KAMPALA INTERNATIONAL UNIVERSITY
EXPLORING THE HEIGHTS

KIU
Journal of Science, Engineering and Technology

KJSET Research Article

Optimization and application of bacterial consortia for enhanced bioremediation of dairy industry effluent using Box-Behnken design

Jimoh F. A*, Ajao A.T., Aborisade W.T. & Abdulsalam, Z.B

Department of Microbiology, Faculty of Pure and Applied Sciences, Kwara State University, Malete, Nigeria

Corresponding Author: fausat.jimoh@kwasu.edu.ng

Paper history:
Received 25 February 2025
Accepted in revised form 27 May 2025

Keywords
Dairy industry effluent; bioremediation; Bacterial consortium; Physicochemical analysis, Box-Behnken design; Optimization

Abstract

This study optimized and evaluated a bacterial consortium for treating dairy industry effluent. Effluent samples collected from a dairy processing facility in Nigeria were analyzed for physicochemical parameters and heavy metal concentrations. Bacterial isolates were screened, identified and tested for compatibility before developing a consortium. Bioremediation conditions were optimized using experimental design, focusing on molasses concentration, temperature, pH and inoculum size. The optimal conditions for total organic carbon degradation were a molasses concentration of 0.60 mg/L, a temperature of 32°C, pH 7.7, and an inoculum size of 17 mL. Under these conditions, total organic carbon decreased from 520 mg/L to 43.6 mg/L, chemical oxygen demand reduced from 1,480 mg/L to 120 mg/L, and biochemical oxygen demand declined from 780 mg/L to 65 mg/L, demonstrating significant pollutant removal. Carbon dioxide evolution increased steadily, reflecting active microbial metabolism, while enzyme activities peaked around day 10, correlating with enhanced bacterial proliferation and subsequent nutrient depletion. The regression model exhibited a high predictive accuracy, with an R^2 value of 0.9997, confirming the reliability of the bioremediation process. These findings underscore the effectiveness of the optimized bacterial consortium in degrading organic pollutants in dairy effluent. The study highlights the potential application of microbial consortia in industrial wastewater treatment, providing a sustainable and efficient approach to reducing environmental pollution.

Copyright© 2025 by authors; licensee KJSET. This publication is open access and can be freely accessed and distributed.

1.0 Introduction

The dairy industry significantly contributes to global food production, but its effluent poses serious environmental challenges (Vaishnav *et al*., 2023). Characterized by high levels of organic matter, nutrients, and suspended solids, dairy effluent can lead to severe water pollution if not treated properly (Permana *et al*., 2023). It is characterized by elevated levels of organic contaminants, including lipids, proteins, and lactose, as well as nitrogenous and phosphorous compounds that facilitate the eutrophication of aquatic ecosystems and hinder the efficiency of standard treatment systems (Dhanker *et al*., 2023). The release of dairy wastewater—either untreated or insufficiently treated—into surface waters constitutes a serious threat to both environmental integrity and public health (Pratap *et al*., 2023).). Traditional treatment methods often fail to completely remove the high organic load and other pollutants (Ghaffar *et al*., 2023). Thus, there is an urgent need for more effective and sustainable bioremediation techniques that can be optimized for maximum efficiency (Navina *et al.,* 2024).

Previous studies have demonstrated the potential of microbial consortia for bioremediation, as these mixed cultures can degrade a wider range of pollutants compared to single strains (Chaudhary *et al.,* 2023). Bacterial consortia have gained attention as a viable solution owing to their capacity to effectively break down intricate organic substances (Chaudhary *et al*., 2023). Optimization of bioremediation has been advanced through statistical tools like Response Surface Methodology (RSM) and Box-Behnken Design, which have demonstrated notable improvements in process efficiency (Aljarad *et al.,* 2023). The Box-Behnken Design, in particular, allows for a thorough examination of the interactions between key variables, ensuring that the most effective conditions are identified (Teuta *et al*., 2024)

Despite the extensive exploration of bacterial consortia for wastewater treatment, limited research specifically focuses on optimizing these consortia for dairy effluent bioremediation using statistical designs like the Box-Behnken Design (Kadam *et al.,* 2024). Additionally, most existing studies do not comprehensively address the combined effects of multiple operational parameters on the bioremediation process (Luo et al., 2024). This study aims to fill this gap by employing a systematic optimization approach to identify the ideal conditions for maximum degradation of organic pollutants in dairy effluent. This study builds upon previous research and provides new insights into the bioremediation of dairy effluent using a novel bacterial consortium and optimized conditions. The findings have the potential to be implemented in real-world scenarios, effectively addressing a critical environmental issue.

2.0 Materials and Methods

2.1 Collection and Pre-Treatment of Samples:
The dairy wastewater was obtained using a clean, dry 5-liter plastic container with a secure stopper from the discharge outlet of a dairy processing plant in Nigeria. Following collection, the sample was promptly transported to the laboratory for subsequent analysis.

2.2 Physicochemical Parameter Analysis:
The chemical parameter like, Total Dissolved Solids (TDS), Electrical conductivities, Total Organic Carbon (TOC), Total Suspended Solids (TSS), Chemical oxygen demand (COD), Biological Oxygen Demand (BOD5), Phosphate, using standard methods (Shakil & Mostafa, 2023) while analyze heavy metal concentrations using atomic absorption spectrophotometry

2.3 Isolation and Screening of Bacterial Isolates

The dairy effluent samples underwent serial dilution and were individually cultured on nutrient agar medium. Distinct colonies, based on morphological characteristics, were subsequently isolated, purified, and preserved on nutrient agar slants at 4 °C. For screening purposes, each bacterial isolate was introduced onto dairy effluent agar (DEA) plates composed of 100 ml of sterilized dairy effluent solidified with 2% agar, without the supplementation of any external nutrients. These culture plates were then incubated at 37 °C for duration of 48 hours (Sonawane & Murthy, 2023).

2.4 Identification of Bacteria

Isolates that exhibited growth on the dairy effluent medium were identified through an examination of their morphological, cultural, and biochemical properties, following the diagnostic criteria outlined in *Bergey's Manual of Determinative Bacteriology* (Andeas *et al*., 2023). Followed by 16S rRNA Gene Sequencing as outline by (Hong *et al*., 2023).

2.5 Mutual Antagonistic Effect

The isolated bacterial strains were evaluated for mutual antagonism to ensure they did not adversely affect one another. Each strain was cross-streaked on separate nutrient agar plates, ensuring that their growth paths did not overlap. Following incubation at 30°C for 48 hours, any antagonistic interactions were assessed in accordance with the procedure described by Güney *et al*. (2024).

2.6 Development of Bacterial Consortium

To prepare the bacterial consortia, individual colonies were cultured on DEA medium and incubated overnight at 37°C. After 12 hours of growth, when the strains had reached the logarithmic phase, they were transferred into 250 mL Erlenmeyer flasks containing 100 mL of nutrient broth and incubated at 37°C with shaking at 200 rpm for another 12 hours. Subsequently, 5 mL from each culture was placed into sterile Falcon tubes and

centrifuged at 5000 rpm for 15 minutes. The resulting supernatant was discarded, and the cell pellets were resuspended in normal saline and thoroughly mixed using a vortex. Then, 0.1 mL of each strain was added to fresh nutrient broth and incubated overnight. This resulting microbial consortium in nutrient broth served as the inoculum for the bioremediation experiment (Kelany *et al.,* 2023).

2.7 Optimization using Box-Behnken Design:

The Box-Behnken factorial design utilized four independent variables: molasses concentration (0.2, 0.4, 0.5, 0.6, and 0.8 mg/L), temperature (25, 30, 32.5, 35, and 40 °C), pH (5, 7, 7.5, 9, and 11), and inoculum volume (5, 10, 12.5, 15, and 20 mL). Each variable was assessed at three coded levels (−1, 0, and +1) across 29 experimental conditions, including one control. The selected levels for each factor were determined based on preliminary experimental findings.

2.8 Bioremediation study of Dairy Industry Effluent

The ideal conditions for maximizing TOC degradation, based on the Box-Behnken design, were implemented in the bioremediation process using a higher molasses concentration (0.60 mg/L), a lower temperature (32°C), an adjusted pH of 7.7, and an inoculum size of 17 mL. A bacterial consortium (10^8 CFU/mL), isolated via wastewater agar medium, was seeded into 100 mL of dairy effluent supplemented with 0.60 mg/L molasses. The flask was incubated at 32°C, 120 rpm, with the pH adjusted to 7.7. The bioremediation process was monitored every 2 days over a 14-day period by measuring colony-forming units, enzyme activities (dehydrogenase, esterase, and lipase), CO_2 evolution, COD, BOD, and TOC.

2.9 Kinetic Study of Enzyme Activities

2.9.1 Esterase Assay:

Esterase activity was quantified by spectrophotometric analysis of p-nitrophenol (p-NP) liberated from p-nitrophenylacetate, with absorbance measured at 410 nm. The procedure followed the method outlined by Distaso et al. (2023). Enzyme activity was reported in terms of micromoles of p-NP released per minute per milliliter of the enzyme preparation. The total protein concentration in the enzyme extract was determined using the Bradford assay, as described by Yan *et al*. (2023).

2.9.2 Dehydrogenase Activity:

Dehydrogenase activity was evaluated based on the reduction of 2,3,5-triphenyltetrazolium chloride (TTC) to triphenyl formazan (TPF), following the method established by Casida *et al.* (1964). To 10 mL of the wastewater sample, 1 mL of 1% TTC solution and 1.5 mL of distilled water were added. After incubating the mixture at 25 °C for 24 hours, TPF was extracted using methanol, and its absorbance was subsequently recorded at 485 nm.

2.9.3 Lipase Activity:

Lipase activity was assessed by quantifying the amount of butyric acid produced from the hydrolysis of tributyrin. A mixture consisting of 10 mL of wastewater, 1.5 mL of toluene, and 1 mL of tributyrin was incubated at 37 °C for 72 hours. Following incubation, butyric acid was extracted using ethyl acetate and measured by titration with 5 mM sodium hydroxide, in accordance with the procedure described by Kuhnert-Finkernagel and Kandeler (Vidal *et al.,* 2024).

2.9.4 Determination of Dissolved CO_2 Evolved from LDPE Degradation

To quantify the dissolved CO_2 released during LDPE degradation, a volumetric alkalinity approach was applied. A 25 mL aliquot of the culture medium was transferred into a conical flask, followed by the addition of 0.05 mL of 0.1 N sodium thiosulfate solution. Subsequently, two drops of methyl orange indicator were added, and the solution was titrated with 0.02 N sodium hydroxide until a color shift from orange-red to yellow was observed. Thereafter, two drops of phenolphthalein indicator were introduced, and titration proceeded until a stable pink hue appeared. The volumes of sodium hydroxide used were documented, and the concentration of dissolved CO_2 was calculated accordingly, following the method described by Nielsen (2024).

$$\text{Dissolve } CO_2 \text{ (mg/l)} = \frac{A \times B \times 50 \times 1000}{V}$$

Where; A = ml of NaOH titrant; B = normality of NaOH; V = ml of the sample

2.9.5 Monitoring of pH Shift

The pH of each experimental setup was recorded at seven-day intervals throughout the duration of the study. A calibrated pH probe was immersed directly into the culture broth to obtain the readings.

2.10 Statistical Analysis

Statistical evaluation was performed using data derived from three independent replicates, with outcomes expressed as the mean ± standard deviation (SD). The Student's t-test was employed to determine statistical significance, with a threshold of $p < 0.05$ indicating significance. All computations and analyses were executed using Microsoft Excel 2021 (Microsoft Corporation, Redmond, WA, USA). The experimental framework

was structured according to the Box-Behnken Design, a Response Surface Methodology (RSM) approach utilized for optimizing bioremediation conditions, refining process variables, and enhancing overall performance outcomes.

3.0 Results

The physicochemical analysis and metal concentrations of dairy effluent samples revealed several key findings as presented in Table 1. The conductivity and pH levels indicate a relatively stable ionic composition and slightly alkaline nature. High Chemical Oxygen Demand (COD) and Biological Oxygen Demand (BOD) values suggest significant organic pollution, while the Total Organic Carbon (TOC) further supports the presence of organic matter. Turbidity levels are moderate, reflecting some particulate matter. The concentrations of suspended solids, both suspended (SS) and total suspended solids (TSS), are notable. The Total Dissolved Solids (TDS) levels highlight a substantial amount of dissolved minerals and salts.

Among the metals, Zinc, Copper, Lead, and Iron were detected, with Iron having the highest concentration. The calcium and sodium levels indicate the mineral content in the effluent. The Total Phosphorus (TP) and Total Nitrogen concentrations were measured, and Chloride levels also indicate the presence of salts. Table I summarizes all the results discussed in this section.

Table 1: Physicochemical Characteristics and Metal Concentration of the Diary Effluent

Parameters	Values (Mean ± SD)
Conductivities	1470 ± 12.48
pH	7.6 ± 0.42
Chemical Oxygen Demand (mg/L)	2432 ± 11.80
Biological Oxygen Demand (mg/L)	1450 ± 7.88
Total Organic Carbon (mg/L)	987 ± 4.89
Turbidity (NTU)	10.89 ± 1.44
Total Nitrogen (mg/L)	320 ± 5.55
Total Suspended Solid(mg/L)	1180 ± 8.55
Total Dissolve Solid(mg/L)	1500 ± 3.87
Zinc (mg/L)	0.67 ± 0.00
Copper (mg/L)	0.97 ± 0.01
Lead (mg/L)	0.09 ± 0.00
Fe(mg/L)	4.2 ± 0.42
Ca(mg/L)	13.04 ± 2.36
Na(mg/L)	76.66 ± 6.48
TP(mg/L)	15.06 ± 0.66
Chloride(mg/L)	630 ± 7.34

Five bacterial species were recovered from the dairy effluent and designated as follows: AMEB-1, AMEB-2, AMEB-3, AMEB-4, and AMEB-5. These isolates were selected based on their prevalence in the effluent and their demonstrated ability to utilize the dairy effluents as a growth medium.

Each of these isolates showed significant growth when cultured in the dairy effluent, indicating their potential effectiveness in breaking down organic and inorganic matter present in the wastewater. This selection process ensured that only the most efficient and robust bacterial species were chosen for further analysis and potential application.

In addition to their individual capabilities, the mutualistic relationship among these isolates was thoroughly investigated. Tests were conducted to observe interactions between the species when cultured together. The results established that there was no antagonism among AMEB-1, AMEB-2, AMEB-3, AMEB-4, and AMEB-5. Instead, the isolates exhibited a cooperative relationship, which is crucial for their combined effectiveness in bioremediation processes. As illustrated in Plate 1.

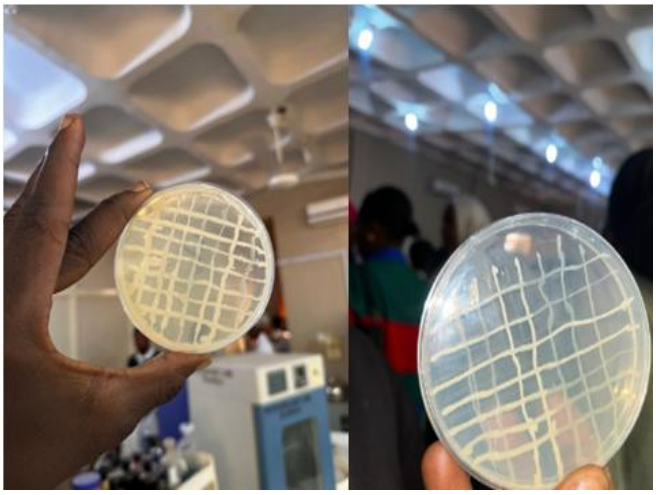

Plate 1: Synergistic Interactions Among AMEB-1, AMEB-2, AMEB-3, AMEB-4, and AMEB-5 Isolates

The synergistic interactions between AMEB-1, AMEB-2, AMEB-3, AMEB-4, and AMEB-5 were observed, supporting their application as a cohesive and effective bioremediation consortium. This figure visually represents the positive interactions among the isolates, further confirming their compatibility and mutual support in the degradation of dairy effluent pollutants. The phylogenetic analysis of the five bacterial isolates recovered from dairy effluent, designated as AMEB-1 to AMEB-5, reveals their close evolutionary relationships with known reference strains. AMEB-1 is closely related to *Bacillus pumilus* isolate M11, indicating it belongs to the *Bacillus pumilus* species. AMEB-2 shows a close relationship with *Alcaligenes faecalis* strain PSD10, identifying it as a member of the

Alcaligenes genus. AMEB-3 is closely associated with *Pseudomonas aeruginosa* strain C-1, classifying it within the *Pseudomonas* genus. AMEB-4 aligns closely with *Arthrobacter nicotianae* strain K5Y, placing it in the *Arthrobacter* genus, while AMEB-5 is closely related to *Flavobacterium aquatile* strain DSM 1132, confirming its classification in the *Flavobacterium* genus. The numbers in parentheses (***PQ056789, ***PQ056790, ***PQ056791, ***PQ056792, and ***PQ056793) are their accession numbers after submitting their 16S rRNA sequences to the NCBI database. The high bootstrap values supporting these relationships indicate strong genetic similarities between the isolates and their respective reference strains, thus confirming their identification and highlighting their potential roles in bioremediation as indicated in Fig. 1

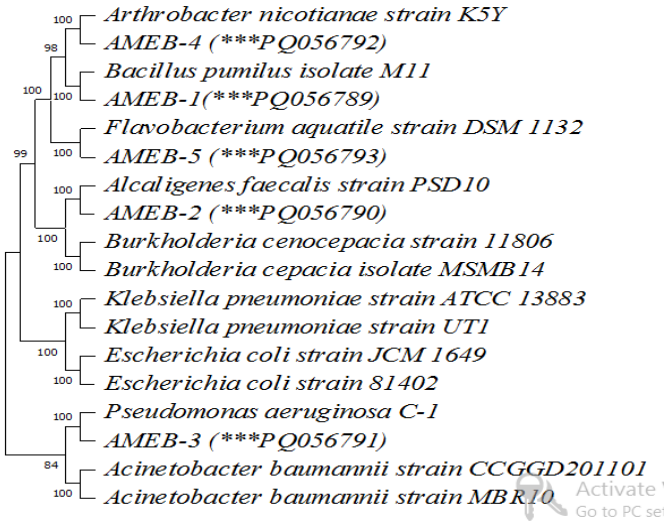

Fig. 1: Phylogenetic Tree of Bacterial Isolates AMEB-1 to AMEB-5 Recovered from Dairy Effluent. Numbers in parenthesis represent GenBank Accession Numbers

The analysis reveals that the model for Total Organic Carbon (TOC) degradation is highly significant, with an F-value of 3669.61 and a p-value < 0.0001. All factors (Molasses, Temperature, pH, Inoculum Size) and their interactions, as well as quadratic terms, are significant ($p < 0.0500$). The model fits the data well, as indicated by low residuals and no significant lack of fit or pure error (Table 2)

Table 2: ANOVA for Quadratic model

Response 1: TOC Degradation (2)

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	3593.16	14	256.65	3669.61	< 0.0001
A-Molasses	88.02	1	88.02	1258.51	< 0.0001
B-Temperature	82.69	1	82.69	1182.26	< 0.0001
C-pH	507.00	1	507.00	7249.02	< 0.0001
D-Inoculum size	252.08	1	252.08	3604.26	< 0.0001
AB	18.06	1	18.06	258.26	< 0.0001
AC	36.00	1	36.00	514.72	< 0.0001
AD	56.25	1	56.25	804.26	< 0.0001
BC	100.00	1	100.00	1429.79	< 0.0001
BD	1.0000	1	1.0000	14.30	0.0020
CD	25.00	1	25.00	357.45	< 0.0001
A^2	77.58	1	77.58	1109.21	< 0.0001
B^2	1783.83	1	1783.83	25504.96	< 0.0001
C^2	1016.89	1	1016.89	14539.43	< 0.0001
D^2	308.45	1	308.45	4410.16	< 0.0001
Residual	0.9792	14	0.0699	-	-
Lack of Fit	0.9792	10	0.0979	-	-
Pure Error	0.0000	4	0.0000	-	-
Cor Total	3594.14	28		-	-

The model equation for Total Organic Carbon (TOC) degradation is given by:

$$\text{TOC Degradation} = 86.00 + 2.71\,A - 2.63\,B - 6.50\,C + 4.58\,D + 2.13\,AB + 3.00\,AC + 3.75\,AD + 5.00\,BC + 0.5000\,BD + 2.50\,CD - 3.46\,A^2 - 16.58\,B^2 - 12.52\,C^2 - 6.90\,D^2$$

In this equation, the baseline TOC degradation is set at 86.00. The effects of factors are described as follows: Molasses (A) is associated with a positive impact, while Temperature (B) and pH (C) are associated with negative impacts, and Inoculum Size (D) is associated with a positive effect. Interaction terms illustrate how combinations of factors influence TOC degradation, with some interactions having positive effects. Non-linear effects are indicated by the quadratic terms, showing that diminishing returns occur at higher levels of Molasses, Temperature, pH, and Inoculum Size. The equation is used to predict TOC degradation based on the levels of these factors and their interactions. The model equation is supported by these visual representations, highlighting the significant factors and their interactions affecting TOC degradation. Maximum TOC degradation can be achieved by optimizing molasses concentration and inoculum size while keeping temperature and pH at lower levels. The presence of quadratic effects is confirmed by the perturbation plot (Graph A), suggesting there are optimal levels for each factor. These optimal conditions are visualized by the 3D surface plots (Graphs B, C, and D) as presented in Fig.2

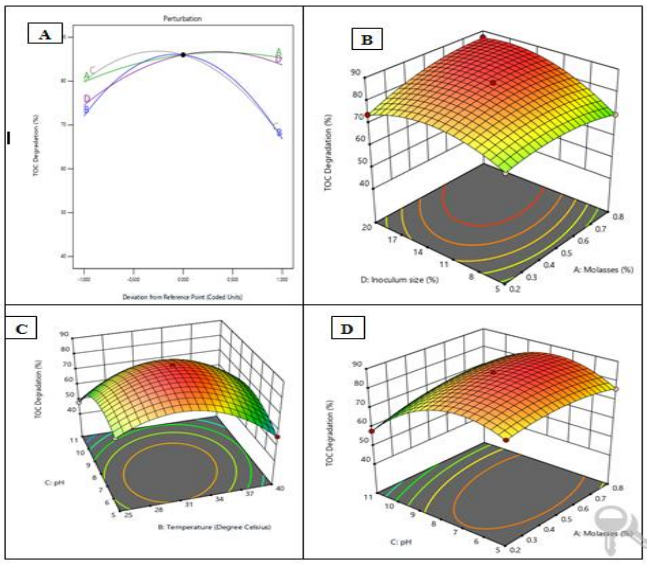

Figure 2: Impact of Various Factors on TOC Degradation. (A) Perturbation plot showing the sensitivity of TOC degradation to deviations in molasses concentration (A), temperature (B), pH (C), and inoculum size (D) from the reference point. (B) 3D surface plot depicting the interaction between molasses concentration (A) and inoculum size (D) on TOC degradation. (C) 3D surface plot illustrating the interaction between temperature (B) and pH (C) on TOC degradation. (D) 3D surface plot showing the interaction between molasses concentration (A) and pH (C) on TOC degradation. Higher TOC degradation is achieved with increased molasses concentration and inoculum size, while lower temperatures and pH values are beneficial.

The regression model shows exceptional performance with an R^2 of 0.9997, indicating that 99.97% of the variance in the dependent variable is explained by the model. The Adjusted R^2, also 0.9997, confirms that the model is well-specified and the addition of predictors hasn't significantly improved the fit. The Predicted R^2, identical at 0.9997, highlights the model's excellent predictive power on new data. Additionally, an Adequate Precision of 203.2946 indicates a very high signal-to-noise ratio, underscoring the model's reliability in making predictions. Overall, these statistics suggest the model is highly effective and reliable as indicated in Fig. 3

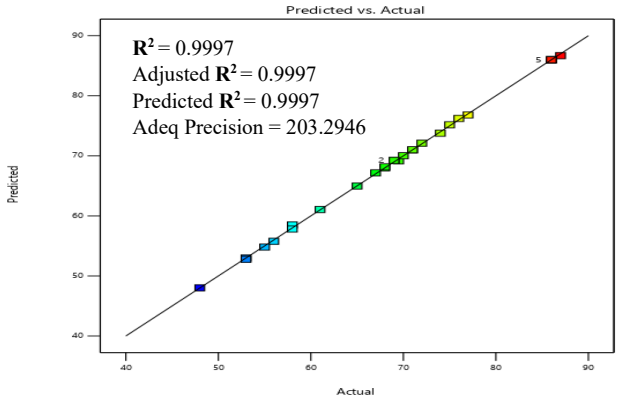

Fig. 3: Comparison of Predicted vs. Actual TOC Degradation Values of Dairy Effluent

Based on the Box-Behnken design, the ideal conditions for maximizing Total Organic Carbon (TOC) degradation in dairy effluent have been identified. To achieve high TOC degradation, it is recommended to increase the molasses concentration, as higher levels provide additional carbon sources that enhance microbial growth and activity. Lower temperatures are preferable, as they optimize microbial enzyme efficiency and reduce metabolic stress. Additionally, maintaining a lower pH creates a more favorable environment for microbial activity, facilitating better degradation of organic pollutants. Finally, increasing the inoculum size boosts the microbial population and enzymatic activity, accelerating the degradation process.

The ideal conditions for maximizing TOC degradation were implemented in the bioremediation process by using a higher molasses concentration (0.60 mg/L), maintaining a lower temperature (32°C), and adjusting the pH. Additionally, a larger inoculum size of 17 mL was used in the experimental setup.

The steady decline in TOC, COD, and BOD over the incubation period indicates that the organic pollutants were effectively degraded over time. Specifically, TOC decreased from approximately 5500 mg/L on day 2 to about 300 mg/L by day 14. COD showed a reduction from roughly 900 mg/L on day 2 to around 100 mg/L by day 14. Similarly, BOD dropped from about 700 mg/L on day 2 to approximately 80 mg/L by day 14. These declines demonstrate the effective breakdown of organic pollutants by microbial activity. The rise and stabilization of CO_2 evolution, which increased from about 50 mg/L on day 2 to nearly 250 mg/L by day 14, suggest that active microbial metabolism was converting organic carbon into CO_2. This indicates ongoing microbial activity and organic matter decomposition throughout the incubation period. The initial increase in TVC from approximately 6 Log CFU/mL on day 2 to a peak of around 9 Log CFU/mL by day 8 indicates robust microbial proliferation as they consumed the available organic matter. The subsequent decline to about 6.5 Log CFU/mL by day 14 suggests that microbial activity

decreased due to nutrient depletion or other environmental stress factors as presented in Fig. 4

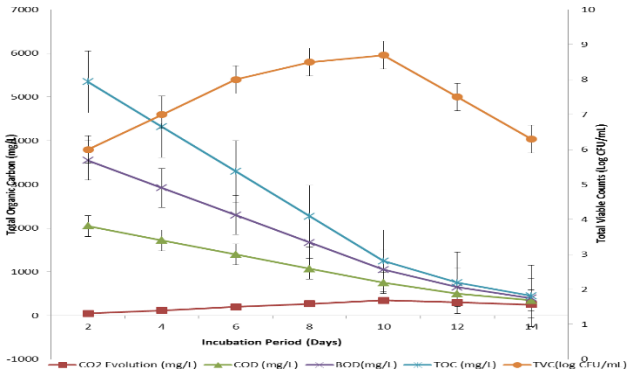

Fig.4: Bioremediation of Dairy Effluent by Bacterial Consortium Over a 14-Day Incubation Period

The dynamics of enzyme activities reveal that dehydrogenase activity initially rise, reaching its peak around day 10, and then slightly declines as the available organic material decreases. Esterase activity exhibits a steady increase, peaking around day 10 before experiencing a slight decline as substrate availability diminishes. Lipase activity follows a similar trend, increasing initially and peaking at day 10, then slightly decreasing as the organic material is depleted. Concurrently, the pH decreases over time due to the metabolic activities of the bacteria, stabilizing after reaching a low point around day 10, when microbial activity is at its peak as presented in Fig.5.

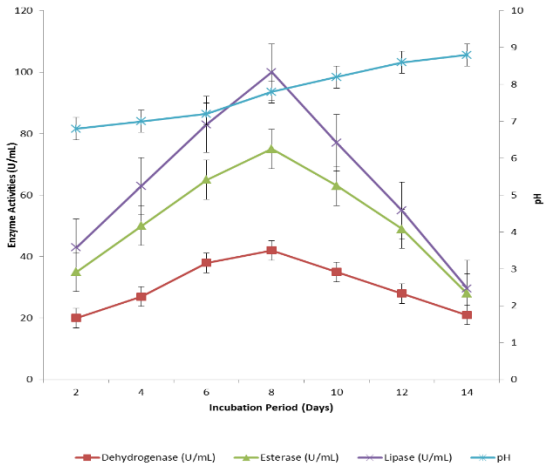

Fig.5: Bioremediation of Dairy Effluent by Bacterial Consortium Enzyme Activities and pH Changes Over a 14-Day Incubation Period

4.0 Discussion

This study highlights the significant environmental implications of untreated dairy effluent, including degraded water quality and harm to aquatic ecosystems. High levels of contaminants like COD, BOD, and suspended solids can lead to oxygen depletion, stressing or killing aquatic organisms. Elevated conductivity and pH levels can alter the chemical balance of receiving waters, negatively affecting biodiversity and aquatic habitats. These findings align with previous research (Porwal *et al*., 2015; Sharma *et al*., 2018; Jindal *et al.,* 2019; Giri *et al*., 2020; Ganta *et al.,* 2022), emphasizing the urgent need for effective treatment strategies to mitigate the environmental impact of dairy effluent. Without proper treatment, discharge can cause long-term ecological damage and pose risks to public health, underscoring the critical need for stringent effluent treatment protocols in the dairy industry to protect water quality and ecosystem health. The predominance of iron, followed by zinc, copper, and lead in the detected metals is significant, reinforcing findings from previous studies like Tabelini *et al*. (2023), which identified iron as a major component in dairy effluent due to its use in processing equipment. This study confirms the typical metal profile of dairy wastewater, highlighting the critical need to address metal contamination in effluent treatment processes to prevent environmental pollution and potential health risks.

In response to the identified contamination levels, this study introduced and characterized five novel bacterial isolates—AMEB-1 (*Bacillus pumilus*), AMEB-2 (*Alcaligenes faecalis*), AMEB-3 (*Pseudomonas aeruginosa*), AMEB-4 (*Arthrobacter nicotianae*), and AMEB-5 (*Flavobacterium aquatile*)—for their potential in bioremediation of dairy effluent. The choice of these strains was informed by their demonstrated ability to thrive in dairy effluent and their potential for degrading complex pollutants. This approach is significant as it expands on existing research by exploring a new bacterial consortium specifically selected for its suitability in treating dairy effluent (Vaishnav *et al*. 2023; Sahu *et al.,* 2023). The observed substantial growth and metabolic activity of these isolates underscore their effectiveness in handling the specific challenges posed by dairy wastewater. Reports have shown that all these bacteria possess adaptability for pollutant degradation, this observation is supported by previous studies: (Li *et al*., 2024) highlighted *Bacillus pumilus* as a versatile bacterium with considerable bioremediation capabilities due to its robust metabolic functions. Similarly, *Alcaligenes faecalis* has been recognized for its ability to degrade a wide range of pollutants, underscoring its potential effectiveness in wastewater treatment (Sahu *et al*., 2023). The bioremediation properties of *Pseudomonas aeruginosa* are well-established, especially regarding its proficiency in breaking down hydrocarbons and other complex organic compounds (Sar *et al*., 2023). Additionally, the *Arthrobacter* genus is noted for its adaptability and efficiency in degrading diverse pollutants, including those present in dairy effluent (Siddiqui & Dahiya, 2023). *Flavobacterium* species also play a crucial role in the

degradation of complex organic materials, enhancing their utility in bioremediation processes (Mukherjee *et al.,* 2024).

The interactions among these bacterial isolates were examined to assess their synergistic potential. The study revealed that the isolates exhibited cooperative relationships rather than antagonistic effects, a crucial finding as positive interactions between microbial strains can significantly enhance bioremediation efforts. The observed synergy among AMEB-1 to AMEB-5 is consistent with previous research, such as Ji *et al*. (2023), which highlighted the benefits of mixed microbial communities in degrading complex wastes. However, the specific interactions within this consortium and their implications for dairy effluent treatment provide new insights into optimizing microbial consortia for wastewater bioremediation. The use of Response Surface Methodology (RSM) to optimize conditions for Total Organic Carbon (TOC) degradation in this study marks a novel application in dairy effluent treatment. By identifying optimal conditions, such as increased molasses concentration, higher inoculum size, and adjusted temperature and pH, the study not only supports previous findings on effective degradation parameters (Sakr *et al.*, 2023) but also extends the knowledge base by successfully applying RSM in this context. The model's exceptional performance, with an R^2 of 0.9997, underscores its robustness and accuracy, offering a reliable tool for optimizing bioremediation processes, as demonstrated by similar methodologies in other contexts (Favier *et al*., 2023). This approach enhances the precision and efficiency of dairy effluent treatment strategies. The significant reductions in TOC, COD, and BOD over the incubation period further validate the effectiveness of the bioremediation process. The observed decrease in TOC from approximately 5500 mg/L to 300 mg/L, COD from 900 mg/L to 100 mg/L, and BOD from 700 mg/L to 80 mg/L underscores the capability of the bacterial consortium in degrading organic pollutants. The increase in CO2 evolution, from 50 mg/L to 250 mg/L, reflects active microbial metabolism and conversion of organic carbon, reinforcing the effectiveness of the bioremediation process. Enzyme activity patterns, including peaks in dehydrogenase, esterase, and lipase activities, confirm the microbial degradation of organic materials. The gradual decline in enzyme activities, which corresponds with the depletion of organic materials and a decrease in pH over time, suggests acidification due to microbial metabolism (Zheng *et al*., 2024). These patterns further validate the active role of the bacterial consortium in the bioremediation process.

The findings of this study demonstrate the critical role of enzyme activity and microbial interactions in the degradation of organic materials in dairy effluent. The use of Response Surface Methodology to optimize TOC degradation highlights the potential for improving bioremediation strategies through precise condition adjustments. The predominance of certain metals further underscores the need for targeted treatment approaches to address contamination. These insights contribute valuable knowledge to the field of wastewater treatment, particularly in optimizing microbial consortia and refining treatment protocols.

5.0 Conclusion

This study advances our understanding of dairy effluent treatment by introducing innovative approaches and insights. The identification of a new bacterial consortium, coupled with the application of Response Surface Methodology (RSM) for process optimization, and a thorough analysis of microbial interactions and enzyme activities, enhances wastewater bioremediation strategies. These advancements not only offer practical solutions for optimizing treatment conditions but also underscore the importance of continued research and innovation in improving environmental management practices within the dairy industry.

Declaration of conflict of interest

All authors contributed jointly to the conceptual development, methodological planning, and execution of this manuscript. They participated in drafting the content and provided critical revisions to ensure the inclusion of substantial intellectual contributions. This article has not been submitted to, nor reviewed by, any other journal or publishing outlet. Furthermore, the authors declare no affiliations with any institutions or entities that hold direct or indirect financial interests related to the subject addressed in this work.

Funding

No funds, grants, or other support was received.

Author Contribution

Each author listed on this submission has made significant contributions to one or more of the following: the conceptualization or design of the study; the collection, analysis, or interpretation of data; or the development of original software applied in the course of the research.

Conflict of Interest Declaration

The authors affirm that no competing interests exist in relation to the publication of this manuscript.

Accession Numbers

The 16S rRNA gene sequences for all isolates have been submitted to the NCBI GenBank database and are available under the following accession numbers: PQ056789, PQ056790, PQ056791, PQ056792, and PQ056793, corresponding to *Bacillus pumilus* strain AMEB-1, *Alcaligenes faecalis* strain AMEB-2, *Pseudomonas aeruginosa* strain AMEB-3, *Arthrobacter nicotianae* strain AMEB-4, and *Flavobacterium aquatile* strain AMEB-5, respectively.

Data Availability Statement

All datasets underpinning the results of this study are comprehensively included within the content of the manuscript.

References

Akinnawo, S.O., Ayadi, P.O. and Oluwalope, M.T. (2023). Chemical coagulation and biological techniques for wastewater treatment. Ovidius Univ *Ann Chem.*;34(1):14–21. https://doi.org/10.2478/auoc-2023-0003

Aljarad, M.R., Kouidhi, B., Ayed, L., Alghamdi, M., Altayeb, H.N. and Chaieb K. (2023). Process optimization of malachite green degradation by mixed biofilm-positive bacteria: application of Box–Behnken designs, RSM, and whole genome sequences analysis. *Biochem Eng J*. 199:109058. https://doi.org/10.1016/j.bej.2023.109058

Andeas, A.F., Wibowo, R.H., Darwis, W., Sipriyadi, N., Supriati, R., Hidayah, T. and Supriyanto, A.P. (2023). Isolation and identification of endophytic bacteria in lempuyang wangi (*Zingiber zerumbeth var. aromaticum Val.*) from Enggano Island, Bengkulu Province. AIP Conf Proc. https://doi.org/10.1063/5.0118435

Chaudhary, P., Xu, M., Ahamad, L,, Chaudhary, A., Kumar, G. , Adeleke, B.S. and Verma, K.K., et al. (2023).Application of synthetic consortia for improvement of soil fertility, pollution remediation, and agricultural productivity: a review. *Agronomy*. 13(3):643. https://doi.org/10.3390/agronomy13030643

Dhanker, R., Khatana, K., Verma, K., Singh, A., Heena, N., Kumar, R. and Mohamed, H.I. (2023). An integrated approach of algae-bacteria mediated treatment of industry-generated wastewater: optimal recycling of water and safe way of resource recovery. *Biocatal Agric Biotechnol*. 54:102936. https://doi.org/10.1016/j.bcab.2023.102936

Distaso, M., Cea-Rama, I., Coscolín, C., Chernikova, T., Tran, H. and Ferrer, M, et al. (2023). The mobility of the cap domain is essential for the substrate promiscuity of a family IV esterase from *Sorghum* rhizosphere microbiome. *Appl Environ Microbiol*. 89(1). https://doi.org/10.1128/aem.01807-22

Favier, L., Simion, A.I., Hlihor, R.M., Fekete-Kertesz, I., Molnár, M.., Harja, M. and Vial, C. (2023). Intensification of the photodegradation efficiency of an emergent water pollutant through process conditions optimization by means of response surface methodology. *J Environ Manag*. 328:116928. https://doi.org/10.1016/j.jenvman.2022.116928

Ganta, A., Bashir, Y. and Das S. (2022). Dairy wastewater as a potential feedstock for valuable production with concurrent wastewater treatment through microbial electrochemical technologies. *Energies*. 15(23):9084.

Ghaffar, I., Hussain, A., Hasan, A. and Deepanraj, B. (2023). Microalgal-induced remediation of wastewaters loaded with organic and inorganic pollutants: an overview. *Chemosphere.* 320:137921. https://doi.org/10.1016/j.chemosphere.2023.137921

Giri, A., Bharti, V.K., Kalia, S., Arora, A., Balaje, S.S. and Chaurasia, O.P. (2020). A review on water quality and dairy cattle health: a special emphasis on high-altitude regions. *Appl Water Sci*. 10(3):1–16.

Güney, İ.G., Derviş, S., Özer, G., Aktaş, H. and Keske, M.A. (2024). Determination of antagonistic activities of endophytic bacteria isolated from different wheat genotypes against *Fusarium culmorum*. Ulus Tarim Ve Yaban Hayati Bilim Derg. 10(1):96–116. https://doi.org/10.24180/ijaws.1386741

Hong, H., Flurin, L., Greenwood-Quaintance, K.E., Wolf, M.J., Pritt, B.S., Norgan, A.P., Patel, R. (2023). 16S rRNA gene PCR/sequencing of heart valves for diagnosis of infective endocarditis in routine clinical practice. *J Clin Microbiol.* 36:234–5. https://doi.org/10.1128/jcm.00341-23

Ji, Y., Wang, N., Yang, N., Chen, X., Liu, Q., Wang, Z., Shi, J. and Liu, L. (2023). Multivariate insights into the effects of inoculating thermophilic aerobic bacteria on the biodegradation of food waste: Process properties, organic degradation, and bacterial communities. *Environ Technol Innov.* 29:102968. https://doi.org/10.1016/j.eti.2022.10296

Jindal, T., Sinha, S., Srivastava, A., Mehrotra, T. and Singh, R. (2019).A review on the dairy industry wastewater characteristics, its impact on environment and treatment possibilities. Emerging Issues in Ecology and Environmental Science: Case Studies from India. 73–84.

Kadam, V., Dhanorkar, M., Patil, S. and Singh, P.(2024). Advances in the co-production of biosurfactant and other biomolecules: Statistical approaches for process

optimization. *J Appl Microbiol.* 135(2). https://doi.org/10.1093/jambio/lxae025

Kelany, M.S., El-Sawy, M.A., El-Gendy, A.R. and Beltagy, E.A. (2023). Bioremediation of industrial wastewater heavy metals using solo and consortium *Enterobacter spp*. *Environ Monit Assess.* 195(11). https://doi.org/10.1007/s10661-023-11951-x

Li, B., Jin, Z., Yang, F., Li, H., Liu, J., Jiang, Z. (2024). Proteomic investigation reveals the role of bacterial laccase from *Bacillus pumilus* in oxidative stress defense. *J Proteomics*. 292:105047. https://doi.org/10.1016/j.jprot.2023.105047

Luo, M., Zhang, X., Zhu, X., Long, T., Cao, S. and Yu, R.(2024). Bioremediation of chlorinated ethenes-contaminated groundwater and the reactive transport modeling – A review. *Environ Res*. 240:117389. https://doi.org/10.1016/j.envres.2023.117389

Manikandan, S. and Saraswathi, R. (2022). Electrocoagulation technique for removing organic and inorganic pollutants (COD) from various industrial effluents: An overview. *Environ Eng Res.* 28(4):220231. https://doi.org/10.4491/eer.2022.231

Mukherjee, R., Banerjee, S. and Das, P. (2024).The role of microbial bioremediation and biodegradation in wastewater treatment. Elsevier eBooks. 337–52. https://doi.org/10.1016/b978-0-443-13884-3.00017-2

Navina, B.K., Velmurugan, N.K., Kumar, P. S, Rangasamy, G., Palanivelu, J., Thamarai, P., Vickram, A., Saravanan, A. and Shakoor, A. (2024). Fungal bioremediation approaches for the removal of toxic pollutants: Mechanistic understanding for biorefinery applications. *Chemosphere*. 350:141123. https://doi.org/10.1016/j.chemosphere.2024.141123

Nielsen, S.S. (2024). Standard solutions and titratable acidity. *Food Sci Text Ser*. 177–82. https://doi.org/10.1007/978-3-031-44970-3_22

Permana, I., Sudirja, R. and Jupesta, J. (2023).Potential of vermifiltration technique to reduce chemical oxygen demand, biological oxygen demand, and total suspended solid of farm dairy effluent in developing countries: Case of Indonesian farm dairy industry. *Green Low-carbon Econ*. 36:234–5. https://doi.org/10.47852/bonviewglce3202933

Porwal, H.J., Mane, A.V. and Velhal, S.G. (2015). Biodegradation of dairy effluent by using microbial isolates obtained from activated sludge. *Water Resour Ind*. 9:1–15.

Pratap, B., Kuma,r S., Nand, S., Azad, I., Bharagava, R.N., Ferreira, L.F.R. and Dutta, V. (2023). Wastewater generation and treatment by various eco-friendly technologies: Possible health hazards and further reuse for environmental safety. *Chemosphere*. 313:137547. https://doi.org/10.1016/j.chemosphere.2022.137547

Sahu, S., Kaur, A., Singh, G., Arya, S.K. (2023). Harnessing the potential of microalgae-bacteria interaction for eco-friendly wastewater treatment: A review on new strategies involving machine learning and artificial intelligence. *J Environ Manag.* 346:119004. https://doi.org/10.1016/j.jenvman.2023.119004

Sakr, E.A.E., Khater, D.Z., Kheiralla, Z.M.H. and El-Khatib, K.M. (2023). Statistical optimization of waste molasses-based exopolysaccharides and self-sustainable bioelectricity production for dual chamber microbial fuel cell by *Bacillus piscis*. *Microb Cell Fact.* 22(1). https://doi.org/10.1186/s12934-023-02216-w

Sar, P., Kundu, S., Ghosh, A. and Saha, B. (2023). Natural surfactant-mediated bioremediation approaches for contaminated soil. RSC Adv. 13(44):30586–30505. https://doi.org/10.1039/d3ra05062a

Shakil, M.S.Z, and Mostafa, M.G.(2023). Characterization of paper mill effluent and its impacts on the environment. *J Chem Environ*. 109–22. https://doi.org/10.56946/jce.v2i01.135

Siddiqui, N. and Dahiya, P. (2023). Key role of microorganisms in industrial wastewater treatment. In Elsevier eBooks. 31–47. https://doi.org/10.1016/b978-0-323-88505-8.00009-7

Sonawane, A.V. and Murthy, Z. (2023). Dairy industry wastewater treatment by MOF and 2D nanomaterial engineered PVDF membranes-based aerobic MBR: Membrane fouling mitigation and stability study. *Process Saf Environ Prot.* 171:680–693. https://doi.org/10.1016/j.psep.2023.01.062

Tabelini, D.B., Lima, J.P.P., Borges, A.C.and Aguiar, A.(2023). A review on the characteristics and methods of dairy industry wastewater treatment in the state of Minas

Gerais, *Brazil. J Water Process* Eng. 53:103779. https://doi.org/10.1016/j.jwpe.2023.103779

Teuta, J.P., Narváez-Cuenca, C., Murillo, M.Á.(2024). A high-performance liquid chromatography method validation and a Box–Behnken experimental design for the extraction optimization of quercitrin from *Nectandra reticulata*. RSC Adv. 14(30):21874–21886. https://doi.org/10.1039/d4ra01723d

Vaishnav, S., Saini, T., Chauhan, A., Gaur, G.K., Tiwari, R., Dutt, T., Tarafdar, A. (2023). Livestock and poultry farm wastewater treatment and its valorization for generating value-added products: Recent updates and way forward. *Bioresour Technol*. 382:129170. https://doi.org/10.1016/j.biortech.2023.129170

Vidal, N., Ventura, M., Martínez, F. and Melero, J. (2024). Selective extraction of high-added value carboxylic acids from aqueous fermentative effluents with new hydrophobic eutectic solvents (HES). Sep *Purif Technol*. 346:127540. https://doi.org/10.1016/j.seppur.2024.127540

Yan, Y., Zhang, M., Gao, J., Qin, L., Fu, X., Wan, J.(2023). Comparison of methods for detecting protein extracted from excess activated sludge. *Environ Sci Pollut Res Int.* 30(21):60967–60975. https://doi.org/10.1007/s11356-023-26455-x

Zheng, X., Wang, Y., Wu, J., Wu, Y., Long, M and Chen, Y. (2024). Enhanced degradation of methanethiol via operational pH regulation in biofilm reactor: Insight of sulfur metabolism pathways and microbial adaptation mechanism. *Chem Eng J*. 153414. https://doi.org/10.1016/j.cej.2024.153414