Recent Advances in Fungal β-Carotene Production: Signalling, Metabolic Engineering, and Bioprocess Development

[1]Ajay Kumar Singh, [2]Akhilesh Kumar Pandey
[1,2] Mycological Research Laboratory, Department of Biological Sciences, Rani Durgawati University, Jabalpur 482001, Madhya Pradesh, India

***Abstract*—β-Carotene ($C_{40}H_{56}$) is one of the most commercially important carotenoids owing to its provitamin A activity, antioxidant properties, and extensive applications in food, feed, nutraceutical, pharmaceutical, and cosmetic industries. Growing consumer demand for naturally derived pigments has intensified interest in microbial fermentation as a sustainable alternative to petrochemical synthesis. Among microbial producers, the heterothallic zygomycete *Blakeslea trispora* remains the dominant industrial platform due to its exceptional β-carotene biosynthetic capacity, efficient submerged fermentation performance, and unique trisporic acid-mediated mating-type signalling system that strongly enhances carotenogenesis.**

This review critically examines recent advances in the biology, metabolism, and industrial exploitation of *B. trispora* for β-carotene production. The taxonomy, morphology, heterothallic life cycle, and trisporic acid signalling network are discussed in relation to carotenoid biosynthesis. Particular emphasis is placed on the complete mevalonate pathway from acetyl-CoA to β-carotene, highlighting key regulatory enzymes including HMG-CoA reductase (hmgR), isopentenyl pyrophosphate isomerase (ipi), geranylgeranyl pyrophosphate synthase (carG), phytoene synthase/lycopene cyclase (carRA), and phytoene desaturase (carB). Advances in fermentation technology, including fed-batch cultivation, oxygen management, lipid supplementation, and process optimisation strategies, are evaluated alongside downstream recovery and purification approaches. The review further analyses recent progress in strain improvement through classical mutagenesis, metabolic engineering, systems biology, and emerging genome-editing technologies. Emerging research directions involving CRISPR-based metabolic engineering, multi-omics-guided strain design, artificial intelligence-assisted bioprocess optimisation, agro-industrial waste valorisation, process analytical technologies, and nanoencapsulation-based formulation systems are also explored.

***Index Terms*—β-carotene; *Blakeslea trispora*; carotenoids; trisporic acid; mevalonate pathway; metabolic engineering; industrial fermentation; CRISPR; bioprocess optimisation; sustainable biotechnology**

I. INTRODUCTION

Carotenoids are a structurally diverse family of isoprenoid-derived natural pigments synthesised constitutively by plants, algae, fungi, and certain photosynthetic bacteria, with more than 750 structures characterised to date [1]. Their characteristic extended π-conjugated polyene chromophore confers both vivid colouration (red, orange, yellow) and potent antioxidant activity, properties that underpin widespread commercial applications across the food, feed, pharmaceutical, and cosmetic sectors [2, 3]. Within the carotenoid family, β-carotene occupies a uniquely important position as the most potent provitamin A compound and a major dietary antioxidant with established roles in human health [4].

Global demand for β-carotene has grown steadily. The world carotenoid market exceeded USD 1.8 billion in 2023, with β-carotene representing the single largest product segment. Projected compound annual growth of 4.5–6.2% through 2030 is driven by expanding nutraceutical, food fortification, and animal feed markets [5]. Historically, synthetic β-carotene produced via petrochemical routes by BASF (Lucarotin®) and DSM (CaroCare®) dominated global supply. However, increasing consumer preference for clean-label, naturally derived ingredients has created strong commercial incentives for bio-based production [6].

Microbial fermentation offers compelling advantages over chemical synthesis and plant extraction: independence from seasonal and geographical constraints, amenability to metabolic engineering, scalability in standard bioreactor infrastructure, and ability to yield the all-*trans* β-carotene isomer predominant in natural sources [7]. Among the microorganisms investigated—including the alga *Dunaliella salina*, the yeasts *Rhodotorula glutinis* and *Yarrowia lipolytica*, and the zygomycetes *Phycomyces blakesleeanus* and *Blakeslea trispora—B. trispora* stands apart for its naturally superior β-carotene accumulation (up to 30 mg g^{-1} dry cell weight in wild-type; >100 mg g^{-1} in engineered strains), robust growth in submerged liquid fermentation, and an extraordinary mating-type-dependent carotenoid amplification mechanism mediated by the apocarotenoid hormone trisporic acid [8, 9].

Despite decades of research, several dimensions of *B. trispora* biology, pathway regulation, and industrial process engineering remain incompletely exploited. The present review provides a comprehensive, critically evaluated synthesis spanning the organism's biology, the complete biosynthetic pathway, process engineering, metabolic engineering, product analysis, health applications, and market considerations, with the goal of identifying key knowledge gaps and guiding future research and industrial development.

II. CAROTENOIDS: CLASSIFICATION, STRUCTURE, AND BIOLOGICAL SIGNIFICANCE

2.1 Chemical Structure and Diversity

All carotenoids share a common C_{40} isoprenoid backbone assembled from eight isoprene (C_5) units. The central feature is a long polyene chain of alternating single and double bonds that constitutes the chromophore responsible for light absorption in the 400–550 nm range [10]. The length and degree of conjugation determine the wavelength of maximum absorbance and, consequently, the observed colour. Structural diversity arises from variations in cyclisation at the terminal ends (forming β-, ε-, γ-, or κ-rings or remaining acyclic), the degree of unsaturation, and post-biosynthetic oxygenation. This gives rise to over 750 characterised natural carotenoids, with additional geometric (*E/Z*) isomers substantially expanding functional diversity [11].

The *E* (*trans*) configuration predominates in freshly biosynthesised carotenoids due to the spatial constraints of the enzymes involved, but *Z* (*cis*) isomers accumulate upon thermal processing, UV irradiation, or acid treatment and may exhibit distinct bioavailability profiles [12]. For example, *cis*-lycopene from cooked tomato products demonstrates enhanced bioaccessibility in human subjects compared with all-*trans*-lycopene from fresh tomatoes.

2.2 Classification: Carotenes versus Xanthophylls

The primary chemical classification of carotenoids distinguishes carotenes (pure hydrocarbons, empirical formula $C_{40}H_x$) from xanthophylls (oxygenated derivatives bearing hydroxyl, keto, epoxide, or allenic functional groups) [13]. Representative carotenes include β-carotene (β,β-carotene), α-carotene (β,ε-carotene), lycopene (acyclic), γ-carotene, and the colourless precursors phytoene and phytofluene. Major xanthophylls include lutein, zeaxanthin, β-cryptoxanthin, astaxanthin, canthaxanthin, and fucoxanthin. A second nutritionally important classification divides carotenoids into provitamin A compounds—α-carotene, β-carotene, and β-cryptoxanthin—which can be converted to retinol in the body, and non-provitamin A carotenoids such as lutein, zeaxanthin, and lycopene [14].

2.3 Antioxidant Mechanisms

The primary antioxidant mechanism of carotenoids is physical quenching of singlet oxygen ($^1O_2$), wherein the excited oxygen species transfers its energy to the carotenoid triplet state (^{3}Car), which subsequently dissipates energy as heat through vibrational relaxation without chemical degradation [15]. Rate constants for $^1O_2$ quenching by β-carotene in organic solvents reach approximately 1.4×10^{10} $M^{-1}s^{-1}$, placing it among the most efficient known biological quenchers [16]. A secondary mechanism involves direct chemical reaction with peroxyl radicals through electron donation or hydrogen atom transfer, forming resonance-stabilised carotenoid radicals [17]. Both mechanisms are relevant to the protective roles of dietary carotenoids in reducing lipid peroxidation, oxidative DNA damage, and protein carbonylation.

2.4 Provitamin A Activity

Vitamin A (retinol) is essential for the visual cycle, regulation of gene expression via retinoic acid receptors, maintenance of mucosal epithelial integrity, and both innate and adaptive immunity [18]. The enzymatic conversion of β-carotene to retinol is catalysed primarily by β-carotene-15,15′-oxygenase 1 (BCO1) in enterocytes and hepatocytes, yielding two molecules of all-*trans*-retinal per molecule of β-carotene, subsequently reduced to retinol [19]. Vitamin A deficiency (VAD) affects approximately 190 million preschool-age children globally and is a leading preventable cause of childhood blindness and mortality in sub-Saharan Africa and South/Southeast Asia [20]. Dietary β-carotene biofortification through fermentation-derived natural sources represents a scalable strategy complementing conventional supplementation programmes.

2.5 Industrial Applications and Market Overview

The commercial applications of β-carotene span five principal sectors: (1) food and beverage colouring and fortification; (2) animal feed and aquaculture; (3) nutraceuticals and dietary supplements; (4) pharmaceuticals; and (5) cosmetics. The approved food additive designation is E160a in the EU and GRAS (Generally Recognized As Safe) under 21 CFR §73.95 in the USA [21]. Global β-carotene production involves an estimated 400 metric tonnes per year, with synthetic routes accounting for approximately 60–70% of supply [5]. Table 1 summarises the major industrial applications, regulatory designations, and estimated market contributions.

Table 1. Industrial applications of β-carotene across major commercial sectors. Market shares are approximate estimates based on published industry reports (2022–2023).

Sector	Primary Application	Regulatory Status	Key Notes
Food & Beverage	Colourant in margarine, cheese, juices, confectionery	E160a (EU); GRAS 21 CFR §73.95 (USA); INS 160a (Codex)	Largest volume application; quantum satis in most categories
Animal Feed	Egg yolk pigmentation; ruminant reproductive performance; salmonid colouration	EU Reg. 2017/1007; 21 CFR §73.90 (USA)	~30% of total β-carotene use; improves conception rates in dairy cattle
Nutraceuticals	Antioxidant supplements; multivitamin tablets; provitamin A products	GRAS / NHP (Canada); food supplement (EU Dir. 2002/46/EC)	Fastest-growing segment; 6–8% CAGR projected to 2030
Pharmaceuticals	Erythropoietic protoporphyria (Lumitene®); topical photoprotection	Prescription drug (USA, EU); OTC photoprotectant	Only approved indication: EPP; supportive evidence for photoprotection
Cosmetics	Self-tanning creams; anti-ageing serums; skin-tone preparations	EU Cosmetics Regulation 1223/2009; FDA cosmetic guidelines	Growing market for natural-origin actives; premiumisation trend

III. BIOLOGY OF BLAKESLEA TRISPORA

3.1 Taxonomy and Phylogenetic Placement

The genus *Blakeslea* was established by Roland Thaxter in 1914, named in honour of Albert Francis Blakeslee (1874–1954), the American botanist who first described heterothallism in Mucoralean fungi [22]. *Blakeslea trispora* (Thaxter) is the type and sole well-characterised species, placed in the order Mucorales, family Choanephoraceae, within the subphylum Mucoromycotina (formerly classified under the now-paraphyletic Zygomycota) [23]. Molecular phylogenetic analyses using 18S rRNA and ITS sequences confirm its close relationship with *Choanephora cucurbitarum* within Choanephoraceae, distinguishable by the characteristic triseptate sporangiophores—the structural feature responsible for the species epithet *trispora*—and differences in sporangiole wall ornamentation [24]. The fully sequenced and annotated genome of *B. trispora* (approximately 36 Mb, ~8,000 predicted genes) provides a foundation for systems-level metabolic analysis [25].

3.2 Colony and Hyphal Morphology

On potato dextrose agar (PDA) at 26 °C, *B. trispora* colonies grow rapidly, expanding at approximately 0.5–1.0 cm h^{-1} to fill a standard 90-mm Petri dish within 48–72 h. Aerial mycelium is initially white and cottony; upon carotenoid induction—especially under mixed-mating-type co-cultivation—the colony transitions progressively through yellow-orange to deep red-orange, with eventual browning of aged mycelium from carotenoid oxidation and melanisation [26]. Vegetative hyphae are coenocytic (aseptate), highly branched, and 5–20 μm in diameter. Numerous lipid bodies (oleosomes) are visible under phase-contrast microscopy; these hydrophobic droplets serve as primary carotenoid storage reservoirs [27]. Asexual reproduction proceeds through the formation of triseptate sporangiophores bearing single-spored sporangioles; sporangiospores are smooth, elongate-ellipsoidal (4–8 × 2–3 μm), and hyaline when freshly released.

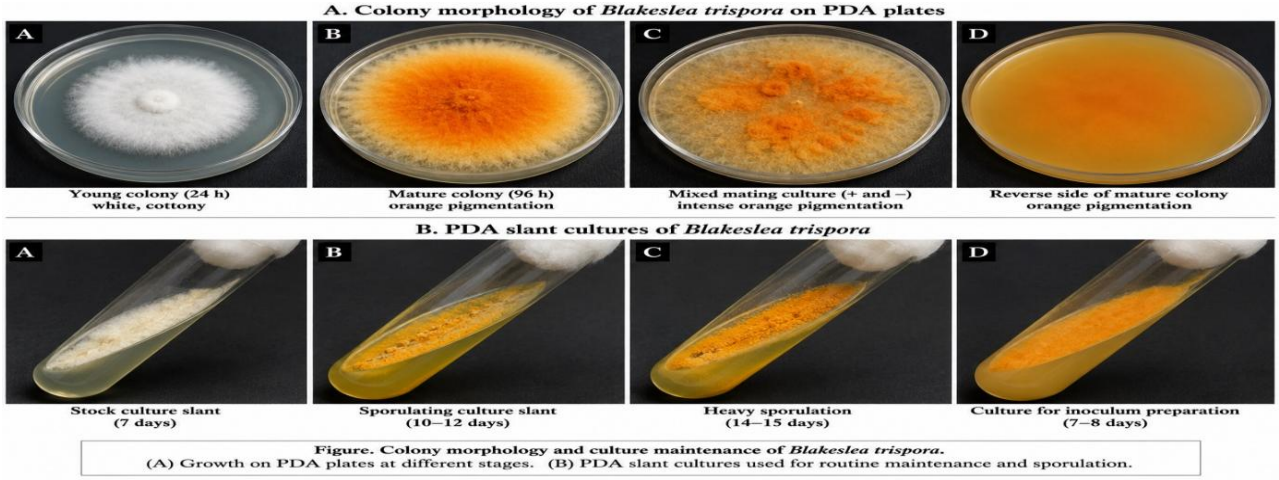

Fig. 1. Morphology of *Blakeslea trispora*. Colony and hyphal morphology showing characteristic orange-red pigmentation on PDA medium under mixed-mating-type cultivation. (Source- Google image)

3.3 Heterothallic Mating System

B. trispora is obligately heterothallic, possessing two morphologically identical but sexually incompatible mating types designated (+) and (−). Physical proximity between opposite mating types triggers directional hyphal growth (zygotropism) mediated by diffusible chemical signals, ultimately resulting in gametangial fusion and the formation of multinucleate zygospores [28]. Zygospores are spherical to slightly flattened (40–80 μm), dark-walled, and lipid-rich (triglycerides and phosphatidylcholine constituting up to 60% of dry zygospore mass). They are metabolically quiescent, with dormancy maintained by cytoplasmic regulatory systems requiring specific environmental cues for germination [29]. The co-cultivation of (+) and (−) strains in industrial fermentations triggers the trisporic acid signalling cascade that amplifies carotenogenic gene expression, forming the cornerstone of the industrial production strategy.

3.4 Trisporic Acid: Molecular Mediator of Carotenogenesis

Trisporic acids (TSAs) are C_{18} apocarotenoid hormones derived from β-carotene via oxidative cleavage catalysed by carotenoid cleavage oxygenases (CCOs) [30]. Three principal forms (TSA-A, -B, -C) have been characterised, with TSA-B as the predominant active species. Biosynthesis of TSA is a collaborative inter-mycelial process: the (+) mating type converts 4-dihydrotrisporin to trisporin, while the (−) mating type oxidises trisporin to trisporic acid; neither mating type alone completes the full pathway [31]. TSAs act as transcriptional activators, inducing *carRA* (encoding phytoene synthase/lycopene cyclase) and *carB* (encoding phytoene desaturase) expression 5–20-fold above basal levels, creating a positive feedback loop: more β-carotene → more TSA precursors → more TSA → more carotenogenic enzyme expression → more β-carotene [32]. This mechanism explains why mixed (+)/(−) fermentations consistently outperform single-mating-type cultures by 5–15-fold.

IV. BIOSYNTHETIC PATHWAY FROM ACETYL-COA TO B-CAROTENE

4.1 Overview

B. trispora synthesises β-carotene exclusively through the cytosolic mevalonate (MVA) pathway for isoprenoid precursor supply, followed by the universal fungal carotenoid pathway from phytoene to the final bicyclic product [33]. Unlike plants, which compartmentalise carotenoid biosynthesis between the cytosol (MVA, sterols) and chloroplasts (MEP pathway, carotenoids), all steps in *B. trispora* occur in the cytoplasm. This co-localisation with the competing ergosterol biosynthetic pathway means that flux partitioning at the geranylgeranyl pyrophosphate (GGPP) branch point is a critical determinant of carotenoid titre. Figure 2 presents the complete integrated pathway.

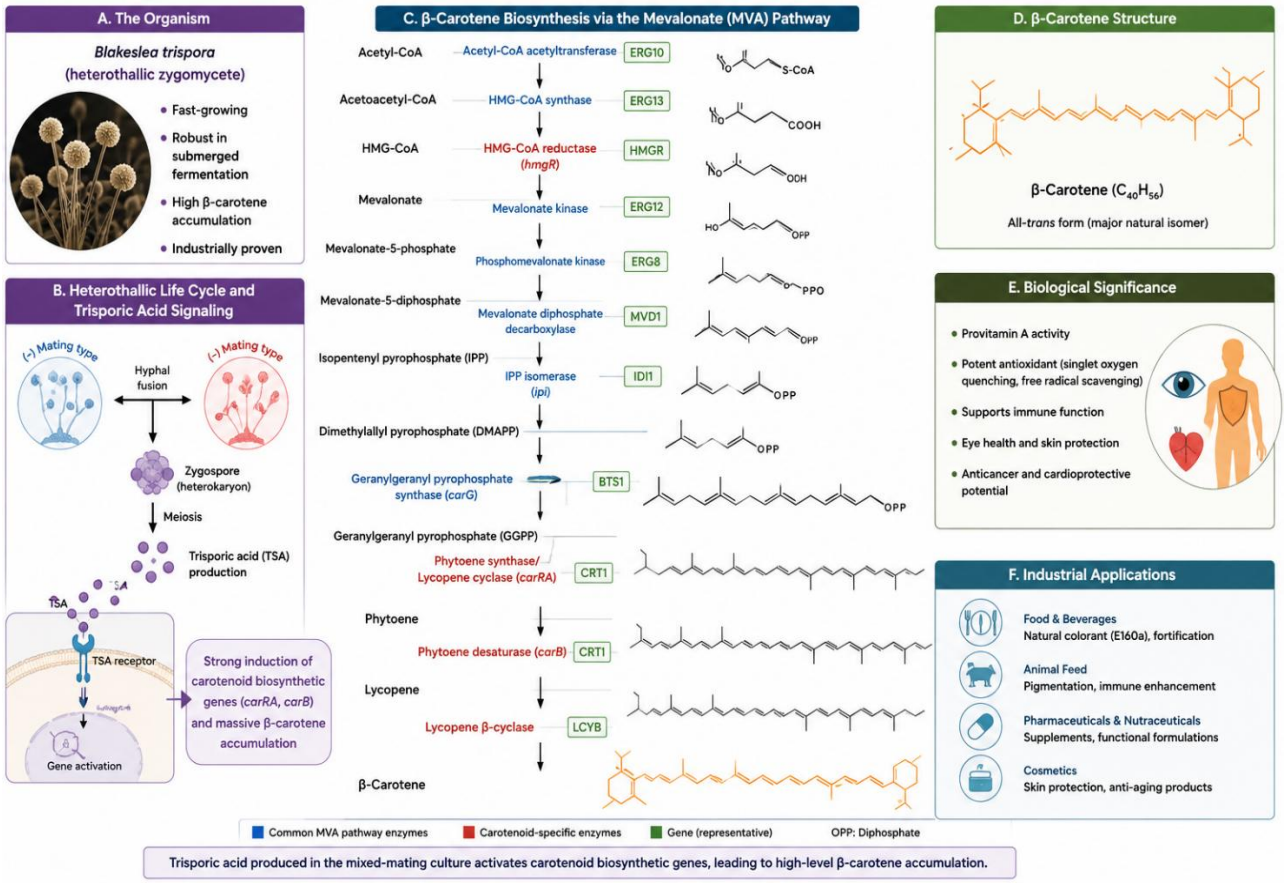

Fig. 2. Complete mevalonate and carotenoid biosynthetic pathway in Blakeslea trispora, showing key enzymatic steps from acetyl-CoA to β-carotene and competing ergosterol branch.

4.2 Mevalonate Pathway: Acetyl-CoA to IPP and DMAPP

Steps 1–2: HMG-CoA formation. Acetyl-CoA acetyltransferase (ACAT) condenses two acetyl-CoA molecules to form acetoacetyl-CoA; HMG-CoA synthase (HMGS) subsequently condenses acetoacetyl-CoA with a third acetyl-CoA to yield (*S*)-3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) [34].

Step 3: Mevalonate formation (rate-limiting). HMG-CoA reductase (HMGR, encoded by *hmgR*) catalyses the two-step NADPH-dependent reduction of HMG-CoA to (*R*)-mevalonate. This is the committed, rate-limiting step of the MVA pathway, subject to end-product feedback inhibition by GGPP and sterols, and strongly induced by trisporic acid during mating [35].

Steps 4–6: Phosphorylation to IPP. Mevalonate kinase (MK), phosphomevalonate kinase (PMK), and mevalonate diphosphate decarboxylase (MVD) sequentially phosphorylate and decarboxylate mevalonate to isopentenyl pyrophosphate (IPP, C_5), consuming three ATP molecules in total [36].

Step 7: Isomerisation. IPP isomerase (IPI, encoded by *ipi*) interconverts IPP and dimethylallyl pyrophosphate (DMAPP), maintaining the ~5:1 IPP:DMAPP ratio required for chain elongation [37].

4.3 Chain Elongation to GGPP

GGPP synthase (GGPPS, encoded by *carG*) catalyses three successive head-to-tail condensations of IPP with the primer DMAPP to yield geranyl pyrophosphate (GPP, C_{10}), farnesyl pyrophosphate (FPP, C_{15}), and finally geranylgeranyl pyrophosphate (GGPP, C_{20}) [38]. GGPP is the critical branch point: squalene synthase (ERG9) condenses two FPP molecules for ergosterol biosynthesis, whereas phytoene synthase channels two GGPP molecules into the carotenoid pathway. The relative activities of these competing enzymes govern isoprenoid flux partitioning [39].

4.4 Carotenoid-Specific Steps: Phytoene to β-Carotene

Phytoene synthesis. Phytoene synthase (PSY), encoded by the 5′ (N-terminal) domain of the bifunctional *carRA* gene, catalyses head-to-head condensation of two GGPP molecules via the intermediate presqualene diphosphate to form 15-*cis*-phytoene (C_{40}), the first committed carotenoid (colourless; three conjugated double bonds) [40].

Phytoene desaturation. In *B. trispora*, a single multifunctional phytoene desaturase (CarB, encoded by *carB*) introduces four successive double bonds into phytoene, extending the conjugation system stepwise through phytofluene (5 double bonds, colourless), ζ-carotene (7, pale yellow), neurosporene (9, yellow), and finally lycopene (11, deep red). CarB requires FAD as cofactor and molecular oxygen as the terminal electron acceptor, explaining the dependence of β-carotene titre on dissolved oxygen during fermentation [41].

Lycopene cyclisation. Lycopene cyclase (CYC), encoded by the 3′ (C-terminal) domain of the same bifunctional *carRA* gene, introduces β-ionone rings at both ends of lycopene through a protonation-cyclisation mechanism, proceeding via the monocyclic intermediate γ-carotene to the bicyclic product β-carotene [42]. The efficiency of this terminal cyclisation determines whether lycopene (CYC-limiting conditions) or β-carotene (CYC-excess conditions) predominates in the culture, a balance amenable to genetic and nutritional manipulation.

4.5 Regulatory Control of the Pathway

Carotenogenesis in *B. trispora* is regulated at transcriptional, post-translational, and metabolic flux levels. At the transcriptional level, *carRA* and *carB* expression is induced 5–20-fold by TSA and is repressed by high glucose concentrations (>80 g L^{-1}) through carbon catabolite repression [43]. Dissolved oxygen below 20% air saturation reduces CarB desaturation activity, causing accumulation of desaturation intermediates at the expense of lycopene and β-carotene [44]. Small-molecule modulators relevant to industrial processes include vegetable oils, which increase acetyl-CoA supply and provide hydrophobic carotenoid storage compartments, and ergosterol synthesis

inhibitors such as ketoconazole (CYP51 inhibitor), which redirects isoprenoid flux from sterols to carotenoids by reducing competition at the GGPP branch point [45].

V. INDUSTRIAL FERMENTATION PROCESS ENGINEERING

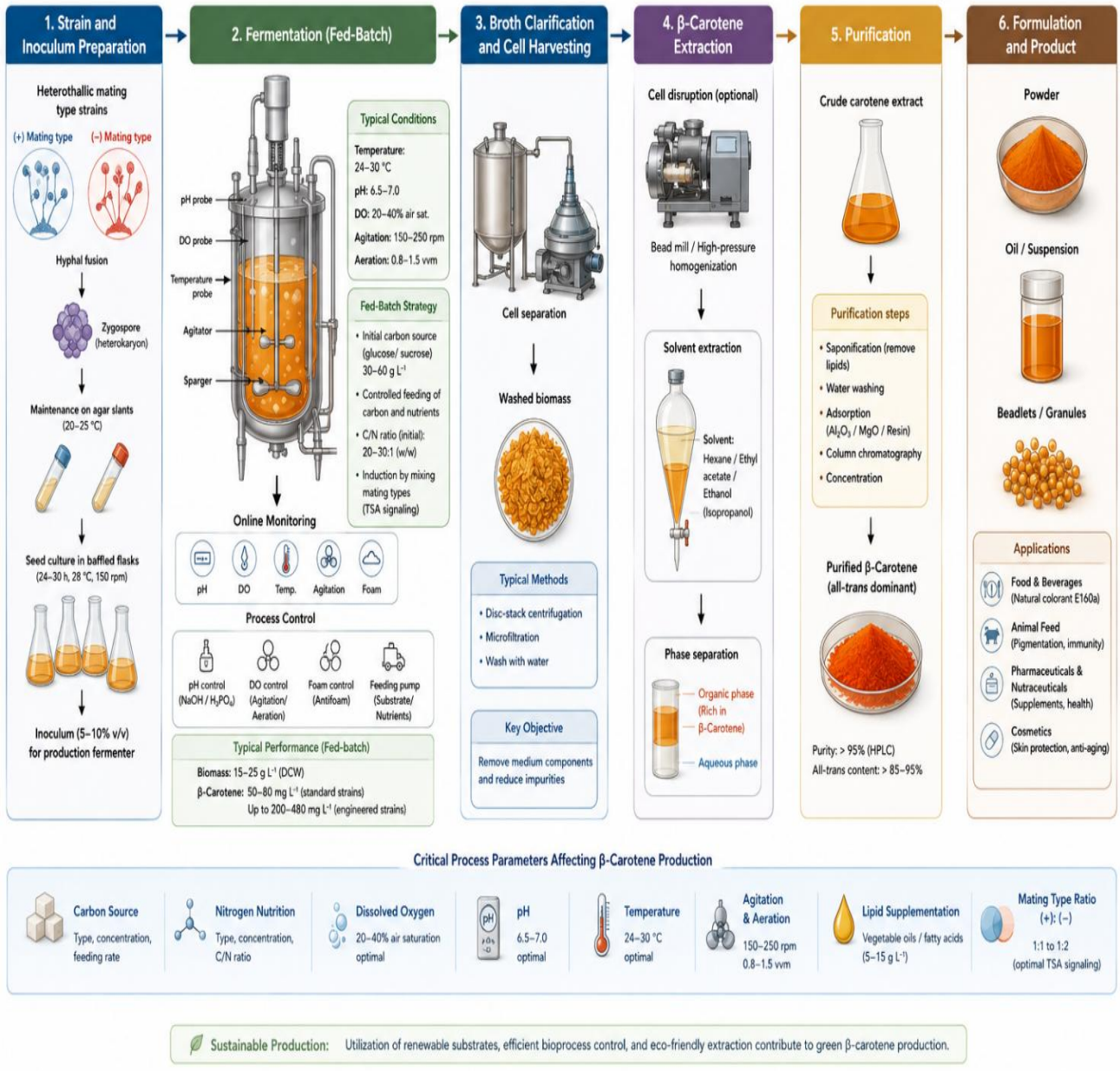

Fig. 3. Industrial bioprocess flow diagram for β-carotene production from Blakeslea trispora. The five-stage process (strain maintenance → medium preparation → fed-batch fermentation → downstream extraction → final product) is shown with key operational parameters at each stage. STRAIN A (+) and STRAIN B (−) are mixed at a 1:1 ratio at inoculation to maximise trisporic acid signalling.

5.1 Strain Selection, Maintenance, and Inoculum Preparation

Industrial production relies on two reference strains: STRAIN A (mating type +) and STRAIN B (mating type −), maintained on PDA slants at 4 °C with subculturing every 4–6 weeks, or cryogenically preserved at −80 °C in 15–20% (v/v) glycerol for long-term storage [46]. Inoculum preparation involves sporulation on fresh PDA at 26 °C for 72 h under diffuse illumination. Sporangiospore suspensions are prepared in sterile 0.1% (v/v) Tween-80, standardised to 10^6–10^7 spores mL^{-1} by haemocytometer, and viability confirmed by methylene blue exclusion (>95% required) [47]. Equal volumes of (+) and (−) suspensions are combined at a 1:1 ratio immediately before reactor inoculation. Ratios outside the range 1:3 to 3:1 significantly reduce trisporic acid accumulation and carotenoid yield [48].

5.2 Culture Medium Composition

Beet molasses is the preferred industrial carbon source due to its low cost, high fermentable sugar content (45–52% w/w), and presence of growth-promoting trace minerals, betaine, and B-vitamins [49]. Because *B. trispora* lacks efficient constitutive sucrose-cleaving activity, molasses media require prior invertase (β-fructofuranosidase) hydrolysis: a 5% (w/v) molasses solution is adjusted to pH 4.5 with HCl, treated with commercial invertase (0.5% w/v) at 55 °C for 2 h, then brought to pH 7.0 with 10 N NaOH and sterilised at 120 °C for 20 min [50]. Initial glucose concentrations of 30–60 g L^{-1} are optimal; concentrations above 80 g L^{-1} invoke catabolite repression of carotenogenic gene expression [51]. Nitrogen sources include ammonium sulphate (2–4 g L^{-1}), corn steep liquor (3–8 g L^{-1}), and yeast extract (1–3 g L^{-1}); a C:N ratio of 20–30:1 (w/w) optimally balances biomass formation and secondary metabolite accumulation [52]. Supplementation with vegetable oils (soybean, corn, or sunflower; 5–15 g L^{-1}) during the stationary growth phase significantly elevates β-carotene titres by providing acetyl-CoA precursors and hydrophobic storage compartments for the pigment [53].

5.3 Bioreactor Configuration and Operating Conditions

Submerged fed-batch fermentation in stirred-tank bioreactors (STRs) equipped with Rushton turbine impellers is the industrial standard. At 10-L pilot scale, 2 L of inoculated medium is fermented for 48 h at 26 °C (exponential phase completion), followed by continuous medium feeding at approximately 27.8 mL h^{-1} until the 6-L working volume is reached. Total fermentation time is approximately 8 days, yielding β-carotene titres of 50–80 mg L^{-1} [54]. Key operating parameters and their physiological rationale are summarised in Table 2. Dissolved oxygen (DO) is maintained above 20–30% air saturation; below 10% DO, CarB desaturation activity is impaired and intermediate carotene accumulation occurs at the expense of final product [55]. Temperature above 30 °C promotes thermal isomerisation of β-carotene from all-*trans* to *cis* forms, reducing both colour intensity and biological activity [56]. Fed-batch cultivation achieves 2–3-fold higher titres than equivalent batch processes by mitigating catabolite repression and extending the productive carotenogenic phase [57].

Table 2. Key fermentation parameters for β-carotene production by Blakeslea trispora in fed-batch stirred-tank bioreactors.

Parameter	Optimal Range	Effect of Deviation	Reference
Temperature	24–28 °C	<20 °C: severely reduced growth; >30 °C: β-carotene isomerisation and degradation	[54, 56]
pH	6.5–7.0	<5.5: inhibited growth; >8.0: lycopene cyclase impaired; lycopene accumulates	[58]
Dissolved oxygen	20–40% air sat.	<10%: intermediate accumulation; >60%: oxidative β-carotene degradation	[55]
Agitation speed	150–250 rpm	<150 rpm: O_2 limitation; >300 rpm: mycelium fragmentation and titre loss	[54]
Aeration rate	0.8–1.5 vvm	<0.8 vvm: O_2 limitation; >2.0 vvm: excessive foaming and high energy cost	[55]
Initial carbon	30–60 g L^{-1}	<20 g L^{-1}: insufficient biomass; >80 g L^{-1}: catabolite repression of carRA and carB	[51]
Vegetable oil	5–15 g L^{-1} (stationary phase)	<5 g L^{-1}: minimal effect; >20 g L^{-1}: mass-transfer limitation and foaming	[53]
Mating type ratio (+:−)	1:1 to 1:2	Ratios >1:4 or <4:1: TSA signalling impaired; titre drops 30–50%	[48]
Fed-batch duration	7–10 days	<6 days: incomplete carotenogenesis; >12 days: product degradation risk	[57]
C:N ratio (initial)	20–30:1 (w/w)	<15:1: biomass growth favoured; >40:1: N limitation slows growth and carotenogenesis	[52]

DO = dissolved oxygen; vvm = volume of gas per volume of liquid per minute.

5.4 Scale-Up Considerations

Scale-up from laboratory (2–10 L) to industrial scale (1,000–50,000 L) introduces significant engineering challenges. The primary criterion is maintenance of the volumetric oxygen mass transfer coefficient (kLa) above approximately 100 h^{-1} to prevent DO limitation [59]. At large scale, higher agitation tip speeds required to achieve adequate kLa can fragment mycelium and reduce viability; solutions include multiple impeller configurations (dual Rushton or combined Rushton/pitched-blade), supplementary bottom aeration through ring spargers, and superficial gas velocity optimisation. Heat dissipation is also limiting; specific heat generation of 0.5–2.0 kW m^{-3} requires adequate jacket cooling capacity, often necessitating chilled water (≥4 °C) at scales above 1,000 L during peak growth [60].

5.5 Downstream Processing

Post-fermentation, β-carotene recovery comprises four operations: biomass recovery, pigment extraction, purification, and formulation. Biomass is separated by centrifugation (5,000–10,000 × g, 15–20 min) or vacuum filtration, washed with distilled water, and dried at 105 °C to constant weight (<5% moisture) [61]. Intracellular β-carotene is extracted from dried biomass with hexane (3 × 5–10 vol g^{-1} biomass, 40–50 °C, N_2 atmosphere), recovering >90% of total pigment [62].

Crude extracts are concentrated by rotary evaporation and purified by silica gel open-column chromatography or, for pharmaceutical grade, by preparative reverse-phase HPLC. Crystallisation from *n*-propanol at 60 °C followed by cooling to 4 °C and vacuum filtration yields crystalline β-carotene of >98% purity (all-*trans* isomer, mp 178–179 °C) [63]. For food-grade applications, crystals are co-processed with gelatin, sucrose, and modified starch to produce water-dispersible beadlets or dissolved in vegetable oil (1–30% w/v) for direct food addition.

VI. METABOLIC ENGINEERING AND STRAIN IMPROVEMENT

6.1 Classical Mutagenesis

Random mutagenesis using UV irradiation, N-methyl-N′-nitro-N-nitrosoguanidine (NTG), or ethyl methanesulfonate (EMS) has historically been the primary strain improvement strategy, yielding mutants accumulating 2–5-fold more β-carotene than wild-type parents [64]. Multi-round mutagenesis–selection programmes and intersexual heterokaryons formed by protoplast fusion of (+) and (−) strains carrying beneficial mutations from separate lineages have produced strains achieving titres exceeding 200 mg L^{-1} [65]. The principal limitation is accumulation of deleterious background mutations and an unpredictable mutational landscape; metabolic engineering now offers rational, targeted alternatives.

6.2 Targeted Gene Overexpression

Agrobacterium tumefaciens-mediated transformation (ATMT) and electroporation-based protoplast transformation have been established for *B. trispora*, enabling precise genomic interventions [66]. Overexpression of *hmgR* under a strong constitutive promoter increases flux to mevalonate, raising β-carotene content 30–40% [67]. Co-overexpression of *ipi* and *carG* improves IPP/DMAPP balance and GGPP supply, alleviating the bottleneck between the MVA pathway and carotenoid chain elongation [68]. Multi-gene co-overexpression strategies combining upstream MVA genes (*hmgR*, *ipi*) with downstream carotenoid genes (*carG*, *carRA*, *carB*) achieved synergistic titre improvements of 60–80% above single-gene overexpression [69]. Introduction of a truncated, deregulated *hmgR* variant lacking the transmembrane sterol-sensing domain further boosted mevalonate production by relieving end-product inhibition [70].

6.3 Suppression of Competing Pathways

The principal competing pathway is ergosterol biosynthesis consuming GGPP/FPP via squalene synthase (ERG9). RNAi-mediated silencing of *erg9* reduced ergosterol content 40–60% and increased β-carotene titre 35–50%, confirming the significance of this competition [71]. Chemical partial inhibition with ketoconazole (CYP51 inhibitor, 0.5–2.0 μg mL^{-1}) achieves equivalent flux redirection without growth arrest, though not approved for food-grade production [45]. A complementary strategy is the overexpression of *carS*, a positive transcriptional regulator of carotenogenic genes, which partially decouples carotenogenesis from TSA-dependent sexual signalling, offering a path toward single-strain high-titre production [72].

6.4 Systems Biology and Genome-Scale Modelling

Genome-scale metabolic models (GEMs) constructed from the sequenced *B. trispora* genome enable flux balance analysis (FBA) to identify non-intuitive engineering targets. FBA predicted that simultaneous overexpression of the pentose phosphate pathway (to increase NADPH supply for HMGR) and attenuation of acetate overflow metabolism could theoretically increase β-carotene yield by 60–80% above wild-type [73]. Transcriptomic comparisons between high- and low-producing fermentations have revealed additional regulatory genes not previously implicated in carotenogenesis, constituting new engineering targets [74].

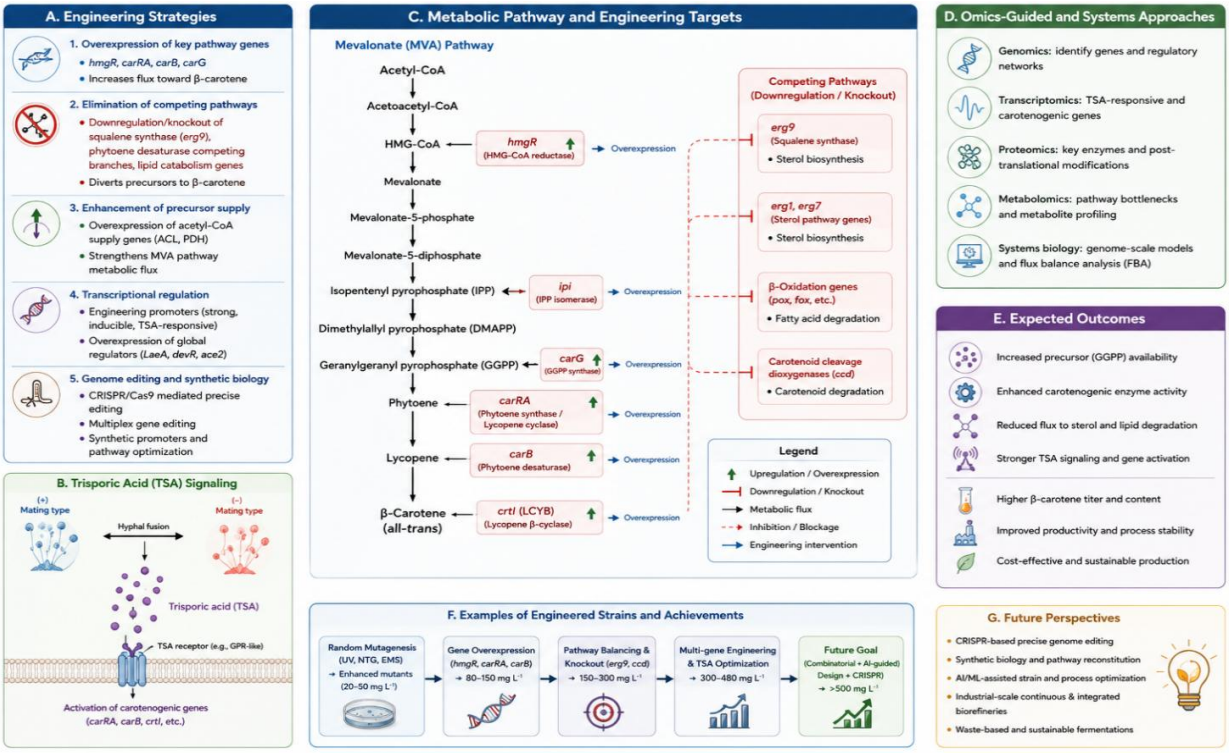

Fig. 4. Metabolic engineering targets for β-carotene overproduction in Blakeslea trispora, highlighting key intervention points in the mevalonate pathway, competing sterol branch, and carotenoid biosynthetic route.

Table 3. Comparative overview of β-carotene production titres and strategies across microbial platforms.

Organism	β-Carotene (mg L^{-1})	β-Carotene (mg g^{-1} DCW)	Cultivation Mode	Key Feature / Reference
Blakeslea trispora (wt)	50–80	10–30	Fed-batch	TSA-driven mating type induction; industrial standard [54, 57]
B. trispora (mutant/engineered)	200–480	~100	Fed-batch	NTG mutagenesis + ketoconazole; best published titre [64, 65]
Dunaliella salina (alga)	14–50	80–120	Open pond	High DCW content; seasonal variability; no fermentation infrastructure [75]

Phycomyces blakesleeanus	1–3	2–5	Batch	Model organism; limited industrial titre [8]
Rhodotorula glutinis	1.8–4.0	1–3	Batch	Yeast; easy genetic tools; low titre [76]
Saccharomyces cerevisiae (eng.)	20.8	—	Batch	Heterologous pathway; academic research platform [77]
Yarrowia lipolytica (eng.)	6.5	—	Fed-batch	Oleaginous yeast; lipid bodies aid carotenoid storage [78]
Escherichia coli (eng.)	0.5–5	—	Batch	Well-characterised host; proof-of-concept titres only [79]

DCW = dry cell weight; Fed-batch = fed-batch stirred-tank; wt = wild type

VII. ANALYTICAL CHARACTERISATION OF B-CAROTENE

7.1 UV-Visible Spectrophotometry

β-Carotene in hexane exhibits characteristic light absorption at 450–455 nm (εmax ≈ 139,900 L mol^{-1} cm^{-1}) with secondary peaks at approximately 425 nm and 480 nm, arising from the all-*trans* polyene chromophore [80]. Beer-Lambert quantification is applied to hexane extracts for rapid titre monitoring during fermentation, though the method cannot resolve β-carotene from co-extracted carotenoids without prior separation. For crude biomass extracts, spectrophotometric readings at A_{450} corrected against a blank hexane extract provide reliable working estimates for process control [81].

7.2 HPLC Analysis

Reversed-phase HPLC on C18 or C30 stationary phases with methanol: methyl tert-butyl ether (MTBE) or acetonitrile: dichloromethane mobile phases is the gold standard for β-carotene quantification in research and regulatory contexts [82]. C30 columns offer superior resolution of *cis/trans* isomers and structural analogues compared with C18. Photodiode array (DAD) detection at 450 nm with UV spectral confirmation (three-peak fingerprint characteristic of all-*trans*-β-carotene) provides definitive peak identification. Quantification against certified reference material (e.g., NIST SRM 3350) ensures metrological traceability [83].

7.3 Mass Spectrometry and NMR

LC-APCI-MS (atmospheric pressure chemical ionisation) provides definitive molecular identification: β-carotene produces $[M+H]^+$ at *m/z* 537.4 and characteristic retro-Diels–Alder fragment ions at *m/z* 444.4, 389.3, and 119.1 confirming the β-ionone ring [84]. High-resolution mass spectrometry (HRMS) on Orbitrap or QTOF instruments enables isomer fingerprinting and ^{13}C metabolic flux analysis. ^{1}H NMR ($CDCl_3$) confirms the all-*E* configuration through olefinic proton chemical shifts (δ 5.9–6.7 ppm) and vicinal coupling constants (*J*HH ~15 Hz for *E* double bonds) [85]. Table 4 provides a comparative summary of analytical methods.

Table 4. Analytical methods for β-carotene characterisation: principles, performance, and applications.

Method	Detection Principle	LOD / LOQ	Primary Application	Key Advantage / Limitation
UV-Vis spectrophotometry	Absorbance at 450 nm	≈0.1 / 0.5 mg L^{-1}	Routine in-process monitoring; total carotenoid estimation	Fast, low-cost; cannot distinguish co-eluting carotenoids
HPLC-DAD (C18/C30)	Chromatographic separation + DAD	≈0.05 / 0.2 mg L^{-1}	Quantification; isomer profiling; regulatory purity testing	Gold standard; C30 resolves cis/trans; calibration required
LC-APCI-MS/MS	Mass fragmentation (m/z 537.4)	~0.01 mg L^{-1}	Structural confirmation; impurity ID; metabolite profiling	Unambiguous molecular ID; high cost; requires expertise
HRMS (Orbitrap/QTOF)	High-resolution accurate mass	~0.001 mg L^{-1}	Isomer fingerprinting; ^{13}C metabolic flux analysis	Highest sensitivity and specificity; not routine-lab accessible
$^{1}H/^{13}C$ NMR spectroscopy	Magnetic resonance ($CDCl_3$)	~1 mg (bulk)	Complete structural elucidation; E/Z geometry assignment	Definitive structure; bulk sample required; relatively slow
Raman / FTIR spectroscopy	Molecular vibrational fingerprint	~0.5 mg (bulk)	Polymorphic form ID; in-line PAT; non-destructive	Excellent for solid-state form ID and in-line monitoring

LOD = limit of detection; LOQ = limit of quantification; DAD = diode array detector; APCI = atmospheric pressure chemical ionisation; HRMS = high-resolution mass spectrometry.

VIII. HEALTH-PROMOTING PROPERTIES: EVIDENCE AND MECHANISMS

8.1 Provitamin A and Vitamin A Deficiency Alleviation

The most firmly established biological role of β-carotene is as an efficacious provitamin A source. Vitamin A deficiency (VAD) affects approximately 190 million preschool children worldwide and is a primary preventable cause of blindness and childhood mortality from infectious diseases [20]. Supplementation studies in high-VAD settings demonstrate 20–34% reductions in child mortality, and fermentation-derived β-carotene provides a scalable natural alternative to synthetic retinol supplements for food fortification in resource-limited settings [86].

8.2 Antioxidant Activity: In Vitro and In Vivo Evidence

β-Carotene is among the most efficient biological $^{1}O_2$ quenchers, with rate constants of 1.4×10^{10} $M^{-1}s^{-1}$ in non-polar environments [16]. Cell culture and animal studies demonstrate reduction of malondialdehyde, 8-hydroxy-2′-deoxyguanosine, and protein carbonyl content following β-carotene supplementation [87]. However, translational evidence from large clinical trials is complex: the ATBC study [89] and CARET trial [88] found no protective—and in some cases adverse—effects of high-dose β-carotene supplements (25–50 mg day^{-1}) in heavy smokers exposed to high oxidative stress, attributed to dose-dependent pro-oxidant behaviour at pharmacological concentrations. These findings underscore that β-carotene is most appropriately

provided at physiological doses through food-matrix-integrated sources rather than isolated high-dose supplements.

8.3 Photoprotection and Skin Health

β-Carotene is the active ingredient in Lumitene® (75–300 mg day^{-1}) for erythropoietic protoporphyria (EPP), reducing phototoxic reactions by increasing the minimal erythema dose to UVA [90]. In healthy individuals, 12–24 mg day^{-1} for ≥10 weeks increases stratum corneum carotenoid deposition and reduces UV-induced erythema, supporting photoprotective nutraceutical and cosmetic applications [91].

8.4 Immune Modulation and Anti-inflammatory Effects

β-Carotene stimulates NK cell activity, enhances macrophage phagocytic capacity, upregulates IL-2 and IFN-γ, and reduces CRP, IL-6, and TNF-α in clinical studies [92]. Anti-inflammatory effects are mediated both through retinoid-dependent gene regulation via retinoic acid receptors and through direct ROS quenching in activated immune cells [93]. Clinical trials in elderly populations show modestly enhanced NK cell activity and lymphocyte proliferative responses with supplementation at 15–50 mg day^{-1} [94].

IX. REGULATORY STATUS AND GLOBAL MARKET

9.1 Regulatory Approvals

β-Carotene from *B. trispora* fermentation has received regulatory approval in all major markets; the pertinent designations and specifications are summarised in Table 5. JECFA (Joint FAO/WHO Expert Committee on Food Additives) established an acceptable daily intake (ADI) of 0–5 mg kg^{-1} bw day^{-1} for β-carotene from all sources [95]. *B. trispora* does not produce mycotoxins under properly controlled fermentation conditions; regulatory specifications require aflatoxin B1/B2/G1/G2 and ochratoxin A testing with undetectable results (<0.1 μg kg^{-1}) in the final product [95]. The principal adverse effect at chronic high intake is reversible carotenodermia (orange skin discolouration) above ~30 mg day^{-1}; no teratogenicity is associated with β-carotene, in contrast to preformed vitamin A [96].

Table 5. Regulatory status of fermentation-derived β-carotene from Blakeslea trispora in major global markets.

Jurisdiction	Regulatory Framework	Designation	Key Specifications
European Union	Regulation (EC) 1333/2008 (food); Regulation (EU) 2017/1007 (feed)	E160a(i) — Approved food colour and feed additive	≥96% all-E β-carotene; residual hexane ≤25 ppm; As ≤1 mg kg^{-1}
United States	21 CFR §73.95 + GRAS Notice GRN 000595	GRAS (food); OTC colour exempt	Purity ≥96%; residual solvents per ICH Q3C; identity by HPLC
China	GB 1886.13-2015 (CFDA/NMPA)	Permitted food colour	Purity ≥96%; Pb ≤10 mg kg^{-1}; As ≤2 mg kg^{-1}; heavy metals per GB standards
Japan	MHLW Food Additives List 2023	Permitted food additive	Japanese Pharmacopoeia specifications; purity >96%; absence of aflatoxins

Codex Alimentarius	FAO/WHO JECFA (2006); GSFA Online	INS 160a; ADI 0–5 mg kg^{-1} bw day^{-1}	JECFA Compendium specifications; accepted globally as reference standard
Australia / New Zealand	FSANZ Food Standards Code	Permitted food additive (code 160a)	JECFA 2006 specifications; max use levels category-specific
Brazil	ANVISA Res. RDC 689/2022	Permitted natural colourant	Same purity specifications as EU E160a(i)

ADI = acceptable daily intake; GRAS = Generally Recognized as Safe; EPP = erythropoietic protoporphyria

9.2 Market Dynamics

The global β-carotene market was valued at approximately USD 370–450 million in 2022 and is projected to reach USD 600–750 million by 2030 (CAGR 5–6%) [5]. Synthetic β-carotene from BASF and DSM dominates by volume (production cost ~USD 200–400 kg^{-1} bulk grade). Natural β-carotene from *D. salina* algae commands a premium (USD 1,000–3,000 kg^{-1}) for consumer naturalness perception. Fermentation-derived β-carotene from *B. trispora* (producers: Chr. Hansen; Zhejiang NHU; BASF legacy processes) occupies an intermediate price point (USD 400–800 kg^{-1}), competing on the basis of natural origin, scalability, and consistent all-*E* isomer profile [97]. Achieving production costs below USD 300 kg^{-1} through titre improvement and process intensification is the key commercial target for competitive displacement of synthetic routes.

X. FUTURE PERSPECTIVES

10.1 Agro-Industrial Waste Valorisation and Biorefinery Integration

Process economics can be substantially improved by replacing purified glucose or refined molasses with low-cost agro-industrial waste streams. Lignocellulosic hydrolysates from sugarcane bagasse, wheat straw, and rice straw, as well as cheese whey permeate and orange peel extracts, have supported *B. trispora* growth and β-carotene production at 80–90% of glucose control titres with 10–30-fold lower substrate costs [98]. Biorefinery concepts integrating β-carotene production with mycelial lipid recovery (for biodiesel) and spent biomass protein utilisation (as animal feed supplement with 35–40% crude protein) improve overall process economics and align with circular economy principles [99].

10.2 CRISPR/Cas9 Genome Editing

CRISPR-based genome editing tools have been adapted for Mucoralean fungi, and their application to *B. trispora* would enable precise multi-gene interventions without the random integration events and marker accumulation inherent in classical transformation approaches [100]. Targeted CRISPR activation (CRISPRa) of *carRA* and *hmgR* could achieve expression levels equivalent to multi-copy overexpression with superior genetic stability, while CRISPR interference (CRISPRi) enables titratable *erg9* attenuation without lethality from complete ergosterol depletion [101].

10.3 Nanoencapsulation and Bioavailability Enhancement

The poor aqueous solubility (log*P* ≈ 15.5) and oxidative lability of β-carotene limit product shelf life and oral bioavailability. Nanoencapsulation using oil-in-water nanoemulsions (droplet size 100–300 nm), solid lipid nanoparticles (SLNs), nanostructured lipid carriers (NLCs), biopolymer-coated nanoparticles (chitosan, whey protein, modified starch), and cyclodextrin inclusion complexes achieves 3–10-fold improvements in in vitro bioaccessibility compared with bulk oil formulations [102, 103]. Preliminary human studies support enhanced plasma β-carotene and retinol responses from nanoemulsion formulations, which will support premium pricing and value differentiation for fermentation-derived material in pharmaceutical and nutraceutical markets [104].

10.4 Process Analytical Technology (PAT) and Continuous Processing

In-line Raman spectroscopy for real-time β-carotene quantification, NIR spectroscopy for biomass estimation, and soft sensors based on CO_2 evolution rate as a metabolic activity proxy are enabling PAT-guided process control for *B. trispora* fermentations [105]. Coupling PAT with model predictive control can dynamically optimise feeding rates, aeration, and pH to maintain carotenogenic conditions, potentially increasing titres by 15–25% over manually controlled processes. Extended perfusion-mode continuous fermentation with periodic re-inoculation of opposite mating type represents an emerging strategy for higher volumetric productivities than current fed-batch modes [106].

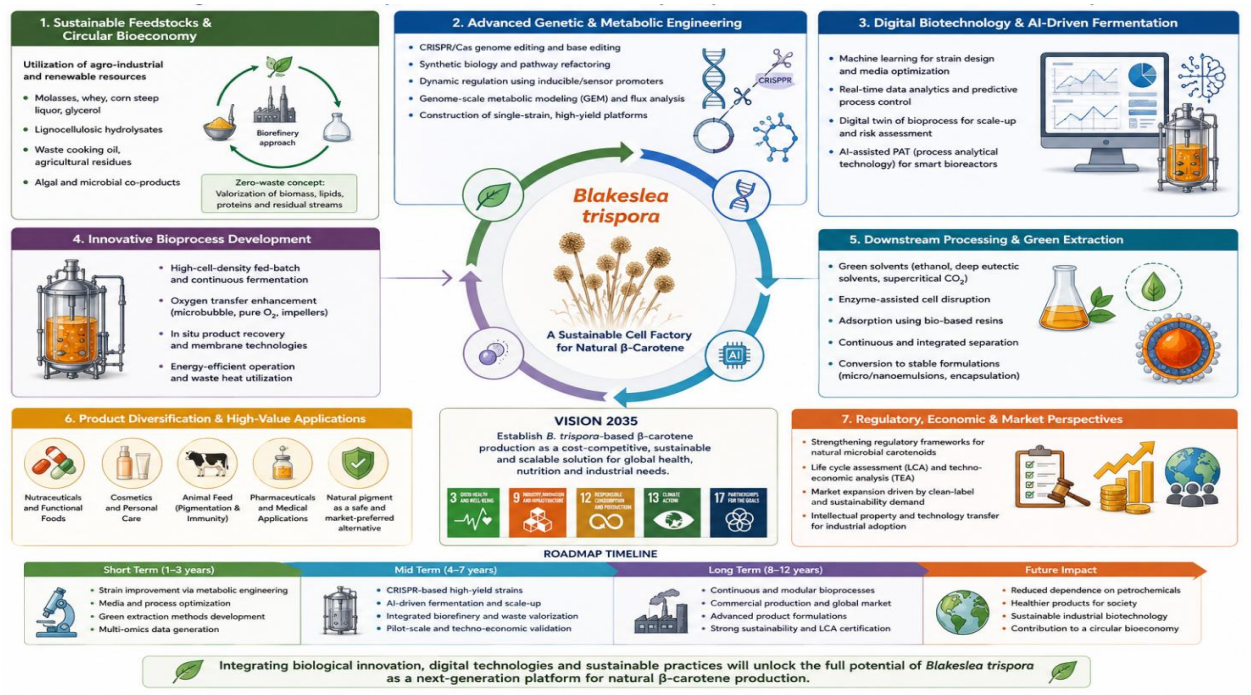

Fig. 5. Future technology roadmap for Blakeslea trispora-based β-carotene production, integrating CRISPR genome editing, AI-assisted bioprocess optimisation, agro-industrial waste valorisation, PAT monitoring, and nanoencapsulation strategies.

XI. CONCLUSIONS AND FUTURE RESEARCH DIRECTIONS

Blakeslea trispora remains the most successful microbial platform for the industrial production of natural β-carotene, combining high intrinsic carotenogenic capacity with the unique trisporic acid (TSA)-mediated signalling system that substantially enhances carotenoid biosynthesis during mixed-mating cultivation. The organism has established a prominent position in the global carotenoid industry because of its compatibility with conventional submerged fermentation systems, ability to accumulate high intracellular β-carotene concentrations, and proven commercial scalability.

Significant advances have been achieved in understanding the molecular basis of β-carotene biosynthesis in *B. trispora*. The complete mevalonate (MVA) pathway, together with the downstream carotenoid biosynthetic network involving *hmgR*, *ipi*, *carG*, *carRA*, and *carB*, has been extensively characterised, providing multiple targets for metabolic engineering and process optimisation. Improvements in fermentation technology, including fed-batch cultivation, optimised oxygen transfer, lipid supplementation, and nutrient management, have substantially increased β-carotene productivity. Furthermore, advances in strain improvement through classical mutagenesis, pathway engineering, and systems biology approaches have demonstrated the feasibility of achieving significantly higher product titres than those obtained with wild-type strains [64, 65, 69, 74].

Despite these achievements, several challenges continue to restrict the full industrial potential of *B. trispora*. Dependence on mixed-mating-type cultivation complicates process control and scale-up, while oxygen-transfer limitations, competing sterol biosynthesis, and costly downstream extraction processes reduce overall production efficiency. In addition, the limited availability of advanced genetic tools and regulatory elements has slowed the implementation of precision metabolic engineering strategies compared with model microbial hosts.

Future research should focus on developing next-generation production platforms capable of overcoming these limitations. CRISPR/Cas-based genome editing, synthetic biology, and multi-omics-guided metabolic engineering offer promising opportunities for constructing high-yield, genetically stable strains with enhanced carbon flux toward β-carotene biosynthesis [100, 101]. Integration of transcriptomics, proteomics, metabolomics, and genome-scale metabolic modelling will facilitate the identification of previously unrecognised regulatory nodes and metabolic bottlenecks. Simultaneously, the application of artificial intelligence, machine learning, digital bioprocess monitoring, and process analytical technologies (PAT) is expected to improve fermentation control, predictive process optimisation, and industrial reproducibility [105, 106].

Sustainability considerations will also play an increasingly important role in future commercialisation efforts. The utilisation of agro-industrial residues, lignocellulosic hydrolysates, food-processing wastes, and circular biorefinery concepts has the potential to significantly reduce production costs and environmental impact [98, 99]. Coupled with green extraction technologies

and advanced nanoencapsulation systems that improve product stability and bioavailability [102–104], these innovations may enhance the economic competitiveness of fermentation-derived β-carotene relative to synthetic alternatives.

Overall, the convergence of metabolic engineering, systems biotechnology, digital bioprocessing, sustainable feedstock utilisation, and advanced formulation technologies is expected to transform *B. trispora* into a next-generation microbial cell factory for natural carotenoid production. Achieving consistent productivities exceeding current industrial benchmarks while reducing production costs below commercially competitive thresholds will be critical for expanding the role of fermentation-derived β-carotene in food, feed, nutraceutical, pharmaceutical, and cosmetic markets.

Key Future Research Priorities

- Development of single-strain β-carotene production systems independent of mating-type interactions.
- CRISPR/Cas-mediated pathway optimisation targeting hmgR, carRA, carB, and competing sterol biosynthesis genes.
- Application of genome-scale metabolic modelling integrated with transcriptomics and proteomics for systems-level strain engineering.
- Development of low-cost agro-industrial feedstock bioprocesses compatible with established industrial infrastructure.
- Adoption of process analytical technologies (PAT) for real-time monitoring and adaptive fermentation control.
- Nanoencapsulation and formulation advances for enhanced β-carotene bioavailability and shelf stability.
- Techno-economic and life-cycle assessments guiding the transition from synthetic to bio-based β-carotene supply chains.

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