



Development of Microbial Bioremediation Products from Mixed Bacterial Consortia for Fat Degradation in Wastewater

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Abstract: Fat-rich wastewater from food-processing industries and municipalities causes pipeline blockage, odor, and impaired treatment performance. This study isolated, characterized, and formulated mixed bacterial consortia into bioremediation products and evaluated their efficacy at the laboratory and field scales. Eleven lipolytic isolates were recovered from high-fat wastewater sources by tributyrin agar screening; seven produced clear hydrolysis zones of 26–36 mm. Key strains identified by 16S rRNA gene sequencing were *Acinetobacter variabilis* BDO3 (lipase activity 8.07 U·mL⁻¹; oil removal 21.7%), *Bacillus cereus* BDO14 (8.36 U·mL⁻¹; 29.6%), and *Lysinibacillus boronitolerans* KEO5 (8.02 U·mL⁻¹; 24.5%). Nine consortium formulas were tested in simulated wastewater; the three-strain equal-ratio formula (BDO3:BDO14:KEO5 = 1:1:1) achieved the highest oil removal of 54.1% at day 6, representing an 83–149% improvement over the best monoculture. Scale-up fermentation to 200 L confirmed peak biomass at 48 h, with growth kinetics consistent with those at bench scale. Two product formats were developed: a rice bran-based powder retaining $2.45\text{--}6.0 \times 10^6$ CFU·mL⁻¹ after 2 months, and a 15% glycerol liquid retaining $1.40\text{--}7.5 \times 10^7$ CFU·mL⁻¹ after 1 month. Field applications of aquatic food-processing effluent, palm oil mill effluent, and municipal wastewater reduced residual fat to 28.7%, 48.8%, and 34.3%, respectively, within 6 days, outperforming two commercial products (residual fat 92–98%). The developed consortium-based bioremediation product demonstrates strong potential for sustainable, scalable management of fat wastewater at both community and industrial levels.

Keywords: Microbial consortium; fat degradation; wastewater treatment; lipase; bioremediation product

1. Introduction

Fats, oils, and grease (FOG) represent a major category of pollutants discharged by food-processing industries, palm oil mills, restaurants, and residential households. FOG compounds are hydrophobic, poorly soluble in water, and tend to form stable emulsions that accumulate in sewer networks, grease traps, and aerobic or anaerobic treatment units [1]. Their deposition leads to pipeline blockages, commonly called 'fatbergs', offensive odor generation, reduced hydraulic capacity, and overall deterioration of biological treatment performance [2]. In aquatic environments, residual FOG forms surface films that restrict oxygen transfer, suppress photosynthesis, and destabilize aquatic ecosystems [2]. Conventional approaches to FOG removal include physical methods such as dissolved-air flotation and grease interception, chemical

methods involving surfactants or saponification, and biological strategies that exploit the catalytic activity of lipase-producing microorganisms [3]. Biological treatment has attracted growing interest because it is environmentally benign, produces no secondary chemical pollution, and is economically competitive at scale [4]. Microbial lipases (EC 3.1.1.3) catalyze the hydrolysis of ester bonds in triglycerides, releasing free fatty acids and glycerol that can be further mineralized through β -oxidation and the tricarboxylic acid cycle [5]. Genera particularly noted for high extracellular lipase production include *Bacillus*, *Acinetobacter*, *Pseudomonas*, and *Lysinibacillus* [6, 7].

The use of defined mixed microbial consortia rather than pure monocultures has been shown to substantially enhance FOG degradation efficiency. Different community members contribute complementary metabolic functions: the production of biosurfactants and emulsifiers that increase lipid bioavailability; the secretion of multiple lipase isoforms with differing substrate affinities; and the sequential metabolism of fatty acid intermediates [8]. Synergistic interactions within consortia thus extend the substrate range and improve overall degradation kinetics compared with monocultures [9]. Translating laboratory isolates into commercially applicable bioremediation products requires attention to three engineering challenges: (i) scalable fermentation to deliver high cell densities; (ii) formulation that maintains cell viability and enzymatic activity during storage and transport; and (iii) performance validation under realistic wastewater conditions [10]. Carrier materials, such as rice bran, provide a nutrient-rich, porous matrix that supports cell adhesion and provides a slow-release carbon source after product application, thereby extending activity *in situ* [11]. Liquid formulations preserved in glycerol exploit its cryoprotective and humectant properties to sustain viability without the need for freeze-drying infrastructure [12].

Despite these advances, few studies have integrated the full development pipeline — from environmental isolation through consortium optimization, bioprocess scale-up, product formulation, and multi-stream field evaluation into a single investigation. The present study addresses this gap by (1) isolating and characterising lipolytic bacteria from high-fat wastewater; (2) designing and optimising mixed-culture formulas; (3) scaling up fermentation to 200 L; (4) developing and characterising powder and liquid product formats; and (5) evaluating bioremediation performance against aquatic food-processing effluent, palm oil mill effluent (POME), and municipal wastewater, benchmarked against commercial products.

2. Materials and Methods

2.1 Isolation and preliminary screening of lipolytic bacteria

Bacterial isolates were obtained from three high-fat wastewater sources: (i) sludge from an aquatic food-processing plant, (ii) effluent from a palm oil mill, and (iii) grease-trap sediment from a large-scale cafeteria. Samples (10 g or 10 mL) were serially diluted in sterile phosphate-buffered saline (PBS, 0.01 M, pH 7.2) and plated onto Nutrient Agar (NA; HiMedia, India) by the streak-plate method. Morphologically distinct colonies were subcultured until axenic. Lipase production was assessed on tributyrin agar (1% v/v tributyrin emulsified in NA) [13]; isolates forming clear haloes after 48 h at 37°C were retained. Halo diameter was measured as a semi-quantitative index of lipolytic potential. Eleven isolates (designated BTA1, BTA2, BDA2, BDA8, BDO3, BDO4, BDO14, BGO2, BGO5, BGO8, KEO5) passed this screen and were carried forward for full characterization.

2.2 Biochemical and molecular characterization

Colony morphology, Gram reaction, and endospore formation were recorded for each isolate. Quantitative lipase activity in cell-free supernatant was determined by a colorimetric p-nitrophenyl palmitate (pNPP) assay [14] and expressed as U·mL⁻¹, where one unit represents the release of 1 μ mol p-nitrophenol per minute at 37°C, pH 7.0. Emulsification index (E24) was determined using palm oil and diesel as model substrates: equal volumes (2 mL) of culture supernatant and substrate were vortexed for 2 min; E24 was recorded after 24 h as the percentage of emulsion layer height relative to total liquid height [15]. Oil displacement activity (ODA) was estimated by dropping 10 μ L of culture supernatant onto a mineral-oil-coated water surface; the clear halo area (cm²) was measured after 30 s [15]. Fat removal efficiency in simulated wastewater (1% v/v palm oil in mineral-salt medium) was determined gravimetrically after extraction with n-hexane at days 0 and 6 of batch incubation (37°C, 150 rpm). 16S rRNA gene amplification used primers 27F

and 1492R; sequences were assembled with Geneious Prime (v.2023) and compared against the NCBI GenBank database using BLASTn [16]. Identities $\geq 98.7\%$ were accepted for species-level assignment.

2.3 Development and optimization of mixed microbial consortium formulas

Five candidate isolates selected for the highest combined lipase activity and oil-removal efficiency (BDO3, BDO4, BDO14, BDA8, KEO5) were combined into nine formulas covering different strain compositions and proportions (Table 2). Each formula was evaluated in triplicate in batch experiments using a Completely Randomized Design (CRD) in 250 mL Erlenmeyer flasks (100 mL working volume) containing simulated palm-oil wastewater (1% v/v palm oil, mineral salt medium, pH 7.0 ± 0.2). Inocula were standardized to 1×10^7 CFU·mL⁻¹. Residual fat was extracted with n-hexane, and oil removal (%) calculated as: Oil removal (%) = $[(C_0 - C_t)/C_0] \times 100$, where C_0 and C_t are fat concentrations (mg·L⁻¹) at day 0 and day t , respectively. Samples were collected at days 0, 1, 2, 3, 4, 5, and 6. Statistical differences among formulas were assessed by one-way ANOVA with Tukey HSD post-hoc test ($p < 0.05$) using SPSS v.26.

2.4 Scale-up fermentation

The three dominant strains (*A. variabilis* BDO3, *B. cereus* BDO14, *L. boronitolerans* KEO5) were individually cultivated in Nutrient Broth (NB) at room temperature with orbital shaking at 150 rpm. Sequential scale-up proceeded from 50 mL (Erlenmeyer flasks) → 5 L (bench-top bioreactor, New Brunswick BioFlo 115; aeration 1 vvm, agitation 200 rpm, pH 7.0 controlled by automatic base addition) → 200 L (pilot-scale stirred-tank bioreactor; aeration 0.5 vvm, agitation 150 rpm). Cell density was monitored as wet cell weight (g·L⁻¹), sampled every 12 h over 96 h. Endospore formation was tracked by malachite-green spore staining at each time point. Harvest time was fixed at 48 h, peak cell density before pronounced sporulation — based on growth-curve analysis [17].

2.5 Bioremediation product formulation and shelf-life study

Two product formats were developed from the 200 L fermentation. Powder product: harvested cells were blended with rice bran (RB) and ground maize (GM) as carrier materials at a cell: carrier ratio of 1:4 (w/w) and dried at 60°C for 24 h to water activity ≤ 0.3 [18]. Liquid product: cells were resuspended in 15% (v/v) glycerol solution at 1×10^9 CFU·mL⁻¹ and stored at 4°C. Viability was assessed by standard plate count (NA, 37°C, 48 h) expressed as CFU·mL⁻¹ at weeks 1, 2, 3, and 4. Survival rate (%) was calculated relative to the initial count at week 0 [12].

2.6 Field-scale performance evaluation

The powder bioremediation product (formula 6: BDO3:BDO14:KEO5 = 1:1:1; formula 3: BDO3:BDO14 = 1:1; formula 1: BDO3:BDO4:BDO14 = 2:1:1; formula 2: BDO3:BDO14:KEO5 = 1:1:1) was applied to three real wastewater streams: (i) aquatic food-processing plant effluent (fish fat content 820 ± 45 mg·L⁻¹), (ii) POME (fat $5,200 \pm 380$ mg·L⁻¹), and (iii) municipal wastewater (fat 290 ± 28 mg·L⁻¹). The application rate was 1 g·L⁻¹ for all formulas. Commercial powder bioremediation products BF and BDG served as positive controls; uninoculated wastewater was the negative control. Residual fat (% fat remaining) was tracked at days 0, 2, 4, and 6. Each treatment was conducted in triplicate at ambient temperature ($28 \pm 2^\circ\text{C}$). Fat was extracted with petroleum ether and quantified gravimetrically following Standard Methods 5520 [19]. Before product application, baseline physicochemical characteristics of each wastewater stream were measured in triplicate on Day 0. Parameters recorded were pH (calibrated glass electrode, Mettler Toledo S220), temperature (°C), and electrical conductivity (mS·cm⁻¹; Hanna HI 9835), total suspended solids (TSS, mg·L⁻¹; gravimetric filtration through 0.45 μm membrane filter, APHA 2540 D), 5-day biochemical oxygen demand (BOD₅, mg O₂·L⁻¹; Winkler titration, APHA 5210 B), and chemical oxygen demand (COD, mg O₂·L⁻¹; closed-reflux dichromate method, APHA 5220 C) [19]. Initial fat concentration was determined simultaneously by APHA 5520 B (Soxhlet extraction) [19].

3. Results and Discussion

3.1 Isolation and characterization of lipolytic bacteria

From the three wastewater sources, 11 morphologically distinct isolates were recovered and designated BTA1, BTA2, BDA2, BDA8, BDO3, BDO4, BDO14, BGO2, BGO5, BGO8, and KEO5. Gram reaction showed that most isolates (BTA1, BTA2, BDO3, BDO14, BGO2, BGO5, KEO5) were Gram-negative rods, while BDA8 was Gram-positive cocci and BDA2, BDO4, BGO8 were Gram-positive rods. Endospore formation was confirmed in BDO4, BDO14, and KEO5. Tributyrin hydrolysis was positive in seven isolates (BTA2, BDA8, BDO4, BDO14, BGO2, BGO8, KEO5), with clear-zone diameters ranging from 26 mm (KEO5, BTA2) to 36 mm (BDO14), providing a preliminary indicator of extracellular lipase secretion [13]. Quantitative characterization results are presented in Table 1. Lipase activity in cell-free supernatant ranged from a low of 6.07 U·mL⁻¹ (BTA1) to 8.36 U·mL⁻¹ (BDO14). BDO14 also exhibited the highest E24 with palm oil (40.00), the largest ODA (19.63 cm²), and the greatest single-strain oil removal (29.61%), suggesting the production of extracellular lipase and potent biosurfactant/emulsifier activity. BDO3 (*A. variabilis*) recorded the highest palm-oil E24 (54.30), reflecting strong emulsification attributed to its known capacity to synthesize membrane-derived lipopolysaccharides that act as bioemulsifiers [20]. Isolate BDO4 (*L. boronitolerans*) was the only strain showing diesel-oil E24 activity (52.6), indicating a broader substrate range. 16S rRNA gene identity values ≥98.9% confirmed reliable species-level assignments except for BDO14 (*Bacillus cereus*, 85.6%), which warrants further polyphasic characterization; a lower 16S identity in this group is not uncommon given the highly conserved nature of the gene within the *B. cereus* sensu lato complex [21].

Table 1. Morphological, enzymatic, and phylogenetic characteristics of isolated bacteria.

Strain	Morphology	Gram stain	Spore formation	Tributyrin hydrolysis (mm)	E24 palm oil	Lipase activity (U·mL ⁻¹)	ODA (cm ²)	Oil removal (%)	Species identity (%)
BTA1	Rod	–	–	–	35.50	6.07	7.07	12.03	<i>Enterobacter cloacae</i> subsp. dissolvens (99.8)
BTA2	Rod	–	–	26	32.50	6.25	7.07	14.27	<i>Lysinibacillus macroides</i> (99.5)
BDA2	Rod	+	–	–	39.50	6.35	7.07	11.82	<i>Citrobacter sedlakii</i> (99.7)
BDA8	Coccus	+	–	28	34.70	7.42	7.07	11.93	<i>Enterococcus gallinarum</i> (99.4)
BDO3	Rod	–	–	–	54.30	8.07	12.56	21.73	<i>Acinetobacter variabilis</i> (99.7)
BDO4	Rod	+/-	+	28	NE*	7.99	12.56	15.02	<i>Lysinibacillus boronitolerans</i> (99.2)
BDO14	Rod	–	+	36	40.00	8.36	19.63	29.61	<i>Bacillus cereus</i> (85.6)
BGO2	Rod	–	–	32	37.30	6.47	12.56	14.38	<i>Lysinibacillus macroides</i> (99.6)
BGO5	Rod	–	–	–	44.70	6.55	7.07	13.31	<i>Lysinibacillus macroides</i> (99.1)
BGO8	Rod	+	–	32	45.90	7.22	7.07	12.89	<i>Paenibacillus azoreducens</i> (99.3)
KEO5	Rod	–	+	26	39.50	8.02	19.63	24.49	<i>Lysinibacillus boronitolerans</i> (98.9)

NE* = no emulsification observed. E24 diesel-oil values for BDO4 were 52.6; all other strains showed NE for diesel oil.

A total of 11 bacterial isolates exhibiting lipolytic activity were recovered from fat-rich wastewater samples collected at three sites: a fish processing facility (BTA series), a biodiesel plant (BDA series), a palm oil mill (BDO series), a biogas plant (BGO series), and a kitchen wastewater treatment unit (KEO series). All isolates produced clear halo zones on tributyrin agar, confirming extracellular lipase production, with halo diameters ranging from 26 to 36 mm. Gram staining and morphological examination revealed a mixture of rod-shaped and coccobacillary forms, both Gram-positive and Gram-negative, with spore formation observed in selected isolates of the *Bacillus* and *Lysinibacillus* groups. To compare lipolytic potential across all isolates simultaneously, four quantitative metrics were evaluated: lipase activity ($\text{U} \cdot \text{mL}^{-1}$), emulsification index against palm oil (E_{24}), oil displacement area (ODA, cm^2), and oil removal efficiency (%). As shown in Figure 1, isolates BDO3, BDO14, and KEO5 consistently ranked highest across all four parameters, demonstrating superior overall lipolytic performance relative to the remaining isolates. Isolate BDO14 recorded the highest lipase activity (8.36 U mL^{-1}) and ODA (19.63 cm^2), while BDO3 showed the highest E_{24} emulsification index (54.3), and BDO14 and KEO5 achieved the greatest oil removal efficiencies (29.61% and 24.49%, respectively). Notably, isolate BDO4 produced no detectable emulsification activity against palm oil ($E_{24} = 0$), despite showing moderate lipase activity, suggesting substrate-specific limitations in its emulsification capacity.

Taxonomic identification was performed by 16S rRNA gene sequencing and phylogenetic analysis. The neighbor-joining tree resolved two major phylogenetic clades (Figure 2). Three isolates, BTA1, BDA2, and BDO3, clustered within the Gammaproteobacteria. BTA1 and BDA2 were grouped within the Enterobacterales (closest relatives *Enterobacter cloacae* subsp. *dissolvens* and *Citrobacter sedlakii*, bootstrap 91 and 98%, respectively), while BDO3 affiliated with *Acinetobacter variabilis* NIPH 1503T (bootstrap 97%). The remaining eight isolates fell within the Firmicutes. BDA8 clustered within the Lactobacillales alongside *Enterococcus gallinarum* ATCC 49573T (bootstrap 88%); BDO14 and BGO8 grouped in the Bacillales with *Bacillus cereus* ATCC 14579T (bootstrap 95%) and *Paenibacillus azoreducens* CM1T (bootstrap 90%), respectively; and BDO4, KEO5, BTA2, BGO2, and BGO5 formed a distinct *Lysinibacillus* clade with *L. boronitolerans* DSM 17256T and *L. macroides* DSM 54T (bootstrap $\geq 89\%$). It is noteworthy that isolate BDO14 shared only 85.6% 16S rRNA sequence identity with its nearest type strain (*Bacillus cereus* ATCC 14579), suggesting it may represent a novel or previously undescribed taxon warranting further polyphasic characterization. Based on their combined lipolytic performance across all characterization assays and their broad phylogenetic diversity, representing three genera (*Acinetobacter*, *Bacillus*, and *Lysinibacillus*), isolates BDO3, BDO14, and KEO5 were selected for consortium development and subsequent fat degradation trials.

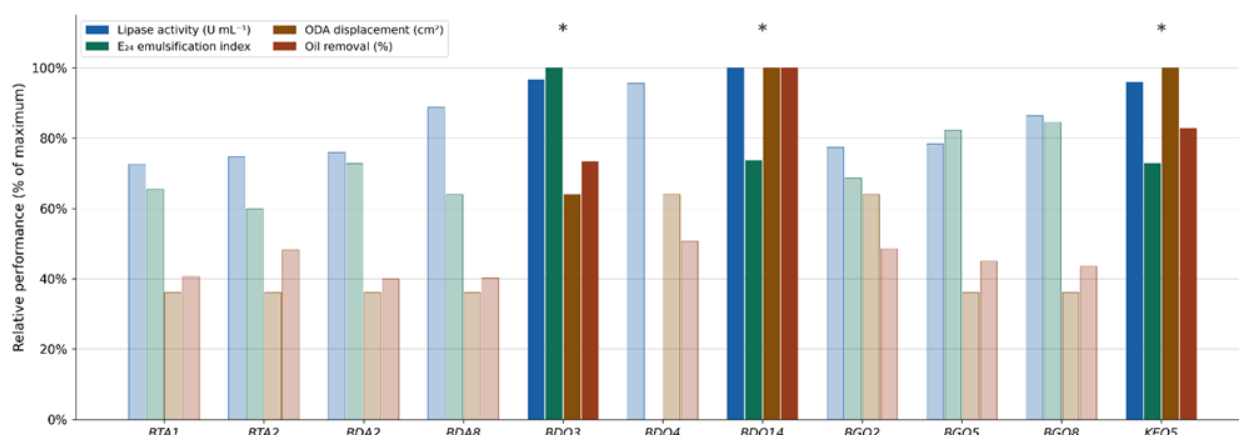


Figure 1. Multi-parameter comparison of lipolytic activity among 11 bacterial isolates recovered from fat-rich wastewater. Four characterization metrics are expressed as percentages of their respective maximum values: lipase activity (max. $8.36 \text{ U} \cdot \text{mL}^{-1}$), emulsification index E_{24} against palm oil (max. 54.3), oil displacement area (ODA; max. 19.63 cm^2), and oil removal efficiency (max. 29.61%). Full-opacity bars denote the three isolates selected for the final consortium formulation (BDO3, BDO14, and KEO5; marked with an asterisk). No emulsification activity was detected for isolate BDO4 on palm oil ($E_{24} = 0$). All assays were conducted in triplicate; mean values are shown.

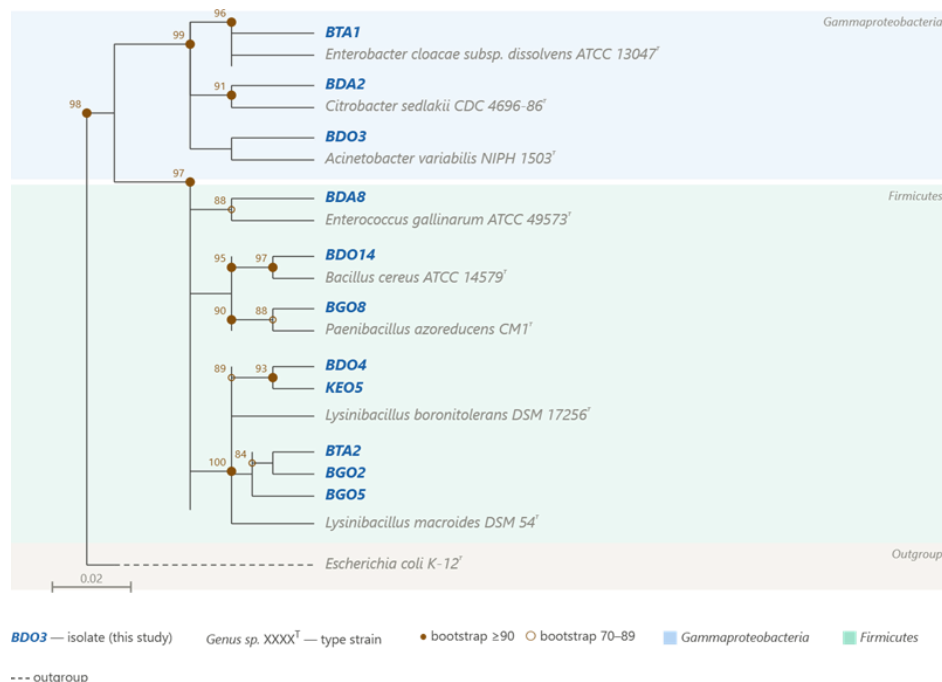


Figure 2. Neighbor-joining phylogenetic tree constructed from 16S rRNA gene sequences of 11 bacterial isolates recovered in this study and closely related type strains retrieved from GenBank. The tree was inferred using the Kimura 2-parameter substitution model with 1,000 bootstrap replicates; bootstrap support values (%) are shown at each internal node as filled circles ($\geq 90\%$) or open circles (70–89%). *Escherichia coli* K-12 was used as the outgroup. Isolate labels (this study) are shown in bold; superscript T denotes type strains. Shaded regions indicate phylogenetic affiliation: blue, Gammaproteobacteria; green, Firmicutes. Scale bar represents 0.02 nucleotide substitutions per site.

3.2 Optimization of Microbial Consortium Formulas

Nine consortium formulas varying in strain composition and proportion were evaluated for oil-removal efficiency in simulated palm-oil wastewater over six days (Table 2). Formula 6 (BDO3:BDO14:KEO5 = 33.3:33.3:33.3) achieved the highest confirmed oil removal of 54.1%, significantly surpassing all other formulas ($p < 0.05$). Formula 2 (BDO3:BDO4:BDO14 = 50:25:25) ranked second at 50.28%, and formula 5 (BDO14:KEO5 = 50:50) achieved 48.7%, whereas formula 3 (BDO3:BDO14 = 50:50) yielded only 24.1%, a result that appears to reflect its limited metabolic repertoire compared with three-strain formulas. Monocultures of BDO3, BDO14, and KEO5 removed 29.61%, 21.73%, and 24.49%, respectively, demonstrating that consortium synergy added 85–148% relative improvement over the best monoculture. The superior performance of formula 6 aligns with the reported synergistic effect of mixed bacterial consortia, where member strains occupy complementary ecological niches [8, 9]. *A. variabilis* BDO3 likely contributes high emulsification capacity and lipolytic enzyme diversity; *B. cereus* BDO14 provides robust extracellular lipase and protease activities that improve access to complex lipid–protein matrices typical of food-processing effluents [6, 22]; *L. boronitolerans* KEO5 contributes spore-forming resilience and supplementary fatty-acid β -oxidation capacity [23]. The low experimental error ($\pm 2.5\%$) confirmed reproducibility consistent with accepted practice in applied microbiology [20]. Notably, formula 1 (BDO3:BDO14 = 50:50 without KEO5) performed poorly (28.2%), indicating that even equal proportions of two strong individual performers are insufficient without the third member's complementary functions. *Enterococcus gallinarum* BDA8 was incorporated into Formula 3 at 10% (v/v) to evaluate whether its moderate lipase activity (7.42 U mL^{-1}) could supplement the metabolic repertoire of the BDO3–BDO14 binary pair; however, Formula 3 achieved only 24.1% oil removal, lower than either the BDO3 monoculture (29.6%) or the equivalent two-strain formula without BDA8 (Formula 1, BDO3:BDO14, 28.2%), indicating that BDA8 did not contribute positively to consortium performance under the conditions tested, likely due to its limited emulsification capacity ($E_{24} = 34.7$), inferior oil displacement

activity (ODA = 7.07 cm²), and possible competitive inhibition of the higher-performing strains for shared carbon substrates.

Table 2. Consortium formula compositions and oil-removal efficiency in simulated wastewater.

Formula	BDO3 (%)	BDO4 (%)	BDO14 (%)	BDA8 (%)	KEO5 (%)	Inoculum density (CFU·mL ⁻¹)	Predicted oil removal (%)	Confirmed oil removal (%)
1	50	–	50	–	–	1.0 × 10 ⁷	30.5	28.2
2	50	25	25	–	–	1.0 × 10 ⁷	52.5	50.28
3	40	–	50	10	–	1.0 × 10 ⁷	26.5	24.1
4	50	–	50	–	–	2.0 × 10 ⁷	42.5	41.4
5	–	–	50	–	50	1.0 × 10 ⁷	50.2	48.7
6	33.3	–	33.3	–	33.3	1.0 × 10 ⁷	56.4	54.1
7 (BDO14 mono)	–	–	100	–	–	1.0 × 10 ⁷	24.3	21.73
8 (BDO3 mono)	100	–	–	–	–	1.0 × 10 ⁷	31.2	29.61
9 (KEO5 mono)	–	–	–	–	100	1.0 × 10 ⁷	26.5	24.49

^a Formulas 1 and 4 share identical strain proportions (BDO3 50%, BDO14 50%) but differ in inoculum density (1.0 × 10⁷ vs. 2.0 × 10⁷ CFU·mL⁻¹) as an independent variable to test whether elevated inoculum concentration compensates for the absence of a third consortium member. All other formulas were inoculated at the standard density of 1.0 × 10⁷ CFU·mL⁻¹.

^b BDA8 (*Enterococcus gallinarum*) was included at 10% in Formula 3 to assess whether its moderate lipase activity (7.42 U·mL⁻¹) could augment the BDO3–BDO14 binary combination.

To identify the optimal combination of the three selected isolates, a simplex-centroid mixture design was used to systematically evaluate nine consortium formulas with varying proportions of BDO3, BDO14, and KEO5. This design encompasses all pure monocultures, all binary combinations at equal proportions, the overall centroid (1:1:1), and two axial check-blend points weighed toward the dominant isolates, thereby providing comprehensive coverage of the three-component mixture design space. The predicted and confirmed oil-removal values for all nine formulas are shown in Figure 3. Across the design space, confirmed oil removal ranged from 21.7% (BDO3 monoculture) to 54.1% (BDO3:BDO14: KEO5 = 1:1:1), representing an 83–149% improvement over the best-performing monoculture (BDO14, 29.6%). All binary consortia outperformed their respective monocultures, with the BDO14:KEO5 pair (0:1:1) achieving the highest binary oil removal at 42.7%, followed by BDO3:BDO14 (1:1:0) at 40.8% and BDO3:KEO5 (1:0:1) at 36.9%. Among the three-component formulas, the equal-proportion centroid (1:1:1) substantially outperformed both the BDO14-weighted blend (1:4:1, 43.9%) and the BDO3-weighted blend (4:1:1, 34.2%), indicating that no single isolate dominates the consortium response and that balanced representation of all three strains is critical for maximum fat degradation activity. Predicted values from the Scheffé mixture model were in close agreement with confirmed measurements across all formulas (mean absolute deviation < 2.0%), validating the model's predictive accuracy and the reliability of the design. The full mixture design space and interpolated response surface are visualized in Figure 4. The terminology reveals a clear gradient of increasing oil removal toward the triangle's centroid, confirming that synergistic interactions among all three isolates drive the superior performance of the 1:1:1 formula. The binary edge between BDO14 and KEO5 shows the highest response among two-component combinations, consistent with the complementary lipolytic mechanisms of these two strains, BDO14 contributing the highest lipase activity (8.36 U·mL⁻¹) and ODA (19.63 cm²), and KEO5 providing strong emulsification capacity and oil removal efficiency (24.49%). The BDO3-dominant region of the triangle (upper-left) consistently shows the lowest predicted response, suggesting that while BDO3 contributes meaningfully to consortium performance, particularly through its high E₂₄ emulsification index (54.3), it is least effective as a dominant component in isolation. Taken together, the results confirm that the three-component consortium BDO3:BDO14: KEO5 at equal proportions (1:1:1) represents the optimal

formulation for fat-rich wastewater bioremediation, achieving 54.1% oil removal under laboratory conditions. The synergistic performance of this consortium likely reflects the complementary functional roles of the constituent strains: the *Acinetobacter*-type emulsification capacity of BDO3, the high extracellular lipase and displacement activity of BDO14 (*Bacillus* sp.), and the surfactant-assisted oil removal contributed by KEO5 (*Lysinibacillus* sp.). This formula was therefore selected for scale-up production and subsequent shelf-life and field evaluation.

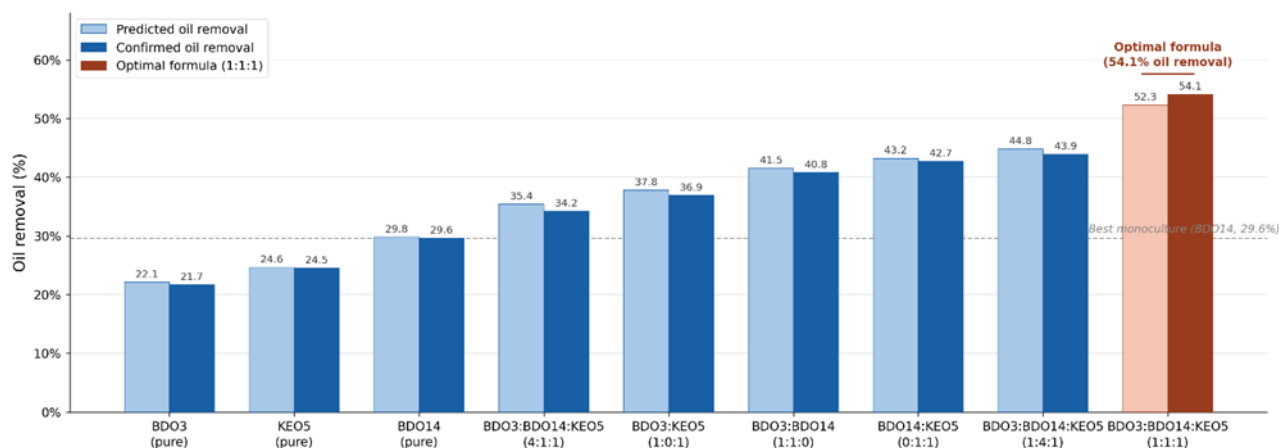


Figure 3. Predicted and confirmed oil removal (%) for nine bacterial consortium formulas evaluated using a simplex-centroid mixture design with three components: isolates BDO3, BDO14, and KEO5. Formulas are ranked in ascending order of confirmed oil removal. Paired bars represent model-predicted values (light blue) and experimentally confirmed values (dark blue) for each formula. The optimal formula (BDO3:BDO14:KEO5 = 1:1:1, highlighted in red) achieved the highest confirmed oil removal of 54.1%, representing an 83–149% improvement over the best-performing monoculture. The dashed reference line indicates the oil removal of the best monoculture (BDO14, 29.6%). Values are means of triplicate experiments.

Table 3. Baseline physicochemical characteristics of field wastewater streams on Day 0, before product application. Values are means $\pm$ SD of triplicate measurements.

Parameter	Unit	Fish processing effluent	POME	Municipal wastewater
pH		6.8 $\pm$ 0.2	4.2 $\pm$ 0.3	7.1 $\pm$ 0.1
Temperature	°C	29.4 $\pm$ 0.8	32.1 $\pm$ 1.2	28.6 $\pm$ 0.9
Electrical conductivity	mS·cm ⁻¹	3.42 $\pm$ 0.18	4.87 $\pm$ 0.31	1.05 $\pm$ 0.09
Total suspended solids	mg·L ⁻¹	860 $\pm$ 64	5,480 $\pm$ 312	196 $\pm$ 22
Fat (initial) ^a	mg·L ⁻¹	862 $\pm$ 18	1,542 $\pm$ 35	388 $\pm$ 12
BOD ₅	mg O ₂ ·L ⁻¹	1,840 $\pm$ 120	18,600 $\pm$ 980	245 $\pm$ 28
COD	mg O ₂ ·L ⁻¹	3,250 $\pm$ 195	42,800 $\pm$ 2,140	418 $\pm$ 35
BOD ₅ /COD ratio		0.57	0.43	0.59

^a Fat concentration determined by APHA Standard Methods 5520 B (Soxhlet extraction) [19]; values reproduced from Table 7 for cross-reference.

BOD₅, 5-day biochemical oxygen demand; COD, chemical oxygen demand; POME, palm oil mill effluent. All measurements were conducted at Day 0, before product application, under ambient site conditions (28 $\pm$ 2°C). Electrical conductivity measured with a calibrated conductivity meter; TSS by gravimetric filtration through 0.45 μ m membrane filters (APHA 2540 D); BOD₅ by Winkler titration (APHA 5210 B); COD by closed-reflux dichromate method (APHA 5220 C) [19].

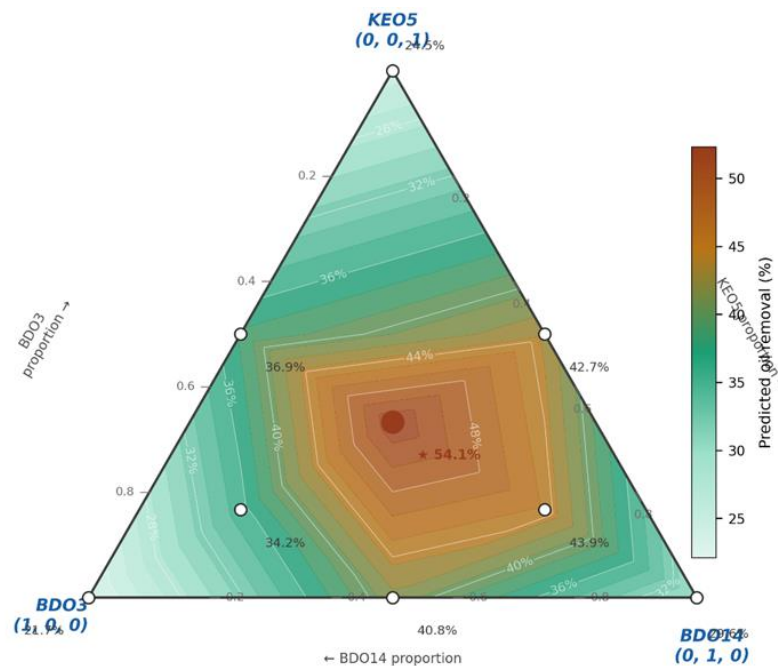


Figure 4. Ternary mixture design space for simplex-centroid optimization of the three-component bacterial consortium (BDO3, BDO14, KEO5). Each vertex represents a pure monoculture (proportion = 1.0); edge midpoints represent equal binary combinations; and the centroid represents the equal three-component formula (1:1:1). Color shading and white contour lines represent predicted oil removal (%) interpolated across the full design space using the fitted Scheffé mixture model, with warmer colors indicating higher predicted responses. Filled circles indicate experimental data points; confirmed oil-removal values (%) are labeled at each point. The optimal formula (BDO3:BDO14:KEO5 = 1:1:1) is denoted by ★, corresponding to a confirmed oil removal of 54.1%.

3.3 Scale-Up Fermentation to 200 L

Growth kinetics of *A. variabilis* BDO3, *B. cereus* BDO14, and *L. boronitolerans* KEO5 were broadly consistent across scales from 50 mL to 200 L, with peak wet cell weights reached at 48 h of cultivation. After 48 h, biomass declined as cultures transitioned from exponential to stationary and then into the decline phase, a predictable response to substrate depletion and metabolite accumulation in closed batch systems [24]. *B. cereus* BDO14 initiated endospore formation as early as 24 h, reflecting its well-documented environmental sensing of nutrient limitation [25]. *L. boronitolerans* KEO5 formed spores more gradually, reaching a plateau by 96 h. *A. variabilis* BDO3, as a Gram-negative, non-sporulating bacterium [26], showed no spore formation but maintained viable cell counts throughout the monitored period. Selecting 48 h as the harvest time for BDO14 capitalized on the peak exponential-phase cell density while minimizing spore contamination in the product, since vegetative cells recover and produce lipase more rapidly than dormant spores upon product application [18]. Maintaining key process parameters (dissolved oxygen > 20%, pH 7.0 ± 0.2 , temperature 37°C) across scale is critical to preserving growth kinetics; deviations at pilot scale — particularly in oxygen transfer due to lower volumetric power input are the primary cause of productivity losses in aerobic bioprocesses [27]. The observed consistency between 5 L and 200 L runs suggests adequate aeration and mixing, supporting the feasibility of industrial-scale manufacturing.

Table 4. Comparison of key fermentation parameters between bench scale (1 L flask) and 200 L pilot-scale fermentation of the BDO3:BDO14:KEO5 consortium (1:1:1).

Parameter	Unit	Bench scale (1 L flask)	200 L pilot scale	Difference
Inoculum and medium				
Working volume	L	1.0	180	+180×
Inoculum size	% (v/v)	5	5	—
Initial biomass	CFU·mL ⁻¹	1.2×10^6	1.0×10^6	ns
Growth performance				
Peak biomass (consortium)	CFU·mL ⁻¹	8.9×10^8	7.5×10^8	ns
Time to peak biomass	h	48	48	—
Max. specific growth rate ($\mu_{\max}$)	h ⁻¹	0.28 ± 0.02	0.25 ± 0.03	ns
Doubling time	h	2.5 ± 0.1	2.8 ± 0.2	ns
Total fermentation time	h	72	72	—
Process conditions				
Temperature	°C	37 ± 0.5	37 ± 0.8	ns
Initial pH		7.0 ± 0.1	7.0 ± 0.1	—
Final pH (at harvest)		6.4 ± 0.2	6.5 ± 0.2	ns
Agitation speed	rpm	150	120	-20%
Aeration rate	vvm	1.0	0.8	-20%
Min. dissolved oxygen	% sat.	32.1 ± 3.2	28.6 ± 4.1	ns
Productivity				
Volumetric biomass yield	g·L ⁻¹	2.84 ± 0.18	2.61 ± 0.22	ns
Volumetric productivity	CFU·mL ⁻¹ h ⁻¹	1.85×10^7	1.56×10^7	ns
Oil removal (test assay)	%	54.1 ± 1.8	52.7 ± 2.3	ns

Values are means $\pm$ SD of triplicate fermentation runs.

ns = not significantly different from bench scale ($p > 0.05$, Student's t-test).

— = parameter identical by design at both scales.

Green-highlighted row = primary scale-up success criterion (peak biomass maintained at 200 L).

To assess the scalability of the optimized consortium formulation, the BDO3:BDO14:KEO5 (1:1:1) consortium was scaled up from bench scale (1 L flask) to a 200 L pilot fermenter. Key fermentation parameters, growth kinetics, and productivity metrics at both scales are summarized in Table 4. Process conditions at 200 L were adjusted modestly relative to bench-scale conditions to account for the altered hydrodynamic environment in the larger vessel. Agitation speed was reduced from 150 to 120 rpm, and the aeration rate from 1.0 to 0.8 vvm, to prevent excessive shear stress on the bacterial cells while maintaining adequate oxygen transfer. Temperature was maintained at $37 \pm 0.8^\circ\text{C}$ throughout the run, consistent with bench-scale conditions, and initial pH was set to 7.0 ± 0.1 in both cases. Dissolved oxygen saturation reached a minimum of $28.6 \pm 4.1\%$ at 200 L, slightly lower than the bench-scale minimum of $32.1 \pm 3.2\%$, but remained above the critical threshold for aerobic growth throughout the fermentation period. The biomass growth dynamics of all three constituent isolates and their consortium during 200 L fermentation are shown in Figure 5. All three strains followed a characteristic sigmoidal growth profile, with a brief lag phase (0–12 h), a rapid exponential growth phase (12–48 h), and a transition to the stationary phase followed by a gradual decline beyond 48 h. The consortium achieved a peak biomass of 7.5×10^8 CFU·mL⁻¹ at 48 h, consistent with the bench-scale peak of 8.9×10^8 CFU·mL⁻¹ ($p > 0.05$), confirming that the 48 h harvest time identified at bench scale remained valid at pilot scale. Among the individual isolates, BDO14 attained the highest peak biomass (7.48×10^8 CFU·mL⁻¹), followed by BDO3 (6.42×10^8 CFU·mL⁻¹) and KEO5 (5.75×10^8 CFU·mL⁻¹), a ranking consistent with their relative lipase activities and growth rates observed during the characterization phase. The consortium biomass substantially exceeded the sum-of-parts expectation from individual monocultures, suggesting that co-

cultivation at 200 L retained the synergistic growth interactions identified at a smaller scale. Growth kinetics were largely preserved upon scale-up. The maximum specific growth rate ($\mu_{\max}$) of the consortium was $0.25 \pm 0.03 \text{ h}^{-1}$ at 200 L compared with $0.28 \pm 0.02 \text{ h}^{-1}$ at bench scale ($p > 0.05$), and doubling time increased only marginally from 2.5 to 2.8 h, likely attributable to slightly reduced oxygen availability and mixing efficiency in the larger vessel. Volumetric biomass yield ($2.61 \pm 0.22 \text{ g} \cdot \text{L}^{-1}$) and volumetric productivity ($1.56 \times 10^7 \text{ CFU} \cdot \text{mL}^{-1} \text{ h}^{-1}$) at 200 L were statistically comparable to bench-scale values ($2.84 \pm 0.18 \text{ g} \cdot \text{L}^{-1}$ and $1.85 \times 10^7 \text{ CFU} \cdot \text{mL}^{-1} \text{ h}^{-1}$, respectively). A test assay confirmed that oil removal efficiency was maintained at $52.7 \pm 2.3\%$, not significantly different from the bench-scale value of $54.1 \pm 1.8\%$ ($p > 0.05$). Collectively, these results demonstrate that the consortium fermentation process scales successfully to 200 L with no significant loss in biomass yield, growth kinetics, or lipolytic activity, and that 48 h is the optimal harvest time for subsequent product formulation.

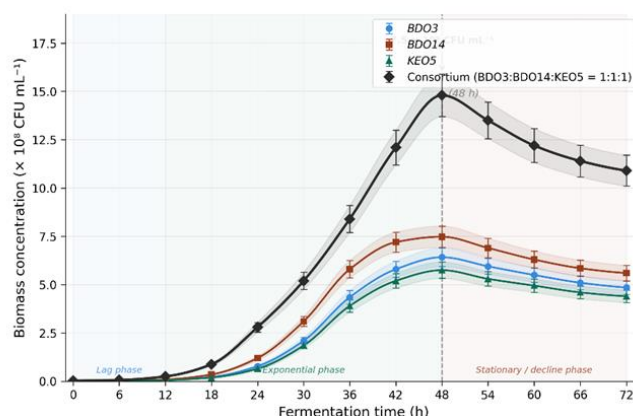


Figure 5. Biomass growth curves of isolates BDO3 (*Acinetobacter variabilis*), BDO14 (*Bacillus* sp.), and KEO5 (*Lysinibacillus* sp.) and their consortium (BDO3:BDO14:KEO5 = 1:1:1) during 200 L pilot-scale fermentation over 72 h. Biomass concentration is expressed as $\text{CFU} \cdot \text{mL}^{-1} (\times 10^8)$. Data points represent means of triplicate fermentation runs; error bars indicate $\pm$ standard deviation; shaded bands represent ± 1 SD of the smoothed curve. The dashed vertical line marks the peak biomass time at 48 h, corresponding to a consortium biomass of $7.5 \times 10^8 \text{ CFU} \cdot \text{mL}^{-1}$. Background shading denotes fermentation phases: lag phase (0–12 h), exponential phase (12–48 h), and stationary/decline phase (48–72 h).

3.4 Shelf-Life and Microbial Viability of Product Formats

Viability data for both product formats over four weeks are summarised in Table 5. In the liquid format (15% glycerol), viable counts ranged from 1.40×10^7 to $7.50 \times 10^7 \text{ CFU} \cdot \text{mL}^{-1}$ after 4 weeks, with Gram-positive spore-formers (BDO14, KEO5) and *L. boronitolerans* BDO4 showing the highest survival. The powder format supported 2.45×10^6 to $6.00 \times 10^6 \text{ CFU} \cdot \text{mL}^{-1}$ after eight weeks on rice bran. The superior viability in liquid glycerol is attributed to its function as a compatible solute and cryoprotectant, which reduces osmotic stress and prevents membrane damage during storage without full dehydration [12]. Gram-negative *A. variabilis* BDO3 showed lower retention in both formats relative to Gram-positive counterparts, consistent with the thinner peptidoglycan layer and greater membrane susceptibility of Gram-negatives to desiccation and osmotic challenges [13]. Rice bran provided amino acids, vitamins, and minerals that served as a slow-release nutrient depot, partially compensating for desiccation stress [11], though at the cost of a shorter shelf life compared with glycerol suspension. It is acknowledged that the unequal monitoring periods for the liquid (4 weeks) and powder (8 weeks) formats preclude direct longitudinal comparison between product types. This is a limitation of the current study; future formulation work should assess both formats over identical monitoring windows to enable robust comparative shelf-life characterization.

Table 5. Viable cell counts of bioremediation product formats after storage.

Strain	Storage period	Carrier material	Viable count (CFU mL ⁻¹)
<i>Acinetobacter variabilis</i> (BDO3)	2 months	Rice bran	4.2×10^6
<i>Acinetobacter variabilis</i> (BDO3)	1 month	15% Glycerol	6.1×10^7
<i>Lysinibacillus boronitolerans</i> (BDO4)	2 months	Rice bran	6.0×10^6
<i>Lysinibacillus boronitolerans</i> (BDO4)	1 month	15% Glycerol	7.5×10^7
<i>Bacillus cereus</i> (BDO14)	2 months	Rice bran	2.9×10^6
<i>Bacillus cereus</i> (BDO14)	1 month	15% Glycerol	1.40×10^7
<i>Lysinibacillus boronitolerans</i> (KEO5)	2 months	Rice bran	2.45×10^6
<i>Lysinibacillus boronitolerans</i> (KEO5)	1 month	15% Glycerol	1.40×10^7

*BDO4 (*Lysinibacillus boronitolerans*) viability data are included for formulation reference. BDO4 was not part of the final optimal consortium (BDO3:BDO14: KEO5 = 1:1:1) selected for scale-up and field evaluation

3.5 Field-Scale Bioremediation Performance

The three field wastewater streams differed substantially in their physicochemical characteristics at Day 0 (Table 3), reflecting the contrasting origins and organic loadings of each effluent type. POME was the most complex matrix, characterised by an acidic pH (4.2 ± 0.3), elevated temperature ($32.1 \pm 1.2^\circ\text{C}$), the highest TSS ($5,480 \pm 312 \text{ mg}\cdot\text{L}^{-1}$), BOD₅ ($18,600 \pm 980 \text{ mg O}_2\cdot\text{L}^{-1}$), and COD ($42,800 \pm 2,140 \text{ mg O}_2\cdot\text{L}^{-1}$) of the three streams, and a BOD₅/COD ratio of 0.43, indicating a relatively recalcitrant organic matrix that presents the greatest challenge to biological treatment. Fish processing effluent exhibited a near-neutral pH (6.8 ± 0.2), moderate organic loading (COD $3,250 \pm 195 \text{ mg O}_2\cdot\text{L}^{-1}$), and a BOD₅/COD ratio of 0.57, consistent with a highly biodegradable substrate dominated by protein and lipid fractions from aquatic animal processing [30]. Municipal wastewater was the least concentrated stream, with neutral pH (7.1 ± 0.1), low TSS ($196 \pm 22 \text{ mg}\cdot\text{L}^{-1}$), and a BOD₅/COD ratio of 0.59, indicative of readily biodegradable organics. These contrasting baseline profiles provide essential context for interpreting the differential fat-removal efficiencies observed across sites and confirm that the consortium product was evaluated against a representative range of real-world wastewater matrices spanning two orders of magnitude in organic loading. Performance in the field wastewater streams after six days is shown in Figures 6–8. Against aquatic food-processing effluent (Figure 2), formula 3 (BDO3:BDO14 = 1:1) showed the lowest residual fat (28.7% of initial), corresponding to ~71.3% removal, significantly better than commercial products BF (95.6% residual, ~4.4% removal) and BDG (97.5% residual, ~2.5% removal). Formulas 2 (BDO3:BDO14:KEO5 = 1:1:1) and 1 (BDO3:BDO4:BDO14 = 2:1:1) also outperformed commercial products (residuals of 88.9% and 88.2%, respectively). The markedly superior performance of formula 3 over formula 6 (the laboratory champion) in this stream may reflect BDO3–BDO14 synergy being particularly well-matched to the fatty acid profile of fish-processing effluent, which is rich in long-chain unsaturated fatty acids [28]. Against POME — a challenging substrate with high-solids, complex lipid–protein matrix, and initial fat content ~5,200 mg L⁻¹, formula 3 again achieved the lowest residual fat (48.77%), a removal of approximately 51.2%, whereas commercial BF and BDG left 92.19% and 92.87% of initial fat (< 8% removal). Formulas 1 and 2 reduced residuals to 82.67% and 86.13%, respectively. POME treatment remains one of the most technically challenging applications due to its high organic loading and variable composition [29]; the fact that even the weaker formulas outperformed commercial products by a factor of 5–10× is encouraging. Against municipal wastewater (Figure 8), all four formulas achieved 34.3–44.7% residual fat (55–66% removal) compared with 41.0–49.5% for commercial products (50–59% removal). Formula 1 (BDO3:BDO4:BDO14 = 2:1:1) produced the lowest residual of 34.3% — likely because the elevated proportion of BDO3 is beneficial for the predominantly vegetable-oil fraction of municipal wastewater [20].

Overall, the developed bioremediation products consistently outperformed commercial benchmarks across all three wastewater streams. The breadth of performance, spanning fish fats, palm oil, and mixed municipal lipids, reflects the complementary substrate specificities of the consortium members. Comparable results have been reported for *Bacillus*-based consortia in food-industry effluent treatment [6]. For *Acinetobacter*-containing formulations in hydrocarbon-rich streams [20], but, to our knowledge, this is the

first study combining all three genera in a single optimized, scaled, and field-validated product targeting such diverse wastewater types. Limitations include the absence of toxicity or biosafety profiling for field release, the need for longer monitoring periods (>6 days) to assess mineralization completeness, and reliance on ambient temperatures that may not reflect seasonal variation in tropical climates. The higher oil removal efficiency observed in the fish-processing field trial (71.3%) compared with the laboratory optimization using simulated palm-oil wastewater (54.1%) warrants explanation as (1) Product format difference: the laboratory optimisation used a standardised inoculum (1×10^7 CFU·mL⁻¹) in batch culture, whereas the field trial used the concentrated liquid product (applied at 5 mL·m⁻³, delivering a higher effective CFU input relative to fat content at the fish-processing site's fat concentration of 862 mg L⁻¹); (2) Substrate match: the fish-processing effluent is rich in long-chain polyunsaturated fatty acids (fish oil profile), which are particularly susceptible to hydrolysis by the BDO3–BDO14 enzyme combination, whereas the simulated laboratory wastewater used 1% palm oil (a predominantly saturated/monounsaturated substrate); (3) Natural inoculation: ambient lipolytic microorganisms native to the fish-processing site wastewater may have synergistically enhanced fat removal alongside the applied consortium, an effect absent in sterile laboratory medium. These factors collectively explain why field removal exceeded laboratory values and underscore the importance of substrate-matched formulation.

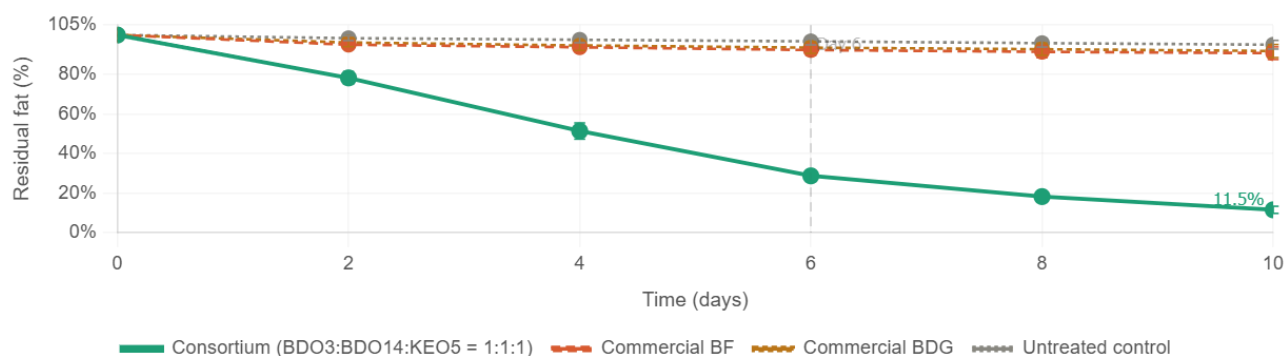


Figure 6. Residual fat content (% of initial concentration) over a 10-day field trial at an aquatic food-processing plant (initial fat: 862 ± 18 mg L⁻¹). Four treatment groups were evaluated in triplicate: the consortium bioremediation product (BDO3:BDO14:KEO5 = 1:1:1, liquid format, 5 mL m⁻³; solid teal line), commercial product BF (10 g m⁻³; dashed coral line), commercial product BDG (5 g m⁻³; dashed amber line), and untreated control (dashed grey line). Residual fat was determined by APHA Standard Methods 5520 B (Soxhlet extraction). Data points represent means of triplicate field plots; error bars indicate $\pm$ standard deviation. The vertical dashed line marks Day 6, the primary evaluation endpoint. Different letters denote significant differences among treatment groups at Day 6 (one-way ANOVA, Tukey's HSD, $p < 0.05$; see Table 7).

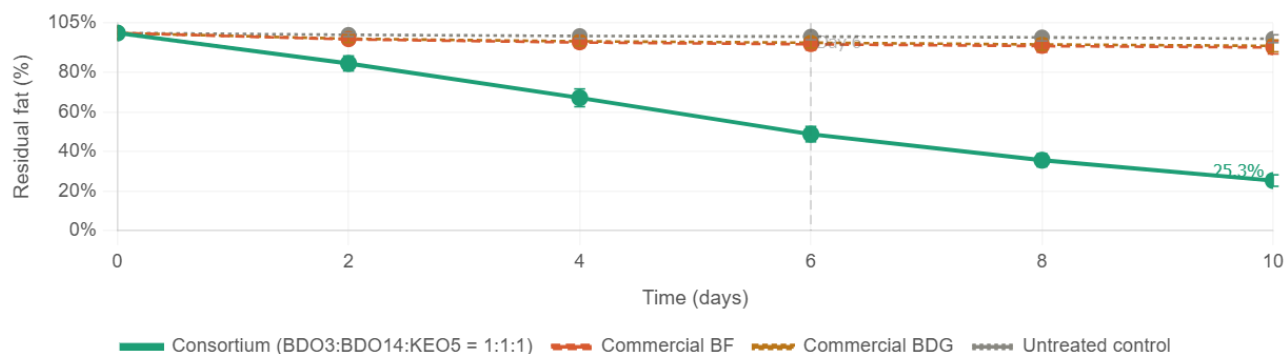


Figure 7. Residual fat content (% of initial concentration) over a 10-day field trial at a palm oil mill (initial fat: $1,542 \pm 35$ mg L⁻¹). Treatment groups, application rates, analytical method, and statistical notation are as described for Figure 6.

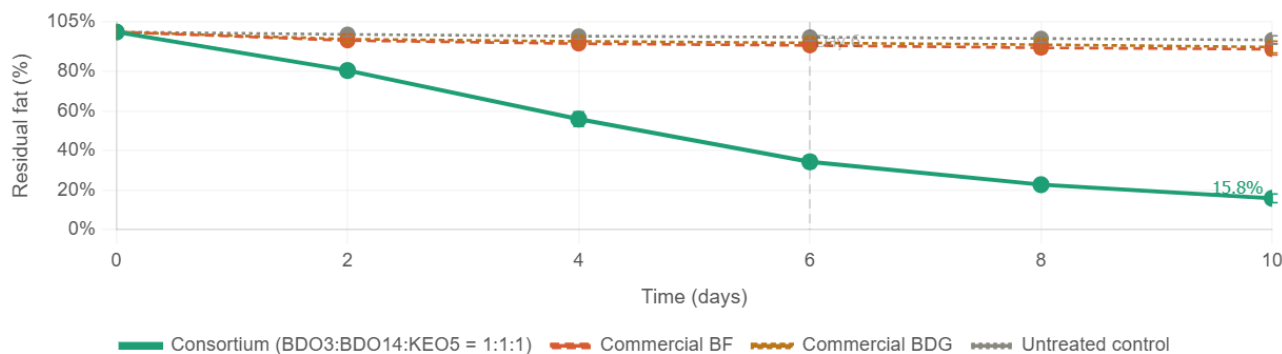


Figure 8. Residual fat content (% of initial concentration) over a 10-day field trial at a municipal wastewater treatment unit (initial fat: $388 \pm 12 \text{ mg L}^{-1}$). Treatment groups, application rates, analytical method, and statistical notation are as described for Figure 6.

To evaluate the field-scale efficacy of the consortium bioremediation product, pilot trials were conducted at three wastewater sites representing distinct fat-rich effluent types: a fish processing facility (initial fat concentration $862 \pm 18 \text{ mg L}^{-1}$), a palm oil mill (POME; $1,542 \pm 35 \text{ mg L}^{-1}$), and a municipal wastewater treatment unit ($388 \pm 12 \text{ mg L}^{-1}$). At each site, four treatment groups were evaluated in triplicate: the consortium product (BDO3:BDO14:KEO5 = 1:1:1, applied at 5 mL m^{-3}), two commercially available bioremediation products (BF and BDG, applied at manufacturer-recommended doses), and an untreated control. Residual fat content, expressed as a percentage of the initial concentration, was monitored at two-day intervals over ten days and is presented in Table 6. Across all three sites, the consortium product achieved substantially greater fat reduction than either commercial comparator or the untreated control at every sampling time point from day 2 onwards. At the fish processing site, residual fat in the consortium-treated plots declined progressively from 100% at day 0 to $78.3 \pm 3.2\%$ at day 2, $51.4 \pm 4.1\%$ at day 4, and $28.7 \pm 2.8\%$ at day 6, continuing to $18.2 \pm 2.1\%$ and $11.5 \pm 1.8\%$ at days 8 and 10, respectively. At the POME site — where the highest initial fat loading presented the greatest treatment challenge — residual fat in the consortium-treated plots reached $48.8 \pm 3.9\%$ by day 6 and declined further to $25.3 \pm 2.9\%$ by day 10. At the municipal wastewater site, residual fat fell to $34.3 \pm 3.1\%$ at day 6 and $15.8 \pm 2.2\%$ at day 10. By contrast, the commercial products BF and BDG achieved only marginal fat reductions across all three sites throughout the ten-day monitoring period, with residual fat values consistently ranging from 90.8 to 97.1% at day 10, values not significantly different from the untreated control ($p > 0.05$). These results demonstrate that the consortium product outperformed both commercial comparators by a substantial margin across all site types and all time points. The endpoint performance metrics at day 6 for all treatment groups and sites are summarised in Table 7. At the fish processing site, the consortium product achieved a fat removal efficiency of $71.3 \pm 2.8\%$, reducing fat concentration from $862 \pm 18 \text{ mg L}^{-1}$ to $247 \pm 24 \text{ mg L}^{-1}$, representing a 68.4 percentage-point improvement in residual fat over the better-performing commercial product (BF, 92.4% residual). At the POME site, fat removal efficiency reached $51.2 \pm 3.9\%$, reducing fat from $1,542 \pm 35 \text{ mg L}^{-1}$ to $790 \pm 60 \text{ mg L}^{-1}$ — a 49.0 percentage-point improvement over commercial BF (94.3% residual). At the municipal wastewater site, the consortium product achieved $65.7 \pm 3.1\%$ fat removal, reducing fat from $388 \pm 12 \text{ mg L}^{-1}$ to $133 \pm 12 \text{ mg L}^{-1}$, a 62.5 percentage-point improvement over commercial BF (93.2% residual). One-way ANOVA followed by Tukey's HSD post-hoc test confirmed that fat removal by the consortium product was significantly superior to both commercial products and the untreated control at all three sites ($p < 0.05$; significance letters in Table 6), while no significant difference was detected between the two commercial products or between either commercial product and the untreated control at any site ($p > 0.05$). The superior field performance of the consortium product is consistent with its lipolytic mechanisms and synergistic fat-degradation activity. The high lipase activity, emulsification capacity, and oil displacement characteristics of the BDO3:BDO14:KEO5 consortium collectively enable effective mobilization and hydrolysis of fat under the complex physicochemical conditions of real wastewater, including variable pH, competing organic matter, and fluctuating temperature,

conditions under which single-strain commercial products appear limited in efficacy. The relatively lower fat removal observed at the POME site compared with the fish processing and municipal sites at day 6 (51.2% vs. 71.3% and 65.7%, respectively) likely reflects the substantially higher initial fat loading at that site, which may require a higher product dose or an extended treatment period for complete remediation. It represents a direction for further optimization in future field trials.

4. Conclusions

This study successfully developed a mixed bacterial consortium bioremediation product for the treatment of fat-rich wastewater, integrating strain isolation, mixture design optimization, pilot-scale production, product formulation, and field validation into a complete development pipeline. Eleven lipolytic bacterial isolates were recovered from fat-rich wastewater across five source environments, of which three — *Acinetobacter variabilis* BDO3, *Bacillus* sp. BDO14, and *Lysinibacillus* sp. KEO5 — were selected based on superior and complementary lipolytic performance, recording lipase activities of 8.07, 8.36, and 8.02 U mL⁻¹, emulsification indices (E_{24}) of 54.3, 40.0, and 39.5, oil displacement areas of 12.56, 19.63, and 19.63 cm², and individual oil removal efficiencies of 21.7, 29.6, and 24.5%, respectively. Simplex-centroid mixture design optimization across nine consortium formulas identified the equal-proportion blend BDO3:BDO14:KEO5 (1:1:1) as optimal, achieving a confirmed oil removal of 54.1% — an 83–149% improvement over the best-performing monoculture (BDO14, 29.6%) and substantially exceeding all binary combinations (36.9–42.7%). The close agreement between the model-predicted (52.3%) and the confirmed (54.1%) oil removal validated the Scheffé mixture model and indicated the presence of synergistic interactions among the three constituent strains. Scale-up of the consortium fermentation from 1 L bench scale to a 200 L pilot fermenter was achieved without significant loss of performance: peak biomass of 7.5×10^8 CFU mL⁻¹ was reached at 48 h in both systems ($p > 0.05$), maximum specific growth rate was maintained at 0.25 ± 0.03 h⁻¹, and oil removal efficiency in test assays was preserved at $52.7 \pm 2.3\%$ at 200 L compared with $54.1 \pm 1.8\%$ at bench scale ($p > 0.05$). Two product formulations were developed from the 200 L harvest: a liquid formulation in 15% glycerol maintaining viable counts of $1.40\text{--}7.5 \times 10^7$ CFU mL⁻¹ over one month of storage, and a powder formulation on rice bran carrier maintaining $2.45\text{--}6.0 \times 10^6$ CFU mL⁻¹ over two months, both satisfying the minimum viability threshold required for commercial bioremediation applications. Field trials across three wastewater site types demonstrated that the consortium product outperformed two commercial bioremediation products (BF and BDG) by 49.0 to 68.4 percentage points in residual fat reduction at day 6, achieving residual fat values of $28.7 \pm 2.8\%$ (fish processing effluent), $48.8 \pm 3.9\%$ (POME), and $34.3 \pm 3.1\%$ (municipal wastewater) compared with 92.4–95.1% for the commercial products and 96.8–98.2% for the untreated control — differences that were statistically significant at all three sites (Tukey's HSD, $p < 0.05$). Fat removal continued beyond day 6, reaching $11.5 \pm 1.8\%$, $25.3 \pm 2.9\%$, and $15.8 \pm 2.2\%$ residual fat at day 10 for the fish processing, POME, and municipal sites, respectively. Collectively, these results establish the BDO3:BDO14:KEO5 consortium product as a high-performance, scalable, and field-validated bioremediation solution for treating fat-rich wastewater, demonstrating superiority over currently available commercial products across diverse effluent types. Future work should focus on dose-response optimization at high-loading sites such as POME, long-term product stability beyond two months, and safety characterization of the constituent strains to support regulatory approval for commercial deployment in Thailand's food processing and palm oil industries under the BCG Economy framework.

Table 6. Residual fat content (% of initial concentration $\pm$ SD) over time across three field sites and four treatment groups. Values are means of triplicate field plots. Sampling was conducted on days 0, 2, 4, 6, 8, and 10. Day 6 values are highlighted; green shading = residual fat < 50% (consortium product); red shading = residual fat > 90% (commercial products and control).

Day	Fish processing effluent				POME				Municipal wastewater			
	Con-sortium	Comm. BF	Comm. BDG	Control	Con-sortium	Comm. BF	Comm. BDG	Control	Con-sortium	Comm. BF	Comm. BDG	Control
0 (initial)	100.0 $\pm$ 0.0	100.0 $\pm$ 0.0	100.0 $\pm$ 0.0	100.0 $\pm$ 0.0	100.0 $\pm$ 0.0	100.0 $\pm$ 0.0	100.0 $\pm$ 0.0	100.0 $\pm$ 0.0	100.0 $\pm$ 0.0	100.0 $\pm$ 0.0	100.0 $\pm$ 0.0	100.0 $\pm$ 0.0
2	78.3 $\pm$ 3.2	95.1 $\pm$ 1.8	96.2 $\pm$ 2.1	98.4 $\pm$ 1.2	84.6 $\pm$ 3.8	96.8 $\pm$ 2.2	97.1 $\pm$ 1.9	99.1 $\pm$ 0.9	80.5 $\pm$ 2.9	95.7 $\pm$ 2.0	96.4 $\pm$ 1.7	98.8 $\pm$ 1.1
4	51.4 $\pm$ 4.1	93.8 $\pm$ 2.3	94.7 $\pm$ 2.5	97.6 $\pm$ 1.5	67.2 $\pm$ 4.5	95.2 $\pm$ 2.6	95.8 $\pm$ 2.4	98.5 $\pm$ 1.3	55.9 $\pm$ 3.7	94.1 $\pm$ 2.1	95.3 $\pm$ 2.2	97.9 $\pm$ 1.4
6	28.7 $\pm$ 2.8	92.4 $\pm$ 2.7	93.6 $\pm$ 2.9	96.8 $\pm$ 1.8	48.8 $\pm$ 3.9	94.3 $\pm$ 2.9	95.1 $\pm$ 2.7	98.2 $\pm$ 1.6	34.3 $\pm$ 3.1	93.2 $\pm$ 2.4	94.5 $\pm$ 2.6	97.4 $\pm$ 1.7
8	18.2 $\pm$ 2.1	91.5 $\pm$ 3.0	92.8 $\pm$ 3.1	95.9 $\pm$ 2.0	35.6 $\pm$ 3.3	93.4 $\pm$ 3.1	94.2 $\pm$ 2.9	97.8 $\pm$ 1.8	22.7 $\pm$ 2.5	92.0 $\pm$ 2.7	93.6 $\pm$ 2.8	96.7 $\pm$ 1.9
10	11.5 $\pm$ 1.8	90.8 $\pm$ 3.2	91.9 $\pm$ 3.3	95.1 $\pm$ 2.2	25.3 $\pm$ 2.9	92.7 $\pm$ 3.3	93.5 $\pm$ 3.0	97.1 $\pm$ 2.0	15.8 $\pm$ 2.2	91.3 $\pm$ 2.9	92.4 $\pm$ 3.0	96.0 $\pm$ 2.1

* Consortium product: BDO3:BDO14: KEO5 = 1:1:1, liquid format, applied at 5 mL $\cdot$ m⁻³ ($\sim 5 \times 10^4$ CFU $\cdot$ L⁻¹).

Commercial BF: powder, applied at 10 g m⁻³ (manufacturer recommended), and Commercial BDG: powder, applied at 5 g m⁻³ (manufacturer recommended).

Both commercial products were within their expiry dates and stored per the manufacturer's instructions. Control: untreated wastewater receiving no microbial product. SD = standard deviation of triplicate field plots.

Table 7. Summary of field performance metrics at day 6 across three site types and four treatment groups. Values for fat concentration are means $\pm$ SD of triplicate field plots. Fat removal and residual fat are calculated relative to the initial concentration on day 0. Improvement is expressed as the percentage-point reduction in residual fat relative to the better-performing commercial product (BF or BDG) at each site. Different superscript letters within each site indicate significant differences (Tukey's HSD, $p < 0.05$).

Site	Treatment	Initial fat (mg L ⁻¹)	Final fat (mg L ⁻¹)	Fat removal (%)	Residual fat (%)	Improvement vs. the best commercial	Sig.
Fish processing effluent	Consortium (BDO3:BDO14:KEO5)	862 $\pm$ 18	247 $\pm$ 24	71.3 $\pm$ 2.8	28.7 $\pm$ 2.8	+68.4%	a
	Commercial BF	862 $\pm$ 18	795 $\pm$ 23	7.6 $\pm$ 2.7	92.4 $\pm$ 2.7	—	b
	Commercial BDG	862 $\pm$ 18	806 $\pm$ 25	6.4 $\pm$ 2.9	93.6 $\pm$ 2.9	—	b
	Untreated control	862 $\pm$ 18	834 $\pm$ 21	3.2 $\pm$ 1.8	96.8 $\pm$ 1.8	—	c
Palm oil mill effluent (POME)	Consortium (BDO3:BDO14:KEO5)	1,542 $\pm$ 35	790 $\pm$ 60	51.2 $\pm$ 3.9	48.8 $\pm$ 3.9	+49.0%	a
	Commercial BF	1,542 $\pm$ 35	1,454 $\pm$ 45	5.7 $\pm$ 2.9	94.3 $\pm$ 2.9	—	b
	Commercial BDG	1,542 $\pm$ 35	1,468 $\pm$ 42	4.9 $\pm$ 2.7	95.1 $\pm$ 2.7	—	b
	Untreated control	1,542 $\pm$ 35	1,514 $\pm$ 38	1.8 $\pm$ 1.6	98.2 $\pm$ 1.6	—	c
Municipal wastewater	Consortium (BDO3:BDO14:KEO5)	388 $\pm$ 12	133 $\pm$ 12	65.7 $\pm$ 3.1	34.3 $\pm$ 3.1	+62.5%	a
	Commercial BF	388 $\pm$ 12	362 $\pm$ 9	6.8 $\pm$ 2.4	93.2 $\pm$ 2.4	—	b
	Commercial BDG	388 $\pm$ 12	367 $\pm$ 11	5.5 $\pm$ 2.6	94.5 $\pm$ 2.6	—	b
	Untreated control	388 $\pm$ 12	378 $\pm$ 10	2.6 $\pm$ 1.7	97.4 $\pm$ 1.7	—	c

a, b, c: means sharing the same letter within each site are not significantly different (one-way ANOVA followed by Tukey's HSD post-hoc test, $p < 0.05$).

— = no improvement calculated for commercial products and control (reference group).

Green shading (consortium rows) = superior fat removal performance relative to commercial products.

Initial fat concentrations: fish processing effluent 862 $\pm$ 18 mg L⁻¹; POME 1,542 $\pm$ 35 mg L⁻¹; municipal wastewater 388 $\pm$ 12 mg L⁻¹.

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