Two-Phase Fermentation Systems for Microbial Production of Terpenes

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Contributor: Tuo Li, Ximeng Liu, Haoyu Xiang, Hehua Zhu, Xuan Lu, Baomin Feng

Microbial cell factories, renowned for their economic and environmental benefits, have emerged as a key trend in academic and industrial areas, particularly in the fermentation of natural compounds. Among these, plant-derived terpenes stand out as a significant class of bioactive natural products. The large-scale production of such terpenes, exemplified by artemisinic acid—a crucial precursor to artemisinin—is now feasible through microbial cell factories. In the fermentation of terpenes, two-phase fermentation technology has been widely applied due to its unique advantages. It facilitates in situ product extraction or adsorption, effectively mitigating the detrimental impact of product accumulation on microbial cells, thereby significantly bolstering the efficiency of microbial production of plant-derived terpenes.

Keywords: two-phase fermentation ; plant-derived terpenes ; microbial cell factory ; in situ extraction

1. Types of Two-Phase fermentation (TPF) Systems

The culture medium, known as the aqueous phase, supports cell growth, while the alternative phase, which can be liquid, solid, or a combination of both, is referred to as the SP. TPF systems are typically classified into liquid–liquid and liquid–solid systems based on the distinctive properties of the SPs [1][2][3][4] (**Figure 1**).

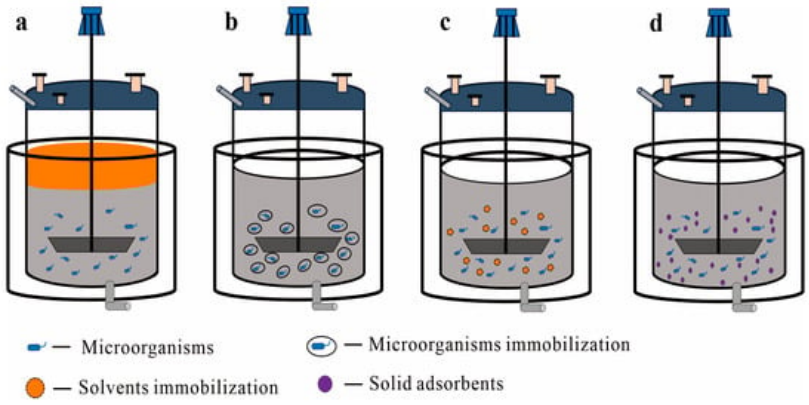

Figure 1. A concept of different types of TPF in a bioreactor: (**a**) liquid–liquid TPF systems, where the orange color on the upper layer represents organic solvents or another aqueous solvent and the dark gray color on the lower layer represents the culture medium; (**b**–**d**) different types of liquid–solid TPF systems: (**b**) immobilized cells as the SP; (**c**) immobilized solvent as the SP; (**d**) solid adsorbents as the SP.

1.1. Liquid–Liquid TPF Systems

Liquid–liquid TPF systems consist of two immiscible phases: an aqueous phase containing the microorganisms and nutrients, and an SP comprising a mixture of compounds, that may be water-insoluble, such as organic solvents or liquid lipophilic compounds, or water-soluble, such as polymers or salts [4][5][6] (**Figure 1**a). Systems with water-insoluble organic compounds are termed aqueous–organic two-phase systems (AOTPS), while those containing water-soluble compounds are referred to as aqueous two-phase systems (ATPS) [5][7].

1.1.1. Aqueous-Organic TPF Systems

Aqueous-organic systems involve an aqueous and an immiscible organic phase, facilitating efficient product separation through in situ extraction [2][8][9]. The interaction between these phases allows for the dispersion of droplets, enhancing the extraction and separation of fermentation products [2][4] (**Figure 2**). Since Inoue and Horikoshi's discovery in 1989 [10], which revealed varying tolerances of microorganisms to organic solvents and led to the isolation of the solvent-tolerant

bacterium *Pseudomonas putida* IH 2000, organic solvents have been increasingly used in fermentation systems for in situ product extraction. For instance, Suzanne Verhoef and colleagues [11] utilized two solvent-tolerant *P. putida* S12 strains, employing glucose as the primary substrate to efficiently produce hydroxystyrene. This led to a final concentration of 21 mM, which was a fourfold increase compared to single-phase fed-batch cultivation. Similarly, Nicola Tan and colleagues [12] focused on trans-nerolidol, a valuable fragrance with antimalarial and anticancer properties, extensively used in cosmetics and agriculture. Under single-phase fed-batch fermentation, the strains produced over 6.8 g/L of nerolidol in 3 days. In contrast, two-phase extractive fed-batch fermentation yielded about 16 g/L of nerolidol in 4 days, with a carbon yield of approximately 9% (g/g), marking the highest yield achieved to date.

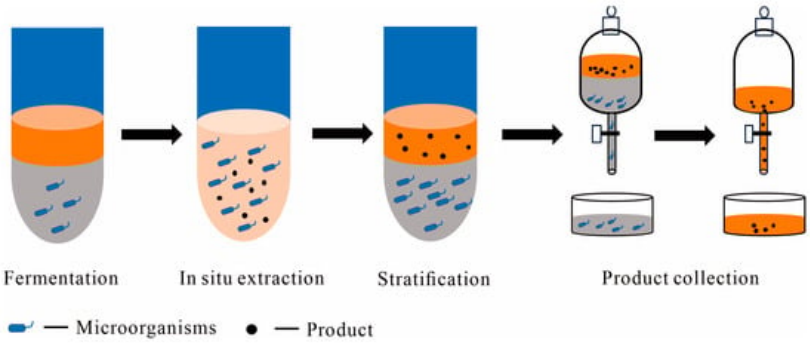

Figure 2. Scheme of aqueous–organic TPF and post-treatment, with main steps including fermentation, in situ extraction, post-fermentation stratification, and product collection; the orange color on the upper layer represents organic solvent or another aqueous solvent, the dark gray color on the lower layer represents the culture medium, and the light orange color of in situ extraction represents two phases mixed during the in situ extractive fermentation.

1.1.2. Aqueous Two-Phase System

The aqueous two-phase system (ATPS), also referred to as an aqueous biphasic system (ABS), represents a biotechnological approach used in both fermentation and extraction processes [13][14]. It consists of two immiscible aqueous phases, usually formed by the combination of two water-soluble polymers, a polymer and a salt or two different salts. This system is environmentally preferable over traditional organic solvent-based TPF systems due to its aqueous nature and nonvolatile components [7][14]. The ATPS is primarily used in ex situ extraction processes, offering an alternative to conventional methods [7][15]. Ionic liquids (ILs), which are salts in the liquid state at low temperatures, have been effectively used for the extraction and purification of fermentation-derived components, showcasing sustainability, efficiency, and eco-friendliness [13][16]. However, the application of ATPSs for in situ extraction remains limited, mainly due to ATPSs' cytotoxicity to cells and the challenge of finding an appropriate formulation for the fermentation process.

H. González-Peñas and coworkers [17] performed a solvent screening for in situ liquid extraction from acetone–butanol–ethanol (ABE) fermentation by *Clostridium acetobutylicum.* They selected methyltrioctylammonium chloride and trihexyl (tetradecyl) phosphonium chloride for their extraction capacity, demonstrating high distribution coefficients. Deep eutectic solvents (DESs) represent an emerging class of eutectic mixtures of Lewis or Brønsted acids and bases, capable of forming a variety of anionic and cationic species [18][19]. Due to the similar characteristics and properties with ILs, DESs are widely known as IL analogs. For example, Liu Jingyang and his team [20] chose a DES composed of choline chloride and urea for the in situ extraction of L-valine produced by *Brevibacterium flavum* XV0505. Optimizing the timing and volumetric fraction of the IL addition, it was shown that adding 0.1% of this IL at the 16th hour of the fermentation process led to the XV0505 strain producing the highest yield of L-valine in both shake-flask and fed-batch fermentation experiments. Similarly, Parul Badhwar and colleagues [21] selected the *Aureobasidium pullulans* strain for cost-effective pullulan production and developed a new ATPS for fermentation. They conducted a comprehensive study of the effects of different molecular weights of polyethylene glycol (PEG) (400, 600, 4000, and 6000) and dextran or mono/di-sodium phosphate salts on the fermentation process. The PEG-dextran ATPS system was found to be suitable for the extractive fermentation of pullulan from *A. pullulans*, achieving a yield of 36.47 g/L. Although ILs showed lower selectivity, their high distribution coefficients indicate a strong potential for targeted extractions, highlighting the need for further optimization in selecting ILs for in situ fermentation processes [22].

1.2. Liquid–Solid TPF Systems

As defined by Sonia Malik et al. [4], liquid–solid TPF systems involve in situ adsorption with an aqueous medium and a solid phase comprising adsorbents or lipophilic materials. This expands on the use of solids as an SP, including the integration of immobilized cells or solutions as the solid phase [23][24] (**Figure 1**b–d). Immobilization refers to the containment or fixation of cells or solutions on or within a matrix [24][25][26]. This process prevents their release during the

fermentation while ensuring adequate permeability for the diffusion of substrates and products [25]. In this context, the immobilized cells or solutions effectively serve as a solid phase (**Figure 1**b,c). Liquid–solid systems show distinct advantages, such as simplified post-fermentation processing and the reusability of immobilized cells.

1.2.1. Immobilized Cells as the Solid Phase

Immobilized cells involve anchoring active cells (as shown in **Figure 3**), serving as biological catalysts, onto a carrier to create a stable structure [23]. Key carriers include solid particles, gels, and membranes [26]. This approach improves cell stability and reusability, thereby streamlining operational and control processes and enhancing their suitability for industrial applications.

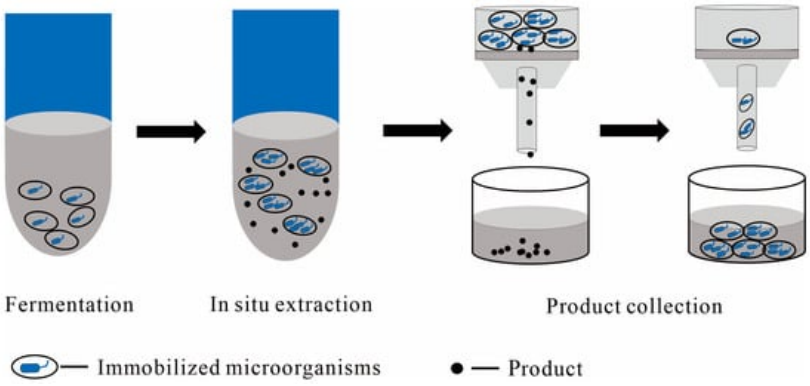

Figure 3. Illustration of a flowchart using immobilized cells as the solid phase in the liquid–solid TPF and downstream processing, with main steps including fermentation, in situ extraction, post-fermentation filtration, and product collection; the dark gray color represents the culture medium.

Cell immobilization techniques encompass a variety of methods [23][27]. Adhesion and adsorptive immobilization entail the physical adsorption of cells onto a carrier's surface. This process depends on the physicochemical properties of both the cells and the carrier. For instance, brewing yeast immobilization onto spent grains involves cell–carrier adhesion, cell–cell attachment, and cell adsorption inside carrier crevices, affected by factors like dilution rate and the hydrophobicity of the carrier [28]. Covalent bonding immobilization involves attaching cells to a carrier via covalent bonds. The efficiency of immobilization is significantly influenced by the quantity and accessibility of reactive groups on the cell surface, which are in turn affected by physiological factors. Thus, covalent coupling is not a major technique used for cell immobilization [27]. Encapsulation immobilization, also known as microencapsulation, involves encapsulating cells within a carrier to create anchored colloidal particles. This technique encompasses coating or entrapping microbial cells with a polymeric material, resulting in the formation of microspheres [29]. Microencapsulation provides several benefits, including increased cell loading capacity, improved cell survival, and a higher production rate of desired microbial products [30]. This technology has been widely applied in various fields involving microbial cells, including the microencapsulation of probiotics [31]. Gel immobilization is characterized by immobilizing cells within a gel-like substance, while entrapment immobilization traps cells within a matrix or polymer. The design of robust matrices, such as macroporous gels with immobilized microbial cells, has shown high efficiency and structural stability [26]. These gels allow for the high retained activity of yeast and *E. coli* cells even after drying and storage, demonstrating their practicality in stirred bioreactors [23][26].

Immobilized cells or enzymes are widely used in biotransformation, with recent papers offering comprehensive overviews of cell or enzyme immobilization techniques in this field [23][26][27]. However, the utilization of immobilized cell technology for producing PDTs remains limited. For example, El-Sayed R. et al. [32] immobilized two mutant strains of *Aspergillus fumigatus* and *Alternaria tenuissima* using five different entrapment carriers of calcium alginate, agar-agar, Na-CMC, gelatin, and arabic gum. Among these, calcium alginate gel beads proved to be the most effective and suitable entrapment carrier for maximum production of paclitaxel. Considering the limited cell immobilization reports, the present part aims to introduce several representative examples involving fermentative production via immobilized cell technology, with the goal of providing useful references and insights for the production of PDTs.

Product inhibition by butanol and acetone is a significant limitation in ABE fermentation. Rizki Fitria Darmayanti and colleagues [33] developed an innovative biobutanol extractive fermentation method using a large volume ratio of extractant with immobilized *Clostridium saccharoperbutylacetonicum* N1-4. The preculture cells of the N1-4 strain were fixed in calcium alginate beads, effectively maintaining a low butanol concentration in the aqueous phase and achieving a total butanol concentration of 64.6 g/L. In a study conducted by Sion Ham and colleagues [34], they utilized engineering techniques and immobilized whole cells of *E. coli* to establish a small-scale reactor system, successfully achieving continuous and efficient production of γ-aminobutyric acid (GABA). Remarkably, these anchored cells maintained high

activity after 15 consecutive uses, whereas free cells lost activity after the ninth reaction. Moreover, after optimizing conditions such as buffer concentration, substrate concentration, and flow rate, the researchers successfully achieved continuous operation for 96 h in a 14 mL scale reactor, producing a total of 165 g of GABA. This not only presents a viable method for producing high concentrations of GABA but also highlights the superior performance of immobilized microbial cells in the process. Weysser Felipe Cândido de Souza et al. [35] utilized an immobilization system comprising 2.0% *w*/*v* alginate, 2.0% *w*/*v* $CaCl_2$, 2.0% *w*/*v* gelatin, and 0.2% *w*/*v* transglutaminase to immobilize *Erwinia* sp. D12 cells. Their experiments revealed that isomaltulose production reached its peak at 327.83 g/L within the first 24 h and that the cells remained stable over 72 h of continuous reaction, maintaining consistent isomaltulose output. This demonstrates that using ionic gelation to immobilize *Erwinia* sp. D12 cells are an effective method for enhancing sucrose-to-isomaltulose conversion. *S. cerevisiae*, a widely favored chassis cell, has shown tremendous potential in producing PDTs [36][37]. Although the technology for immobilizing *S. cerevisiae* cells has not yet been applied in the aforementioned fermentation field, the techniques for immobilizing or encapsulating *S. cerevisiae* cells are already quite mature in other biotransformation areas. These studies provide valuable experience and reference for future use of immobilized yeast in the production of terpenoid compounds.

1.2.2. Immobilized Solvent as the Solid Phase

The solution immobilization system involves integrating solution chemical substances with solid carriers to create solid particles or agglomerates, as illustrated in **Figure 4**. This technology is commonly used in separation and purification processes, enhancing product purity and minimizing waste. The previous discussion of liquid–liquid TPF introduced several biphasic systems, with a focus on the immobilization of ILs [24][38]. ILs are commonly immobilized onto materials like silica or polymers through physical confinement or covalent grafting, mainly enhancing organic catalysis and separation.

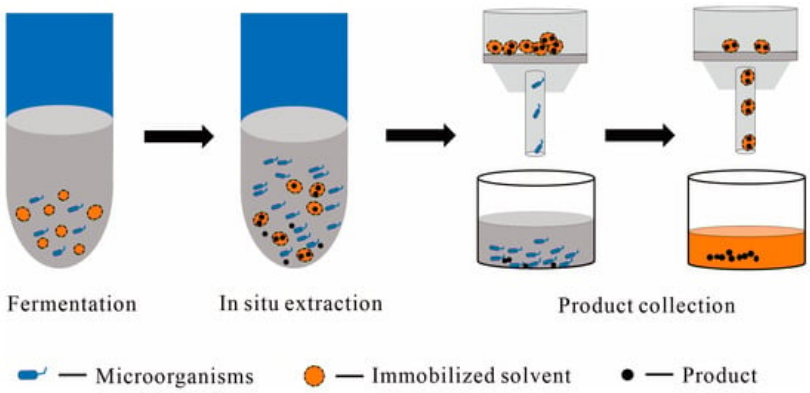

Figure 4. A conceptual scheme of using immobilized solvent as the solid phase in the liquid–solid TPF and downstream processing, with main steps including fermentation, in situ extraction, post-fermentation filtration, and product collection; the dark gray color represents the culture medium and the orange color represents solvents, like ILs.

For example, Changhee Lee and colleagues [39] immobilized the lipase B (CALB) from *Candida Antarctica* and 1-octyl-3-methylimidazolium tetrafluoroborate in a polymeric hybrid monolith, obtaining an enzyme-SILP (e-SILP) catalyst. This catalyst was effective in continuous gas-phase transesterification of vinyl propionate and 2-propanol. Additionally, ILs were anchored on silica as a stationary phase for compound separation and purification. Another application of solution immobilization involved the same CALB in ester enzyme reaction systems. To address the solubility mismatch between enzymes and substrates, a Pickering gel emulsion stabilized by enzyme-modified polymer nanomaterials was developed, facilitating biphasic biocatalysis. These nanomaterials, produced surfactant-free via emulsion polymerization and covalently attached to CALB, were mixed with heptane to create an aqueous dispersion, enhancing nanoparticle decoration. Impressively, CALB immobilized in this emulsion achieved a 96.5% conversion rate and retained 92.5% of its activity after 10 reaction cycles [40]. Similarly, Susanne Wiese et al. [41] employed microgels in emulsions to improve the interaction between oil and water phases, forming droplets encapsulating both enzyme- and substrate-containing oil. The microgels positioned at the droplet interface facilitated substrate conversion. Post-reaction, heating beyond the microgels' volume phase transition temperature induced emulsion breakdown, which allowed for product recovery via macroscopic phase separation.

1.2.3. Solid Adsorbents as the Solid Phase

Solid adsorbents like polymer beads and resins are preferred for in situ extractive fermentation due to their ability to efficiently adsorb and remove products from the aqueous phase, simplifying the process by eliminating extra separation

steps (**Figure 5**). These adsorbents, particularly effective for volatile compounds, offer a nontoxic alternative to organic solvents.

Figure 5. A conceptual flowchart using solid adsorbents as the solid phase in the liquid–solid TPF and downstream processing, with main steps including fermentation, in situ extraction, post-fermentation filtration, and product collection; the dark gray color represents the culture medium.

As early as 2009, Guillermo Quijano et al. [3] detailed the use of solid-phase adsorbents in TPF for environmental biotechnology, notably in wastewater treatment. Sonia Malik et al. [4] provided insights into the application of adsorbent resins in plant cell fermentation, with a dedicated chapter focusing on the selection of appropriate adsorbents and operating conditions. Furthermore, Thomas Phillips and colleagues [42] explored the use of adsorbent resins in the microbial fermentation of natural products, employing in situ solid-phase adsorption techniques. Their paper not only delved into the underlying mechanisms but also examined the influence of in situ adsorption on the biosynthesis of microbial natural products.

Jianxu Li and coworkers [43] evaluated an integrated in situ fermentation and in situ product recovery process aimed at enhancing the output of the antibiotic compound beauvericin (BEA) in *Fusarium redolens* Dzf2 mycelial cultivation. For this purpose, they employed macroporous polystyrene resin (X-5) as the adsorbent (encased in nylon bags), introducing it into flasks containing fungal mycelia. The findings indicated a significant increase in BEA volumetric production, from 194 mg/L to 265 mg/L by Day 7, with 65% of BEA adsorbed onto the resin. Renewing the resin and adding glucose on Day 7 further elevated BEA output to 400 mg/L by Day 9, effectively doubling the yield compared to the batch control culture. Haishan Qi et al. [44] introduced adsorbent resin HP20 during the fermentation of *Streptomyces hygroscopicus* var. *ascomyceticus* FS35. Following a metabolic profiling analysis and subsequent rational fermentation optimization, the production of ascomycin by *S. hygroscopicus* var. *ascomyceticus* FS35 significantly increased to 460 mg/L in a 168 h fermentation period. This represents a 53.3% enhancement compared to the yield under initial fermentation conditions. These case studies highlight the potential of solid adsorbent strategies, particularly adsorbent resins, in amplifying the production of significant natural products and refining processes.

2. The Advantages of TPF Systems

In microbial fermentation, increased yield is often hindered by the accumulation of fermentation products. Integrating fermentation with in situ extraction presents an effective strategy to mitigate this issue. This integrated approach accelerates product formation, boosts yield, and simplifies downstream processing. Among various two-phase systems, aqueous–organic and liquid–solid (resin) TPF technologies are particularly prominent and mature in microbial fermentation. Thus, subsequent chapters will extensively discuss the benefits of these TPF systems.

2.1. Enhance Productivity

Numerous studies have demonstrated that in situ product extraction, employing either a liquid (organic solvent) or solid (resin) phase, significantly enhances production. Microbial cells in the culture medium synthesize products, which are then extracted or adsorbed by the SP, disrupting the equilibrium and promoting product release. For instance, in β-elemene biosynthesis by *E. coli*, strategies like efflux protein enhancement and the use of n-dodecane as an organic phase in fermentation increased the β-elemene yield to 3.52 g/L [45].

2.1.1. Reducing Toxicity to Microbial Cells

Targeted products and harmful metabolites released during fermentation can inhibit microbial growth and production. Some monoterpenes and phenolic compounds can impair cell walls, membranes, and organelle membranes, diminish the

activity of specific enzymes within the cells, obstruct normal cellular functions, and ultimately result in microbial death. TPF technology, by enabling simultaneous production and separation, efficiently extracts or adsorbs both products and nontarget metabolites, enhancing microbial tolerance and productivity. For example, Wei Liu and colleagues [46] discovered that during batch-fed fermentation of an engineered *E. coli* strain producing geraniol, introducing isopropyl myristate to establish an aqueous–organic two-phase system significantly prevented the volatilization of the target product and diminished its cellular toxicity. This method resulted in a notable increase in product yield.

2.1.2. Alleviating Feedback Inhibition

Product accumulation may lead to feedback inhibition, impeding the activity of enzymes in the biosynthetic pathway. While most secondary metabolites produced by microbial cells are hydrophobic with low solubility in the culture medium, even minimal concentrations can inhibit enzymes involved in their biosynthesis. TPF is instrumental in facilitating in situ extraction or adsorption of products, effectively alleviating feedback inhibition in the biosynthetic pathway or affecting cell membrane transport. For example, in the study conducted by Jorge H. Santoyo-Garcia et al. [47], it was found that the resin could remove the products/reactive oxygen species' (ROS) effects in the production of paclitaxel by *Taxus baccata* vascular stem cells. This removal is crucial as it prevents the activation of secondary undesired pathways, inhibits cell growth, or diverts the metabolic flux towards side products.

2.1.3. Preventing Product Degradation and Loss

In the fermentation process, some enzymes in microbial cells or acidic substances in the fermentation system can degrade certain metabolites, particularly at high concentrations. The TPF systems can ensure that secondary metabolites are maximally protected from degradation by the microorganisms' own enzymes, effectively limiting the loss of products in cell culture. Taking salinosporamide A as an example, this natural molecule is produced by marine actinomycete *Salinispora tropica* and has a half-life of 140 min. Adding 2% (*w*/*v*) XAD-7 resin at 24 h of fermentation increased the yield from 5.7 mg/L to 278 mg/L, suggesting that the resin may protect the product from degradation [48]. The hydrophobic and volatile characteristics of some terpenes primarily contribute to product loss in microbial production processes. To mitigate the volatile losses of terpenes, a prevalent strategy is employing TPF with organic solvents. These solvents not only decrease the volatility of terpenes but also reduce their toxicity to cells, thereby enhancing productivity.

2.2. Industrial Application: Cost-Effective and Downstream Processing

The primary goal of microbial fermentation research is scaling to industrial production, often hindered by complex and costly downstream processes. TPF technology can address this challenge, reducing post-fermentation costs and facilitating scale-up.

2.2.1. Increase in Cell Biomass and Recycling of the Second Phase

In TPF systems, the SP extracts or adsorbs cellular products, fostering cell growth and increasing microbial cell biomass and yield compared to traditional approaches. On the other hand, the recycling of the SP is another key feature of the TPF system. In industrial production, effective separation and recycling techniques allow multiple uses of the SP, minimizing downstream processing costs.

2.2.2. Reduction of Post-Processing Steps

Utilizing TPF systems obviates the need for intricate product harvesting procedures, preserving cell integrity and not interfering with the culture process, thereby minimizing the costs and time. Traditional fermentation typically necessitates numerous steps such as organic solvent extraction, concentration, and distillation to isolate the product. TPF technology streamlines these processes. For instance, in liquid–liquid TPF, the product, extracted by the SP organic solvent during fermentation, eliminates the need for extraction. The fermentation broth is centrifuged, and the organic solvent is directly concentrated, followed by distillation to retrieve the product. In solid–liquid TPF, a concentration step is unnecessary; organic solvents are directly employed to elute and extract from adsorbents like macroporous resins, followed by distillation, reducing industrial post-processing steps.

3. Applications of TPF in Microbial Production of Terpenes

Terpenes represent a very important class of secondary metabolites in plants, with over 80,000 structural types identified to date [49]. These compounds, composed of isoprene units (C5 units), vary in the number of isoprene units they contain [50], leading to classifications such as monoterpenes (C10), sesquiterpenes (C15), diterpenes (C20), sesterterpenes (C25), triterpenes (C30), sesquarterpenes (C35), tetraterpenes (C40), and polyterpenes (C > 40). Currently, there are

several marketed plant-derived terpenoid drugs, such as paclitaxel, β-elemene, and artemisinin. Additionally, terpenes are highly favored in the fragrance and cosmetic industry, featuring components like menthol and ambergris [51].

The biosynthesis of terpenes in plants is complex but well understood [51][52][53][54]. As shown in **Figure 6**, isoprene isopentenyl diphosphate (IPP) and dimethyl allyl phosphate (DMAPP) are common precursors for all terpenes. One molecule of DMAPP and varying numbers of IPP condense under the influence of prenyltransferases to produce different terpene precursors. These precursors are then converted into various terpene skeletons under the action of various terpene synthases (TPs). The mevalonate (MVA) and 2-C-methyl-d-erythritol 4-phosphate (MEP) pathways are two distinct metabolic routes for the biosynthesis of terpenes in plants. The MVA pathway starts with acetyl-CoA and proceeds through six enzymatic steps to produce IPP and DMAPP, the basic building blocks for isoprenoid synthesis [55]. The MEP pathway uses pyruvate and glyceraldehyde 3-phosphate (G3P) as substrates and involves seven enzymatic reactions to produce IPP and DMAPP [56]. Given the well-characterized biosynthetic pathways of PDTs, the utilization of microbial engineering holds significant potential as an effective alternative for the production of desired terpenes.

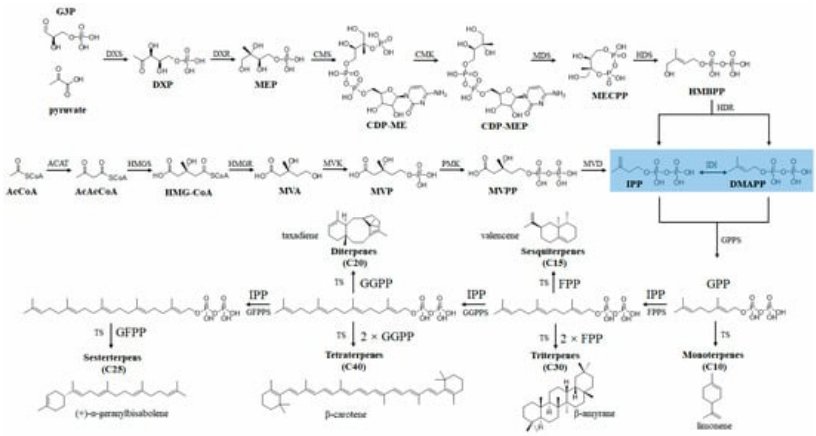

Figure 6. Metabolic pathway of terpene biosynthesis. The terpenes' skeletons are formed by the condensation of multiple units of IPP and its isomer, DMAPP. MEP biosynthetic pathway starts with pyruvate and G3P. Through a series of enzyme-catalyzed reactions, it ultimately produces IPP and DMAPP (shown on a light blue background). This process involves a variety of enzymes, DXS (1-deoxy-d-xylulose-5-phosphate synthase) and DXR (1-deoxy-d-xylulose-5-phosphate reductoisomerase), CMS (2-C-methyl-d-erythritol 4-phosphate cytidyltransferase), CMK (4-diphosphocytidyl-2-C-methyl-d-erythritol kinase), MDS (2-C-methyl-d-erythritol 2,4-cyclodiphosphate synthase), HDS (4-hydroxy-3-methylbut-2-enyl diphosphate synthase), and HDR (4-hydroxy-3-methylbut-2-enyl diphosphate reductase). The MVA biosynthetic pathway, which is another pathway for terpene biosynthesis, distinct from the MEP pathway, starts with acetyl-CoA. The primary enzymes involved in the MVA pathway are acetyl-CoA acetyltransferase (ACAT), hydroxymethylglutaryl-CoA synthase (HMGS), hydroxymethylglutaryl-CoA reductase (HMGR), mevalonate kinase (MVK), phosphomevalonate kinase (PMK), mevalonate-5-pyrophosphate decarboxylase (MVD), and isopentenyl-diphosphate delta-isomerase (IDI). Geranyl diphosphate synthase (GPPS), farnesyl diphosphate synthase (FPPS), geranylgeranyl diphosphate synthase (GGPPS), and farnesylgeranyl diphosphate synthase (GFPPS) TPs convert the basic terpene precursors, IPP and DMAPP, into various terpene compounds. Abbreviation of metabolites: DXP, 1-deoxy-d-xylulose-5-phosphate; MEP, 2-C-methyl-d-erythritol-4-phosphate; CDP-ME, 4-diphosphocytidyl-2-C-methyl-d-erythritol; CDP-MEP, 4-diphosphocytidyl-2-C-methyl-d-erythritol 2-phosphate; MEcPP, 2-C-methyl-d-erythritol-2,4-cyclodiphosphate; HMB-PP, 4-hydroxy-3-methylbut-2-enyl-diphosphate; AcAc-CoA, acetoacetyl-CoA; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; MVA, mevalonate; MVP, mevalonate-5-phosphate; MVPP, mevalonate-5-pyrophosphate; IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl pyrophosphate; GPP, geranyl pyrophosphate; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl diphosphate; GFPP, farnesylgeranyl diphosphate.

3.1. Microbial Production of Plant-Derived Terpenes

Microorganisms with a short growth cycle and minimal environmental impact are an ideal choice for PDT biosynthesis [57]. The microbial synthesis of terpenes, particularly with *E. coli* and *S. cerevisiae*, aligns with the goals of green and sustainable development due to their well-characterized metabolic pathways, genetic tractability, and suitability for large-scale fermentation [58]. These organisms offer the benefits of operational simplicity and cost-effectiveness, leveraging inexpensive substrates for efficient growth [59]. Further, the advancement in molecular and synthetic biology has led to the successful utilization of other microbial chassis such as *Yarrowia lipolytica* and *Rhodosporidium toruloides* in terpenoid biosynthesis. *Y. lipolytica*, recognized for its lipid production and "Generally Recognized as Safe" (GRAS) status [60], excels at utilizing renewable carbon sources and exhibits a high acetyl-CoA flux, making it a potent producer of acetyl-CoA-derived products [61]. *R. toruloides*, known for its broad substrate range and inhibitor tolerance, emerges as another

viable host for high-value compound production [62]. TPF technology, encompassing both aqueous–organic solvent and aqueous–solid TPF systems, is pivotal in the microbial production of terpenes, enhancing the efficiency and quality of products such as β-elemene [63] and artemisinic acid [64]. The modifications of terpenes' biosynthetic pathways in the microbial cell factory and the key enzymes engineering this process have been discussed by other comprehensive reviews.

3.1.1. Monoterpenes

Monoterpenes, the simplest terpenes comprising two isoprene units, are key to the aromatic profiles of many plants' essential oils [51]. Recent TPF applications have shown significant promise in enhancing monoterpene production. Taking geraniol as an example, it is not only an acyclic monoterpene isolated from plant essential oils that has been extensively utilized in the flavor industry for the past few decades but has also garnered considerable interest in recent years as a potential biofuel [46]. By overexpressing the synthase and heterologous MVA pathway in *E. coli* and subsequently employing fed-batch fermentation with isopropyl myristate as the organic phase, the production of geraniol was significantly enhanced, resulting in a yield of 2 g/L compared to the initial 78.8 mg/L obtained after basic fermentation in a bioreactor [46]. In another study, by adding the same SP in fed-batch fermentation, the production of geraniol was further increased to 13.19 g/L [65]. Different organic solvents, such as n-decane [66] and n-dodecane [67], had been employed as the SP in the TPF, which also increased the production of geraniol.

The demand for linalool, particularly as a flavoring agent, has been escalating, especially in the realm of processed foods and beverages. Achieving stable and cost-effective production of linalool is essential, as current extraction methods yield limited quantities and are not economically viable. To overcome the volatility of linalool in aqueous solutions and its high toxicity to microorganisms during fermentation production, an in situ extraction fermentation using isopropyl myristate as the SP was developed, resulting in 5.60 g/L (*S*)-linalool and 3.71 g/L (*R*)-linalool [68].

Limonene, the principal monoterpene in citrus fruit essential oils, is also found in oak, pine, and spearmint. Recently, it has garnered attention as a potential alternative or additive for jet fuel [69][70]. Although limonene is currently produced mainly as a by-product of orange juice manufacturing, the low concentration in natural sources makes its isolation economically unfeasible. Willrodt et al. [71] constructed an *E. coli* strain carrying a dual-plasmid system and performed a two-phase fed-batch operation in a bioreactor. The addition of an inert organic solvent prevented product inhibition, toxic effects, and limonene evaporation losses [72]. Diisononyl phthalate (DINP) has a good partition coefficient and has no detectable effect on *E. coli* growth [73]. When using DINP as the organic phase, the final limonene concentration reached 3630 mg/L [72]. The above examples showed that the appropriate use of biphasic fermentation technology and selection of suitable organic solvents as the SP can effectively enhance the monoterpenes' productivity.

In the TPF systems, beyond utilizing organic solvents like n-dodecane as the SP, the resin can also be employed as an adsorbent in this phase to enhance monoterpene production. For example, by inducing cells with IPTG and arabinose to increase P450 expression levels and using Amberlite resin to extract the product, the production of perillyl alcohol can be boosted to 105 mg/L [74].

3.1.2. Sesquiterpenes

Sesquiterpenes, with their 15-carbon backbone derived from three isoprene units, exhibit a remarkable diversity in chemical structures and shapes, making them a significant class of terpene known for various structures and functions [75]. These compounds, found in numerous plants, contribute to the unique scents and flavors of many essential oils and have notable biological activities, including the antimalaria drug artemisinin. Unfortunately, the yield of artemisinin extracted from plant extracts is low, increasing the cost of treatments, and is affected by weather and environmental factors. Keasling's team [76] focused on engineering microbes for the biosynthesis of artemisinin. By reconstructing the MVA pathway from *S. cerevisiae* into *E. coli* and regulating the relevant genes, they successfully produced amorphadiene in *E. coli*, which is the precursor of artemisinin. Using TPF technology, they innovatively used n-dodecane [64], isopropyl myristate [77], and methyl oleate [77] as the organic phase, providing new ideas and methods for the microbial metabolic synthesis of terpenoid compounds.

The isomers α-farnesene and β-farnesene play a crucial role in plant–insect interactions and possess significant economic value in pharmaceuticals, cosmetics, seasonings, and bioenergy [78]. Recent studies have successfully leveraged microbial metabolic engineering for the heterologous production of farnesene. *E. coli*, *S. cerevisiae*, and *Y. Lipolytica* have been successfully engineered for farnesene production [79][80][81][82][83]. Produced through batch fermentation with n-dodecane as the SP in a bioreactor, the production of α-farnesene reached 10.4 g/L [84]. An optimized S. cerevisiae strain in a 20,000 L bioreactor, with polymers and olefins as extractants, significantly increased α-farnesene

yield to 130 g/L [85]. You et al. [83] engineered an *E. coli* strain overexpressing β-farnesene with IDI and FPPs, minimizing IPP accumulation. Using n-decane as the organic phase, this strain achieved a final titer of 8.74 g/L. In *Y. lipolytica*, the fusion expression of farnesene synthase and FPPs enhanced α-farnesene synthesis, reduced intracellular accumulation of mevalonate, and yielded 25.55 g/L of α-farnesene in TPF with n-dodecane [86]. Although the yield of α-farnesene through flask fermentation, employing resin as an adsorbent and *Anabaena* sp. as the cell factory, is presently limited to 305.4 μg/L [87], this observation suggests the viability of resin as an SP within the TPF system for sesquiterpene production.

β-elemene is a sesquiterpene extracted from *Curcuma aromatica* Salisb. '*Wenyujin*' and is one of the most widely used antitumor drugs for the treatment of various cancer tumors in China [88]. The heterologous MVA pathway and cyanobacterial enzyme genes were concurrently introduced into *E. coli*, resulting in a β-elemene yield of 6325.5 μg/L in shaking bottles [89]. However, this yield is insufficient for industrial production. Recent studies have identified efficient synthases from algae and integrated key pathway enzymes, export genes, and translational engineering to implement TPF technology in bioreactors with n-dodecane as the SP, achieving a β-elemene yield of 3.52 g/L [45]. *Y. lipolytica*, serving as an exceptional cell factory, has been engineered to reconstruct the endogenous mevalonate pathway and regulate lipid metabolism, resulting in a β-elemene titer of 39 g/L in a bioreactor containing an isopropyl myristate organic phase [63].

3.1.3. Diterpenes

Diterpenes, with 20 carbon atoms from four isoprene units, exhibit vast structural diversity and a wide range of biological activities, making them crucial in pharmaceuticals, food additives, fragrance synthesis, and agriculture [90]. Paclitaxel (Taxol®), a compound found in the bark of the Pacific yew tree, stands out for its effectiveness against breast and ovarian cancers. The overexpression of enzymes in the MEP pathway and paclitaxel synthase in *E. coli* led to the successful creation of a strain capable of producing taxadiene, a key precursor of paclitaxel, with a yield of 1 g/L achieved through two-phase fed-batch fermentation using n-dodecane as the SP [91]. In the case of *S. cerevisiae*, both liquid–liquid TPF with n-dodecane and solid–liquid fermentation using silica gel as an adsorbent were effective in enhancing taxadiene yield, which gave the yield of 129 mg/L [92] and 8 mg/L [47], respectively. These findings underscore the efficacy of TPF technology in enhancing productivity.

Another example of fermentation paclitaxel has been discussed above, where the mutant strains of *A. fumigatus* and *A. tenuissima* were immobilized by five different entrapment materials and successfully applied for production enhancement of paclitaxel. The paclitaxel titers obtained by the immobilized mycelia of the respective mutants, 694.67 and 388.65 μg/L, were promising for fungal production of paclitaxel [32]. Thus, the immobilized cell technology has shown considerable potential for application in the industrial-scale production of paclitaxel through biotechnological processes.

In the production of miltiradiene using *S. cerevisiae* as the cell factory, the use of n-dodecane as the SP increased the yield by up to ten times, archiving at 3.5 g/L [93]. By strengthening upstream pathways, regulating central carbon metabolism and cofactor supply, fusing and truncating terpenoid synthase genes, knocking out related regulatory factors, and using TPF technology with n-hexane, the yield of sclareol in the bioreactor reached 11.4 g/L [94].

3.1.4. Triterpenes and Tetraterpenes

Triterpenes and tetraterpenes, composed of six and eight isoprene units, respectively, play diverse roles in nature and human applications [95][96][97]. Triterpenes are recognized for their biological activities, often utilized in traditional medicine for their anti-inflammatory, antiviral, and anticancer properties. Tetraterpenes are best known for their presence in colorful plant pigments, such as carotenoids [97]. Most economically valuable triterpenoids are water-soluble, featuring hydrophilic groups like carboxyl or sugar moieties, enabling their dissolution in the aqueous phase during microbial fermentation such as ginsenosides, thus bypassing the need for an extraction solvent phase. This eliminates the need for an extract SP. Conversely, lipophilic triterpenoids, such as squalene—used in cosmetics, dietary supplements, and as a vaccine adjuvant—require TPF for biosynthesis. Employing yeast with n-dodecane as the solvent phase has yielded significant squalene production (207.02 mg/L) [84]. Similarly, protopanaxadiol, the precursor to ginsenosides, has been biosynthesized using yeast as a cell factory, with methyl oleate or n-dodecane serving as in situ extraction solvents, resulting in a yield of 1189 mg/L [98]. In contrast, tetraterpenoids, particularly plant pigments, are fat-soluble substances due to their hydrophobic structures. β-Carotene, a naturally occurring red-orange pigment and one of the important tetraterpenoids, plays a crucial role in maintaining vision, skin health, and a properly functioning immune system owing to its conversion into vitamin A. In general, β-carotenoids produced by microorganisms are intracellularly stored, not

released outside the cell. For instance, *Y. lipolytica* has a large intracellular organelle for lipid storage, referred to as the lipid body.

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