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Safe Cosmetic Actives via Two-liquid Phase Fermentation: Optimization of Oil Phase and Emulsifier Selection

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1. Introduction

The demand for sustainable and skin-safe cosmetic ingredients continues to grow alongside increasing consumer awareness and stricter regulatory requirements. In response, biotechnological approaches such as microbial fermentation have emerged as promising alternatives to conventional extraction methods, which often rely on environmentally harmful technologies such as high-power microwave, ultrasonic, or chemical reagent extraction [1]. Fermentation offers several advantages, including enhanced bioavailability of actives, controlled production conditions, and reduced environmental footprint.

Two-liquid phase fermentation (TLPF) is an innovative fermentation technique that incorporates an immiscible oil phase into an aqueous microbial culture, often accompanied by the use of oxygen carriers. This dual-phase system enhances oxygen transfer efficiency, supports microbial growth, and facilitates the selective partitioning of fermentation metabolites [2,3]. Compared to traditional single-phase fermentation systems, TLPF has demonstrated significant potential in improving both strain propagation and product yield, particularly in industrial applications such as pharmaceuticals and bioenergy [4].

Despite these advantages, the application of TLPF in the cosmetics industry remains underexplored. Current cosmetic fermentation systems primarily rely on conventional aqueous fermentation, which can suffer from limitations such as poor oxygen solubility and suboptimal biomass conversion. These challenges restrict the full realization of fermentation-derived actives with multifunctional skin benefits.

In this study, we evaluated the applicability of TLPF for producing safe and effective cosmetic actives using *Saccharomyces cerevisiae* as the model strain. We focus on optimizing key parameters including oil phase type, emulsifier selection, and water-to-oil ratio to enhance yeast growth and fermentation efficacy. The fermentation products were assessed for their biochemical activity, safety (via Human Repeated Insult Patch Test, HRIPT), and functional performance in topical formulations.

By establishing a comprehensive optimization strategy and validating product efficacy and safety, this research contributes to a deeper understanding of TLPF as a sustainable and scalable solution for the next generation of cosmetic actives.

2. Materials and Methods

2.1 Design of the Two-Liquid Phase Fermentation System

The yeast strain *Saccharomyces cerevisiae* was isolated from distiller's grains collected in Wuyishan, Fujian Province, China. Yeast extract peptone dextrose (YPD) medium was used as the aqueous phase for fermentation. A single-factor experimental design was adopted to investigate the effect of four variables on yeast growth in the TLPF system: (1) oil phase type, (2) emulsifier type, (3) oil-to-aqueous phase ratio, and (4) emulsifier-to-oil ratio, as described by Cao et al. [5].

(1) Oil Phase Selection
Three oils representing mineral, synthetic, and vegetable origins were selected to ensure cosmetic safety: White mineral oil (mineral oil), Hydrogenated polyisobutylene (synthetic oil), Jojoba seed oil (vegetable oil). These are commonly used in industrial TLPF due to their immiscibility and biocompatibility [6–8].

(2) Emulsifier Selection
Two emulsifiers representing different emulsion systems were chosen: Polyglycerol-3 methylglucose distearate (O/W emulsifier), Polyglycerol-3 diisostearate (W/O emulsifier)

(3) Oil-to-Aqueous Phase Ratio
Four ratios were tested: 2:8, 4:6, 5:5, and 6:4, based on pre-screening results and their reported influence on oxygen mass transfer and fermentation rate [9].

(4) Emulsifier-to-Oil Ratio
Emulsifiers were blended with oil at four different ratios: 1:2, 1:6, 1:10, and 1:14, based on emulsion stability and dispersion efficiency in TLPF systems.

All fermentations were performed in 250 mL Erlenmeyer flasks with 100 mL working volume at 28°C and 160 rpm for 24 h. Colony-forming units (CFUs) were assessed using the dilution plate method (10–12× dilution) to evaluate yeast proliferation [6].

2.2 Growth Curve Determination

The optimized TLPF condition was further analyzed by sampling at 12, 24, 48, 60, 72, and 96 h. Yeast growth was quantified using the same dilution plating method to establish the microbial growth curve [7].

2.3 Comparison of Fermentation Systems

Four groups were prepared to compare fermentation performance:
L1: TLPF under optimized conditions.
L2: Traditional single aqueous-phase fermentation.
L3: TLPF without emulsifier.
L4: L1 system further emulsified following a cosmetic formulation process.
CFUs were measured across groups. Emulsified systems (L1, L3) were also visualized using light microscopy to observe oil droplet distribution and stability.

2.4 Total Sugar Content

Total sugar concentration in L1, L2, and L3 fermentation broths was measured using the phenol-sulfuric acid method [10].

2.5 Antioxidant Activity Assays

Fermentation broth from group L1 was subjected to low-temperature micro-jet extraction [13], followed by membrane filtration to prepare a clear filtration. A cosmetic raw material system was formulated containing 25% fermentation filtrate, preservatives, and deionized water. Antioxidant activities were measured by DPPH and ABTS radical scavenging assays using UV-visible spectrophotometry at 2% test concentration [11,12].

2.6 Human Skin Efficacy Evaluation (VISIA® Imaging)

An essence was prepared using 3% of the TLPF extract (L1) and tested on 30 healthy volunteers divided into a test group and control group in a 2:1 ratio. The test group applied the TLPF-containing essence, while the control group used a placebo formulation. All subjects applied to the product once every night.

Skin condition was assessed at baseline, day 7, day 14, and day 28 using VISIA® 7 (Canfield Scientific, USA). Red zone and pore area images were quantitatively analyzed [14].

2.7 Safety Assessment: Human Repeated Insult Patch Test (HRIPT)

To assess safety, HRIPT was conducted on 30 volunteers using a 5% solution of the TLPF filtrate. The left arm served as the test site and the right arm as the control. Test patches were applied and removed after 24 h, and skin responses were recorded at 30 min, 24 h, and 48 h post-removal. Skin reaction grades were evaluated by a board-certified dermatologist using standard dermatological grading scales [17].

3. Results

3.1 Optimization of Single-Factor Parameters in Two-Liquid Phase Fermentation (TLPF)

3.1.1 Oil Phase Selection

The impact of different oil phases on *S. cerevisiae* growth is shown in Figure 1. Among the three tested oils, jojoba seed oil supported the highest colony formation, followed by hydrogenated polyisobutylene. The lowest growth was observed with white mineral oil. These findings suggest that jojoba seed oil provides a more favorable microenvironment for yeast growth in the TLPF system.

(a) (b) (c)

Figure 1. Growth of *S. cerevisiae* on agar plates in TLPF systems with different oil phases.
(a) White mineral oil, (b) Hydrogenated polyisobutylene, (c) Jojoba seed oil.

3.1.2 Emulsifier Selection

Figure 2 illustrates yeast growth in systems containing different emulsifiers. The W/O emulsifier, polyglycerol-3 diisostearate (b), produced significantly more colonies compared to the O/W emulsifier, polyglycerol-3 methylglucose distearate (a), indicating its superior compatibility with the TLPF environment.

(a) (b)

Figure 2. Growth of *S. cerevisiae* on agar plates with different emulsifiers. (a) Polyglycerol-3 methylglucose distearate group, (b) Polyglycerol-3 diisostearate group.

3.1.3 Oil-to-Aqueous Phase Ratio

As shown in Figure 3, increasing the oil phase content promoted yeast growth. Among the four tested ratios, the 6:4 water-to-oil ratio (d) produced the most colonies. The 2:8 ratio (a) showed the weakest performance, highlighting the importance of oil volume for oxygen availability.

(a) (b) (c) (d)

Figure 3. Growth of *S. cerevisiae* on agar plates with different oil-to-aqueous phase ratios.
(a) 2:8, (b) 4:6, (c) 5:5, (d) 6:4.

3.1.4 Emulsifier-to-Oil Phase Ratio

The influence of emulsifier concentration on yeast growth is shown in Figure 4. The 1:6 ratio (B) led to the highest colony formation, suggesting an optimal balance between dispersion stability and microbial oxygen exchange. Extremely low (D) or high (A) emulsifier levels diminished growth.

(a) (b) (c) (d)

Figure 4. Growth of *S. cerevisiae* on agar plates at different emulsifier-to-oil ratios.
(a) 1:2, (b) 1:6, (c) 1:10, (d) 1:14.

In Conclusion: The optimal medium composition for TLPF was determined to be jojoba seed oil + polyglycerol-3 diisostearate, with a 4:6 oil-to-water ratio and 1:6 emulsifier-to-oil ratio.

3.2 Growth Curve of *S. cerevisiae* in the Optimized TLPF System

According to the optimized medium conditions for the TLPF system, the growth trend of *S. cerevisiae* over different fermentation times is shown in Figure 5. The colony counts exhibited

a continuous increase from 0 to 48 hours, reaching the maximum at 48 hours. After 48 hours, a gradual decline in colony numbers was observed, indicating the transition from the exponential growth phase to the stationary and decline phases.

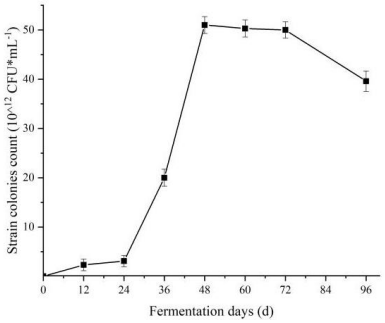


Figure 5. Growth curve of *S. cerevisiae* in the optimized TLPF system over 96 hours.

3.3 Comparison of Fermentation Strategies

The fermentation appearances of groups L1, L2, and L3 are shown in Figure 6a, and the corresponding colony counts on agar plates are presented in Figure 6b. In the L3 group, clear stratification between the aqueous and oil phases was observed, although the oil phase appeared less distinct compared to previous observations. L3 also exhibited the lowest colony counts among all groups. In contrast, the L1 group displayed no visible oil phase separation, forming a uniform suspension, and achieved the highest colony counts. The total sugar contents of the fermentation broths are summarized in Figure 6c. The L1 group had the highest total sugar content, which was 25% higher than the L2 group and 42.8% higher than the L3 group, further reflecting the differences in fermentation efficiency between the groups.

L1 L2 L3

Figure 6a. Appearance of fermentation broths (L1, L2, L3).

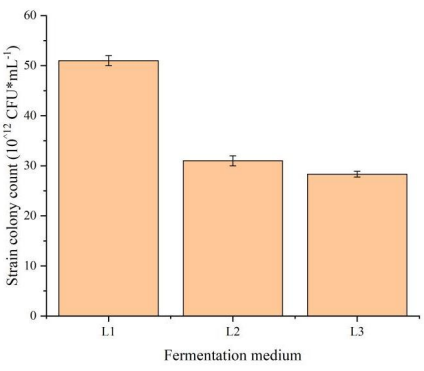


Figure 6b. Colony counts on agar plates.

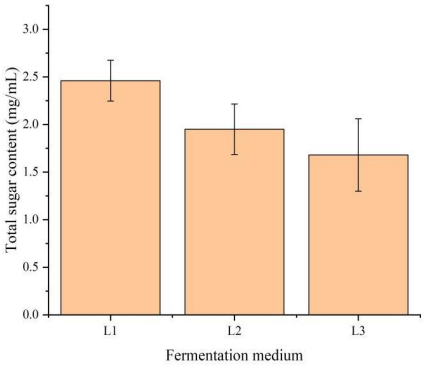


Figure 6c. Total sugar content of fermentation broths.

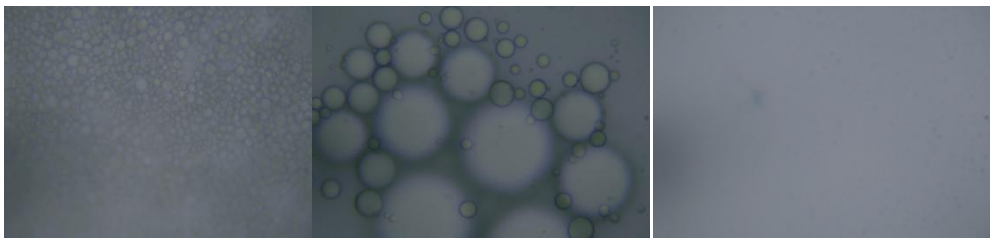
3.4 Oil Droplet Distribution in Fermentation Broth

The appearances of the L1, L3, and L4 dilutions are shown in Figure 7a. L1 exhibited a stable suspension, while L3 showed visible oil droplet distribution with clear phase separation. In contrast, L4 presented a milky, cloudy appearance, indicating incomplete emulsification.

The microscopic images of the oil droplet states are depicted in Figure 7b.In the L3 group, oil droplets were unevenly distributed with significant size variation across the fermentation broth. The L4 group exhibited poorly defined oil droplets with low optical clarity. Conversely, the L1 group displayed oil droplets of uniform size that were evenly dispersed throughout the medium, suggesting superior emulsion stability and system homogeneity.

L1 L3 L4

Figure 7a. Appearance of diluted fermentation broths (L1, L3, L4).



L1 L3 L4

Figure 7b. Microscopic images of oil droplet distribution in L1, L3, and L4.

3.5 Free Radical Scavenging Activity

The results of the DPPH and ABTS radical scavenging assays are presented in Figure 8. The L1 group exhibited the highest scavenging activity for both DPPH and ABTS radicals, significantly exceeding the levels observed in the single-phase fermentation group (L2) under identical conditions.In contrast, the L3 group demonstrated the lowest scavenging activities in both assays, indicating reduced antioxidant potential compared to the optimized TLPF system.

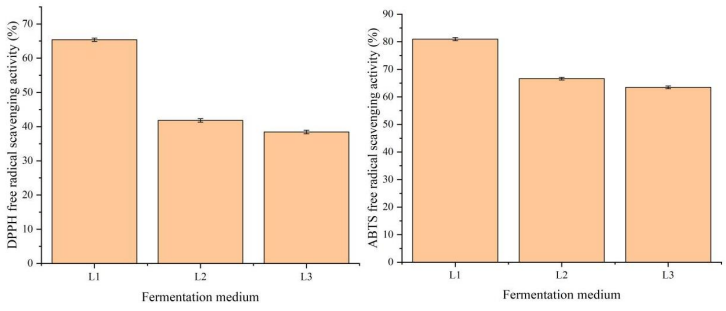


Figure 8. (a) DPPH radical scavenging activity. (b) ABTS radical scavenging activity.

3.6 Clinical Efficacy: VISIA® Imaging

The red zone images and quantitative test values after 14 days of TLPF essence application are presented in Figures 9a and 9b. After 14 days, no significant improvement was observed in the control group, with minimal differences between baseline and post-treatment scores.
In contrast, the test group exhibited a marked reduction in red zone values,dropping by 26.79%, as indicated by the visibly lighter coloration in the corresponding images. A similar trend was observed in pore score measurements, which decreased by 9.73% in the test group,, while changes in the control group remained negligible. These results suggest that the TLPF filtrate possesses both soothing and astringent effects, contributing to overall skin condition improvement.

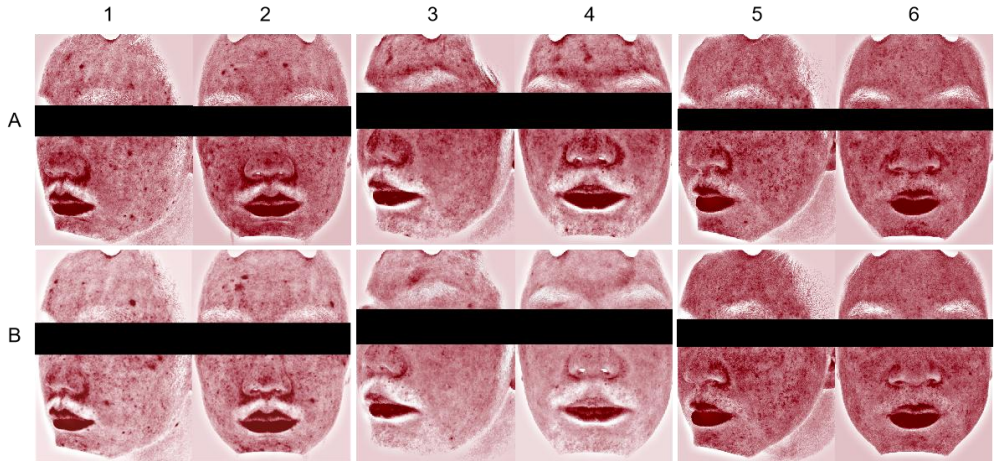


Figure 9a. Red zone images of subjects before and after 14 days (test and control groups).

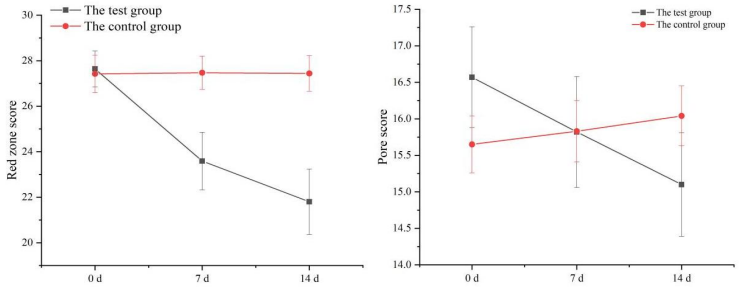


Figure 9b. Quantitative analysis of red zone and pore scores.

3.7 Human Safety Assessment

The results of the Human Repeated Insult Patch Test (HRIPT) demonstrated that none of the subjects experienced any adverse skin reactions at 30 minutes, 24 hours, or 48 hours following the removal of the test substance containing 5% TLPF filtrate. All observations were confirmed by a board-certified dermatologist, with all participants showing negative reactions throughout the evaluation period, thereby confirming the excellent skin compatibility of the TLPF filtrate.

4. Discussion

TLPF is an innovative psychochemical platform for cosmetic ingredient development, which however has not yet been utilized in the beauty market. Although TLPF for the generation of nanofibers for pharmaceutical and biotechnological applications is well reported in literature [3,7], no studies so far have focused on a systematic optimization and application of TLPF with the yeast S. cerevisiae for the production of cosmetic-acceptable calming actives.

Taking advantage of the dual-phase mixture established by the choice of vegetable oil and emulsifier, we showed that TLPF does not only contribute to boosting microbial growth and metabolite yield but also results in bioactives with direct dermocosmetic properties. This provides a novel method for the delivery of high potency actives via a fermentation platform optimized for topical applications.

Unlike conventional fermentation processes which depend entirely on aqueous system, the biphasic system would allow oxygen to diffuse through the oil phase [9] and, at the same time, exhibit emulsion-like property that corresponds to better stability of raw materials and possible delivery.

These results suggested that not only oil phase type (jojoba seed oil) but also emulsifier (W/O type: polyglycerol-3 diisostearate) was not formulation tools but functional variables in fermentation system design. These results also provide an innovative coupling of formulation science and bioprocess technology, providing a new perspective for active ingredient innovation.

This study provides a scalable and sustainable alternative to traditional actives production. Many fermentation-derived ingredients currently on the market are limited by low yields or high production costs [2]. Our TLPF model not only enhances metabolite output (25% increase in sugar content) but also improves biological efficacy, as confirmed by DPPH and ABTS assays and human skin evaluations. These advantages position TLPF as a next-generation technology for clean, effective, and green activities, which aligns with growing consumer demand for multifunctional and scientifically supported skincare solutions.

It also provides formulators with additional flexibility in the selection of raw materials – we can mix 'n' match plant oils and emulsifiers to suit regional preferences, sustainability claims or skin compatibility. This paves the way for bespoke fermentation regimes, fitting a brand story or regional ingredient momentum, providing yet another level of product differentiation.

This study fills a number of important gaps in literature:

Gas-Liquid Oxygen Transfer in Fermentation Systems: We show how oil-phase control can address an enduring issue in aerobic microbial fermentation – poor O2 solubility in

water media [9]. Through optimizing the ratio of oil-to-water and emulsifier-to-oil, we offer a feasible approach for promoting oxygen transportation and metabolism.

Absence of Cosmetics-Oriented Fermentation Design: Although many ingredients are "fermented," few are formulated to become cosmetics with skin safety being the priority in the fermentation process. Here we fill this gap, evidencing not only enhanced bioactivity, but also clinical safety and dermal tolerability (HRIPT grade 0).

Stability and Change of Oil Droplets: We demonstrate that emulsification in the course of processing leads to stable products while post-formulation emulsions phase separation with time. This uncovers an alternative way of stabilizing bio-ferments in the absence of traditional surfactant systems – a question that until now has remained unanswered for clean-label cosmetic development.

5. Conclusion

The present study reports for the first time the successful scale-up of two-liquid phase fermentation (TLPF) technology to produce non-toxic and efficacious cosmetic actives. We chose jojoba seed oil together with W/O type emulsifier using an optimized dute-phase system, we markedly enhanced the yeast growth, metabolite production, and antioxidant activity. These fermentation-derived actives were also shown to be safe (HRIPT) and effective (13.6% decrease in facial redness) in human trials.

Our results indicate that TLPF is not a simple fermentation optimization tool but an innovative concept to create high efficient and environmental friendly cosmetics ingredients. It provides a new approach to the above problems that are encountered in conventional ferment processing, such as fermentation efficiency, skin friendliness and formulating stability.

This work paves new options for next-level fermentation customization, enabling raw material suppliers and brand R&D teams to co-develop fermented ingredients with specific functionality and market resonance.

Conflict of Interest Statement

The authors declare no conflict of interest.

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