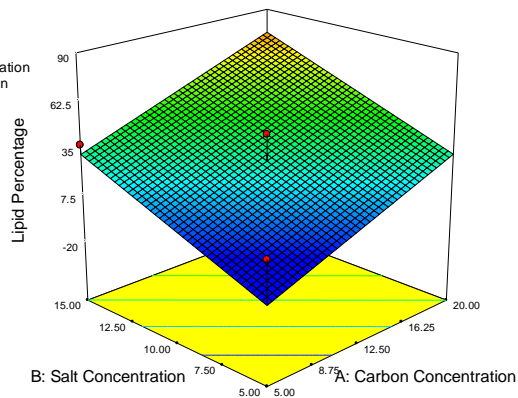X2 = B: Salt Concentration

Figure 6 3D showing the lipid production in respect to varying salt and carbon concentration

Fatty acid analysis by GC-MS

Throughout the duration of our research, we have observed particular fatty acids that predominate in the lipid composition. 12-methyltetradecanoic acid (aiC15:0, 41%), palmitic acid (C16:0, 21%), and stearic acid (C18:0, 16%) are the fatty acids that most significantly predominate. Additionally, we identified myristic acid (C14:0, 5%), pentadecanoic acid (C15:0, 7%), 14-methylhexadecanoic acid (aiC16:0, 8%), and 7-hexadecanoic acid (C17:1, cis7, 1.89%) in minute quantities. Result is shown in Figure 7. It is worth noting that the predominant fatty acids possess the desirable properties of length, branching, and saturation, which render them highly suitable for the production of biofuels. Given these characteristics, a prospective biofuel can be developed that possesses a reduced melting point and a substantial energy discharge. Therefore, strain DAA1 exhibits considerable potential as a viable substitute for lipid synthesis and showcases the ability to scale up industrial applications.
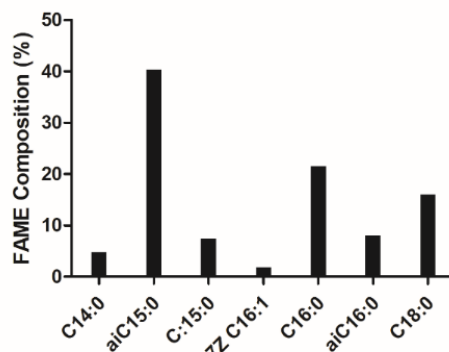

Figure 7 showing the fatty acid composition in *Bacillus velezensis*, DAA1. The most abundant fatty acid is 14-methyltetradecanoic acid (aiC15:0)

DISCUSSION

Soil is frequently used as a medium for isolating microorganisms owing to the abundance of microbial life that it contains in its natural habitat. The exponential growth of industry in coastal and urban regions has resulted in a pressing concern—the improper disposal of untreated industrial refuse into adjacent bodies of water (**Murínová & Dercová, 2013**). The discharge of waste without regulation has resulted in substantial alterations to the ecosystems of rivers (**Marathe *et al.*, 2017**). These changes have affected not only the river itself, but also the sediment and the microbes that inhabit it (**Kumar *et al.*, 2020**). On the contrary, the Ithikkara River has experienced a comparatively minor degree of contamination, predominantly attributable to its remote location from significant industrial centers. Consequently, soil samples were procured from four discrete locations along the riverbank to guarantee that the isolated strains remained free from contaminants and preserved as closely as possible to their natural state.

Polyhydroxyalkanoates acids (PHAs) and poly (3-hydroxybutyric acid) [poly(3HB)] are among the storage lipids that certain bacterial species are capable of producing in the context of lipid accumulation (**Hiroki, 1992**). Triacylglycerides (TAGs) are biosynthesized and accumulated intracellularly by a wide variety of bacteria, including the Gram-positive and Gram-negative taxa Streptomyces, Nocardia, Mycobacterium, Rhodococcus, Gordonia, and Bacillus. *Bacillus velezensis* is an aerobic, Gram-positive, endospore-forming bacterium that is renowned for its activities beyond lipid storage (**Adeniji *et al.*, 2019; Rabbee *et al.*, 2019; Ye *et al.*, 2018**). It is also known for facilitating plant growth, producing antibiotics, enzymes, iron chelation, phytohormones, and antitumor agents.

The present investigation effectively isolated *Bacillus velezensis* DAA1 from soil samples, and preliminary assessment revealed that it possesses the capacity to synthesise lipids at a rate of 47.5% by dry weight of its cells. In order to achieve optimal growth conditions for this strain, the Response Surface Methodology (RSM) was implemented. The utilization of this statistical instrument enabled us to examine the carbon-to-salt concentration ratio. The results indicated that run 3, which utilized 12.5 g/L of carbon (glucose) and 17.07 g/L of salt (sodium chloride) as a source, generated the most lipids (79.13%). In order to substantiate our results, we computed the p-value and F-value of the ANOVA test. These values of 0.0224 and 6.34, respectively, validated the statistical significance of our model. RSM has been shown to be an effective instrument for resource conservation and experimentation reduction, as exemplified by the study conducted by **Bagewadi *et al.*, 2018**, wherein they improved endoglucanase production in *Trichoderma harzianum* strain HZN11 to enhance bioethanol production.

Another investigation by **Towijit** *et al* used Response Surface Methodology (RSM) to improve the culture parameters, namely temperature and shaking speed, in order to enhance lipid production by *Rhodotorula graminis*. The maximum lipid yield recorded in the experiment was 17.04 g/L under the conditions of a temperature of 28°C and a shaking speed of 239 rpm. (**Umaporn Towijit *et al*, 2014**). The lipid synthesis in *Scendesmus* shows a significant increase of 54.64% subsequent to the optimization of media composition by the implementation of Response Surface Methodology (RSM)(**Yang *et al.*, 2014**). It is essential to minimize the costs of medium components and solvents used in extraction in order to reduce the price of biodiesel(**Subramaniam *et al.*, 2010**). The process of resource allocation is effectively supported by RSM, which in turn enables achieving of desirable results.

The ability of *Bacillus velezensis* DAA1 to generate and retain lipids, amounting to 79.13% of its dry weight in cells, has been demonstrated in this study. The analysis conducted using Gas Chromatography-Mass Spectrometry (GC-MS) identified significant concentrations of palmitic acid, 12-methyltetradecanoic acid, and stearic acid. In the same way, **Nair *et al.*, 2020** reported that an alternative strain of *B. velezensis*, designated ASN1, grown in waste paper hydrolysate, displayed a fatty acid profile of pentadecanoic acid (19.97%), palmitic acid (47.72%), stearic acid (27.56%), and myristic acid (4.72%). It has also been discovered that branching fatty acids enhance the properties of biodiesel by reducing its melting point (**Azad *et al.*, 2024**). **Knothe & Dunn, 2009** conducted a study which observed that the incorporation of branched fatty acids derived from

Bacillus sp. into biodiesel resulted in an augmentation of its low-temperature flow. The lipid profile of *B.velezensis DAA1* exhibits the presence of branched fatty acids, which indicates its potential as a substitute strain for lipid synthesis. The strain's fatty acid profile demonstrates a blend of saturated and branched fatty acids, providing further evidence of its capacity to function as an alternative fuel source.

CONCLUSION

Through the use of Response Surface Methodology (RSM) and optimizing growth conditions, this study was able to isolate *Bacillus velezensis* DAA1 from soil samples along the Ithikkara River and demonstrate its potential for generating lipids with a significant yield of 79.13% by dry weight. The technique effectively identified the ideal ratio of carbon to salt concentration for maximum lipid formation, demonstrating the value of RSM in promoting biological processes while preserving resources. The potential of *B. velezensis* DAA1 as a sustainable source for biodiesel production is highlighted by its lipid profile, which is rich in branched fatty acids like stearic and palmitic acid. The study provides important insights into the application of microbial biotechnology for renewable energy, specifically in the production of biodiesel with improved low-temperature flow properties due to the presence of branched fatty acids. This is achieved by highlighting the strain's lipid biosynthesis capabilities and optimizing growth conditions.

Conflict of Interest: The authors have no competing interests to declare that are relevant to the content of this article.

Data Availability: All data generated or analyzed during this study are included in this published article and its supplementary information files.

REFERENCES

Adeniji, A. A., Loots, D. T., & Babalola, O. O. (2019). *Bacillus velezensis*: phylogeny, useful applications, and avenues for exploitation. *Applied Microbiology and Biotechnology*. https://doi.org/10.1007/s00253-019-09710-5

Agrawal, D., & Kumar, V. (2023). Recent progress on sugarcane-bagasse based lactic acid production: Technical advancements, potential and limitations. *Industrial Crops and Products*, *193*. https://doi.org/10.1016/j.indcrop.2022.116132

Alvarez, H. M., Pucci, O. H., & Steinbüchel, A. (1997). Lipid storage compounds in marine bacteria. *Applied Microbiology and Biotechnology*, *47*(2), 132–139. https://doi.org/10.1007/s002530050901

Alvarez, Héctor M., Roxana A. Silva, M. H., Hernández, Martín A. María, A., & Villalba, S. (2013). Metabolism of triacylglycerols in Rhodococcus species: insights from physiology and molecular genetics. *Journal of Molecular Biochemistry*, *2*(1), 69–78.

Andreou, L. V. (2013). Preparation of genomic DNA from bacteria. *Methods in Enzymology*, *529*, 143–151. https://doi.org/10.1016/B978-0-12-418687-3.00011-2

Azad, V. K., De, K., & Majumder, S. (2024). Ethanol blending and its environmental impacts: A case study of India. *Energy for Sustainable Development*, *79*, 101385. https://doi.org/10.1016/J.ESD.2024.101385

Bagewadi, Z. K., Mulla, S. I., & Ninnekar, H. Z. (2018). Optimization of endoglucanase production from *Trichoderma harzianum* strain HZN11 by central composite design under response surface methodology. *Biomass Conversion and Biorefinery*, *8*(2), 305–316. https://doi.org/10.1007/s13399-017-0285-3

Bligh, E. G., & Dyer, W. J. (1959). A rapid method of total extraction and purification. *Canadian Journal of Biochemistry and Physiology*, *37*(8), 911–917.

Bradbury, J. F. (1970). Isolation and preliminary study of bacteria from plants. *PANS Pest Articles and News Summaries*, *16*(4), 632–637. https://doi.org/10.1080/09670877009413430

Demirbas, A. (2007). Progress and recent trends in biofuels. *Progress in Energy and Combustion Science*, *33*(1), 1–18. https://doi.org/10.1016/j.pecs.2006.06.001

F. Breidt, T.L. Romick, & H.P. Fleming. (1994). *A rapid method for the determination of bacterial growth kinetics.pdf*. *3*(919), 59–68.

Gautam, R., Ansari, N. A., Thakur, P., Sharma, A., & Singh, Y. (2022). Status of biofuel in India with production and performance characteristics: a review. *International Journal of Ambient Energy*, *43*(1), 61–77. https://doi.org/10.1080/01430750.2019.1630298

Ghosh, P., Swati, & Thakur, I. S. (2014). Enhanced removal of COD and color from landfill leachate in a sequential bioreactor. *Bioresource Technology*, *170*, 10–19. https://doi.org/10.1016/j.biortech.2014.07.079

Greenspan, P., Mayer, E. P., & Fowler, S. D. (1985). Nile red: A selective fluorescent stain for intracellular lipid droplets. *Journal of Cell Biology*, *100*(3), 965–973. https://doi.org/10.1083/jcb.100.3.965

Guo, Y., Yeh, T., Song, W., Xu, D., & Wang, S. (2015). A review of bio-oil production from hydrothermal liquefaction of algae. *Renewable and Sustainable Energy Reviews*, *48*, 776–790. https://doi.org/10.1016/j.rser.2015.04.049

Hiroki, M. (1992). Effects of Heavy Metal Contamination on Soil Microbial Population. *Soil Science and Plant Nutrition*, *38*(1), 141–147. https://doi.org/10.1080/00380768.1992.10416961

Knothe, G., & Dunn, R. O. (2009). A Comprehensive Evaluation of the Melting Points of Fatty Acids and Esters Determined by Differential Scanning Calorimetry. *JAOCS, Journal of the American Oil Chemists' Society*, *86*(9), 843–856. https://doi.org/10.1007/s11746-009-1423-2

Kumar, V., Sharma, A., Kumar, R., Bhardwaj, R., Kumar Thukral, A., & Rodrigo-Comino, J. (2020). Assessment of heavy-metal pollution in three different Indian water bodies by combination of multivariate analysis and water pollution indices. *Human and Ecological Risk Assessment*, *26*(1), 1–16. https://doi.org/10.1080/10807039.2018.1497946

Leong, W. H., Lim, J. W., Lam, M. K., Uemura, Y., & Ho, Y. C. (2018). Third generation biofuels: A nutritional perspective in enhancing microbial lipid production. *Renewable and Sustainable Energy Reviews*, *91*, 950–961. https://doi.org/10.1016/j.rser.2018.04.066

Lestari, S., Mäki-Arvela, P., Beltramini, J., Lu, G. Q. M., & Murzin, D. Y. (2009). Transforming triglycerides and fatty acids into biofuels. *ChemSusChem*, *2*(12), 1109–1119. https://doi.org/10.1002/cssc.200900107

Marathe, N. P., Pal, C., Gaikwad, S. S., Jonsson, V., Kristiansson, E., & Larsson, D. G. J. (2017). Untreated urban waste contaminates Indian river sediments with resistance genes to last resort antibiotics. *Water Research*, *124*, 388–397. https://doi.org/10.1016/j.watres.2017.07.060

Maza, D. D., Viñarta, S. C., Su, Y., Guillamón, J. M., & Aybar, M. J. (2020). Growth and lipid production of *Rhodotorula glutinis* R4, in comparison to other oleaginous yeasts. *Journal of Biotechnology*, *310*, 21–31. https://doi.org/10.1016/j.jbiotec.2020.01.012

Murínová, S., & Dercová, K. (2013). Bacterial cell membrane adaptation responses on stress caused with the environmental pollutants. *Acta Chimica Slovaca*, *6*(1), 106–114. https://doi.org/10.2478/acs-2013-0017

Nair, A. S., Al-Bahry, S., & Sivakumar, N. (2020). Co-production of microbial lipids and biosurfactant from waste office paper hydrolysate using a novel strain *Bacillus velezensis* ASN1. *Biomass Conversion and Biorefinery*, *10*(2), 383–391. https://doi.org/10.1007/s13399-019-00420-6

Nair, A. S., Al-Battashi, H., Al-Akzawi, A., Annamalai, N., Gujarathi, A., Al-Bahry, S., Dhillon, G. S., & Sivakumar, N. (2018). Waste office paper: A potential feedstock for cellulase production by a novel strain *Bacillus velezensis* ASN1. *Waste Management*, *79*, 491–500. https://doi.org/10.1016/j.wasman.2018.08.014

Neeti, K., Gaurav, K., & Singh, R. (2023). *The Potential of Algae Biofuel as a Renewable and Sustainable Bioresource*. 22. https://doi.org/10.3390/ecp2023-14716

Prabhu, A. A., Gadela, R., Bharali, B., Deshavath, N. N., & Dasu, V. V. (2019). Development of high biomass and lipid yielding medium for newly isolated *Rhodotorula mucilaginosa*. *Fuel*, *239*, 874–885. https://doi.org/10.1016/j.fuel.2018.11.088

Qadeer, S., Mahmood, S., Anjum, M., Ilyas, N., Ali, Z., & Khalid, A. (2018). Synchronization of lipid-based biofuel production with waste treatment using oleaginous bacteria: A biorefinery concept. *Process Safety and Environmental Protection*, *115*, 99–107. https://doi.org/10.1016/j.psep.2017.10.011

Rabbee, M. F., Sarafat Ali, M., Choi, J., Hwang, B. S., Jeong, S. C., & Baek, K. hyun. (2019). Bacillus velezensis: A valuable member of bioactive molecules within plant microbiomes. *Molecules*, *24*(6), 1–13. https://doi.org/10.3390/molecules24061046

Ratledge, C. (1994). Yeasts, moulds, algae and bacteria as sources of lipids. *Technological Advances in Improved and Alternative Sources of Lipids*, *1993*, 235–291. https://doi.org/10.1007/978-1-4615-2109-9_9

Ruiz-García, C., Béjar, V., Martínez-Checa, F., Llamas, I., & Quesada, E. (2005). Bacillus velezensis sp. nov., a surfactant-producing bacterium isolated from the river Vélez in Málaga, southern Spain. *International Journal of Systematic and Evolutionary Microbiology*, *55*(1), 191–195. https://doi.org/10.1099/ijs.0.63310-0

Sarsekeyeva, F. K., Usserbaeva, A. A., Zayadan, B. K., Mironov, K. S., Sidorov, R. A., Kozlova, A. Yu., Kupriyanova, E. V., Sinetova, M. A., & Los, D. A. (2014). Isolation and Characterization of a New Cyanobacterial Strain with a Unique Fatty Acid Composition. *Advances in Microbiology*, *04*(15), 1033–1043. https://doi.org/10.4236/aim.2014.415114

Subramaniam, R., Dufreche, S., Zappi, M., & Bajpai, R. (2010). Microbial lipids from renewable resources: Production and characterization. *Journal of Industrial Microbiology and Biotechnology*, *37*(12), 1271–1287. https://doi.org/10.1007/s10295-010-0884-5

Talha Akhtar, E. (2023). *"Biofuels: A Renewable Solution for Energy Security and Climate Change Mitigation."* https://ssrn.com/abstract=4475528

Umaporn Towijit , Chompoo Amponpiboon , Malinee Sriariyanum, and S. K. (2014). *Optimization of Lipid Production by Oleaginous Yeast Using Response Surface Methodology*. January, 0–13. https://doi.org/10.14456/sjst.2014.34

Wältermann, M., Hinz, A., Robenek, H., Troyer, D., Reichelt, R., Malkus, U., Galla, H. J., Kalscheuer, R., Stöveken, T., Von Landenberg, P., & Steinbüchel, A. (2005). Mechanism of lipid-body formation in prokaryotes: How bacteria fatten up. *Molecular Microbiology*, *55*(3), 750–763. https://doi.org/10.1111/j.1365-2958.2004.04441.x

Yang, F., Long, L., Sun, X., Wu, H., Li, T., & Xiang, W. (2014). Optimization of medium using response surface methodology for lipid production by Scenedesmus sp. *Marine Drugs*, *12*(3), 1245–1257. https://doi.org/10.3390/md12031245

Yao, L., Gerde, J. A., & Wang, T. (2012). Oil extraction from microalga Nannochloropsis sp. with isopropyl alcohol. *JAOCS, Journal of the American Oil Chemists' Society*, *89*(12), 2279–2287. https://doi.org/10.1007/s11746-012-2124-9

Ye, M., Tang, X., Yang, R., Zhang, H., Li, F., Tao, F., Li, F., & Wang, Z. (2018). Characteristics and Application of a Novel Species of Bacillus: *Bacillus velezensis*. *ACS Chemical Biology*, *13*(3), 500–505. https://doi.org/10.1021/acschembio.7b00874