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NEWSLETTER

EUROCAROTEN

EUROPEAN NETWORK TO ADVANCE CAROTENOID RESEARCH AND APPLICATIONS IN AGRO-FOOD AND HEALTH

PRODUCTION OF CAROTENOIDS – Case *Blakeslea trispora*

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Blakeslea trispora as an industrial source of β-carotene and lycopene

The world market for carotenoids reached 1.5 billion dollars in 2019 and is projected to reach 2.0 billion dollars in 2026, highlighting them as an emerging market with very good growth prospects and many business opportunities. Although carotenoids produced by chemical synthesis dominate the global market, consumer's demand for natural and clean label products is the driving force for the natural carotenoids sector, which is growing rapidly (an average annual rate of ~ 4% for the period 2016-2023)[1].

Natural carotenoids are mainly produced from plants. Penetration of microbial carotenoids into the food/feed sector has been accelerated during the last years despite challenges and limitation regarding scale-up strategies, manufacturing costs and price, and regulatory approval processes. Among the types of microbes that can produce commercially vital carotenoids, *Blakeslea trispora* is of primary industrial interest as a source of β-carotene and lycopene for commercial exploitation[2]. The latter is partially related to the fact that β-carotene from *B. trispora* is the first microbial pigment approved for use as food additive by the European Commission (E160a (ii))[3]. Also, lycopene derived from this fungus has been placed on the EU market as a novel food ingredient (Commission regulation 2006/721/EU)[4] and more recently under the entry E160d (Commission directive 2011/3/EU)[5]. From the technological point of view, *B. trispora* is beneficial in carotenoid production processes because of (a) its high specificity toward the biosynthesis of the end-product of the carotenogenesis pathway (all-*trans* β-carotene) at the expense of other structurally related carotenoids (**Figure 1**) and (b) its ability to accumulate intracellularly high amount of carotenoids[6,7].

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The viability of the bioprocess is influenced by the manufacturing and commercialization costs of the final product and its intended utilization. Process innovations concerning microbial upgrading of residues and wastes for carotenoid production will reduce the manufacturing costs and make it more environmentally friendly.

Carotenogenesis in *Blakeslea trispora* cells

Blakeslea trispora is classified as Zygomycetes and belongs to the order Mucorales and the family Choanephoraceae. This fungus undergoes both sexual and asexual reproduction that leads, respectively, to the production of zygospores and sporangiospores. *B. trispora* is present in two sexual forms, namely plus (+) and minus (-) mating types[8,9]. The fungus is non-pathogenic and does not produce toxic compounds[10].

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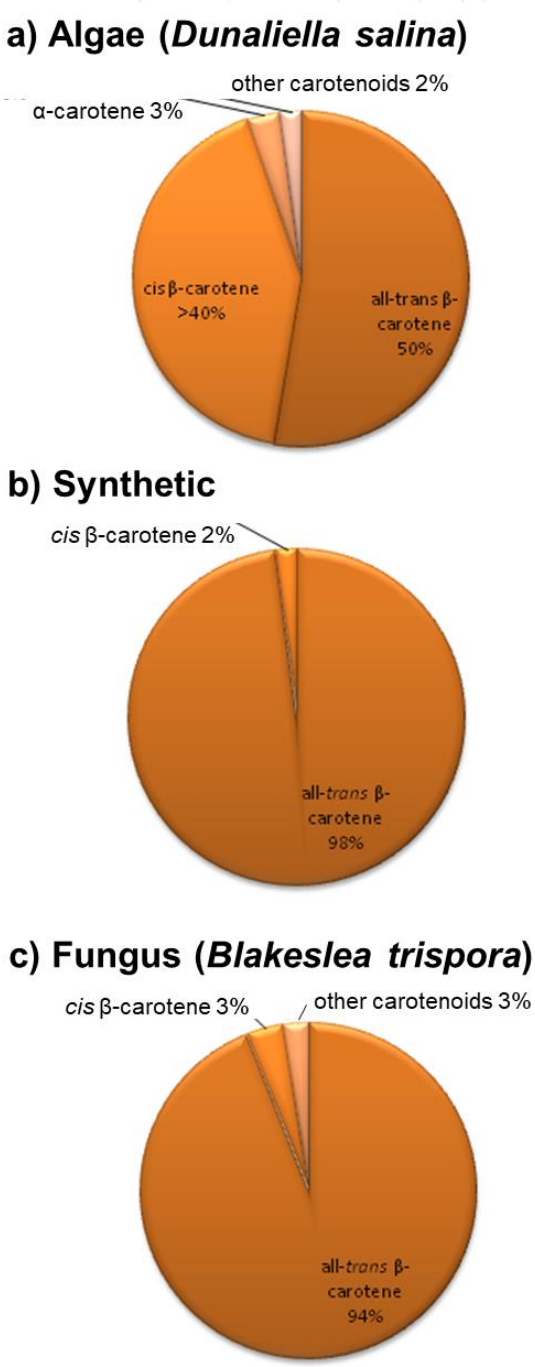
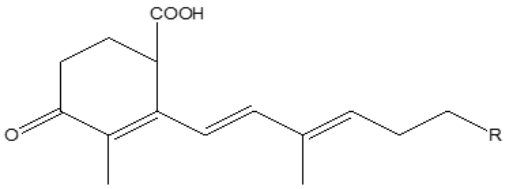


Figure 1. The carotenoid profiles of the three main sources for β-carotene (a) Algae (*Dunaliella salina*), (b) Synthetic and (c) Fungus (*Blakeslea trispora*)[6,7].

Excessive production of the fungal β-carotene can takes places only when mycelia of the two mating types are co-cultivated (mating). During mating the synthesis of trisporic acids, a family of apocarotenoids (**Figure 2**), is favoured, which in turn regulates the mating procedure and stimulates carotenogenesis[11]. *B. trispora* contains β-carotene as the main carotenoid and its precursor forms that are phytoene, phytofluene, lycopene, and γ-carotene under the standard aerobic cultivation conditions in media rich in cereal products, carbohydrates, mineral salts, surfactants, vitamins and vegetable oils (**Figure 3**)[12,13]. In this non-photosynthetic fungus, carotenoids are synthesized in specialized mycelia characterized by large lipid globules, the hydrophobic core of which is mainly composed of triacylglycerols (TAGs) (**Figure 4**)[14].



Trisporic acid B: R = -C-CH_3
Trisporic acid C: R = -$CHOCH_3$

Figure 2. Chemical structure of trisporic acids.

B. trispora is able to accumulate lycopene in the presence of inhibitors that block the cyclization of the open ends of acyclic lycopene into β-ionone rings located in β-carotene molecule. Inhibition of lycopene cyclase activity by chemical (amines and nitrogenous heterocyclic bases) or genetic means promotes lycopene accumulation in fungal cells[15]. Accordingly, lycopene formation results in the interruption of sexual reproduction processes (**Figure 5**). The activity of lycopene cyclase inhibitors is positively related to their basicity expressed as pKa values. Electronic effects together with steric hindrance of bulky substituents in the molecules are limiting factors for their activity[15].Carotenogenesis in fungal cells is stimulated by oxidative stress reflecting the antioxidant role of carotenoids and protection of the fungal cells[17].

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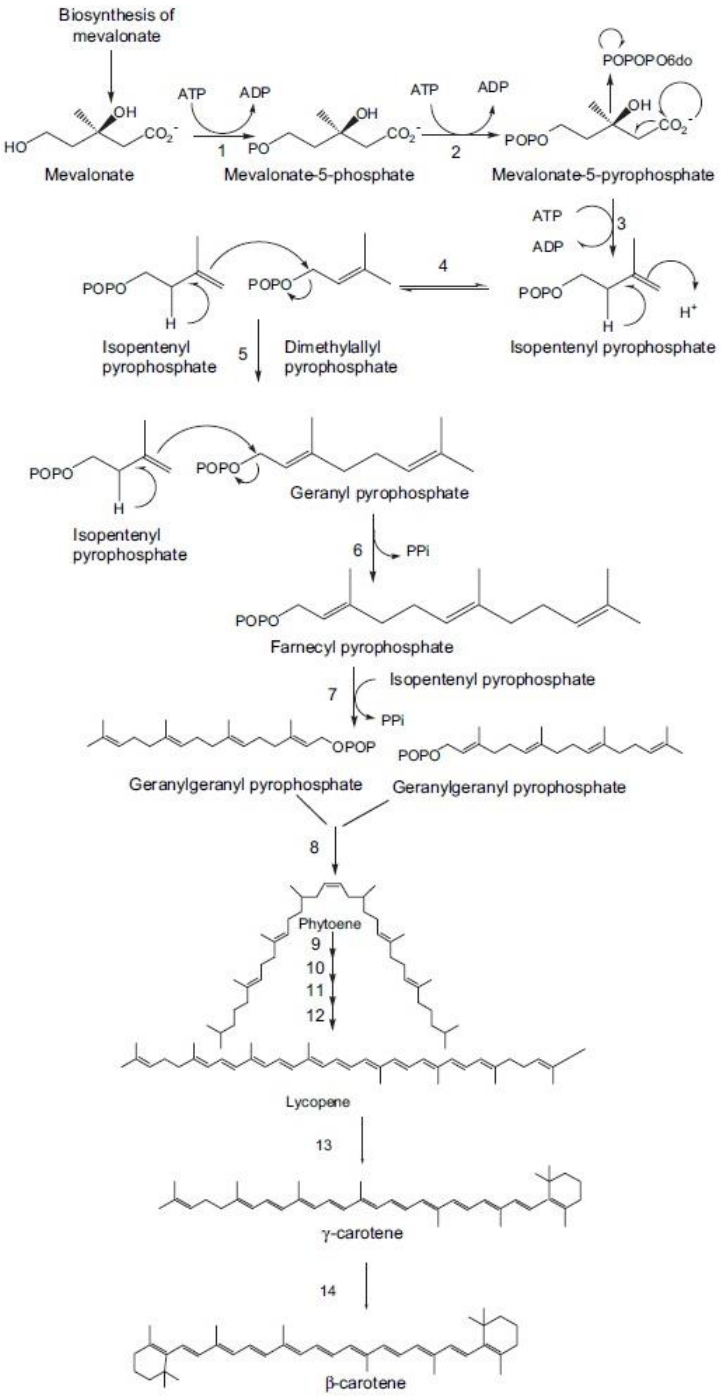

Figure 3. Biosynthetic pathway for lycopene and β-carotene formation in *Blakeslea trispora* cells[15].

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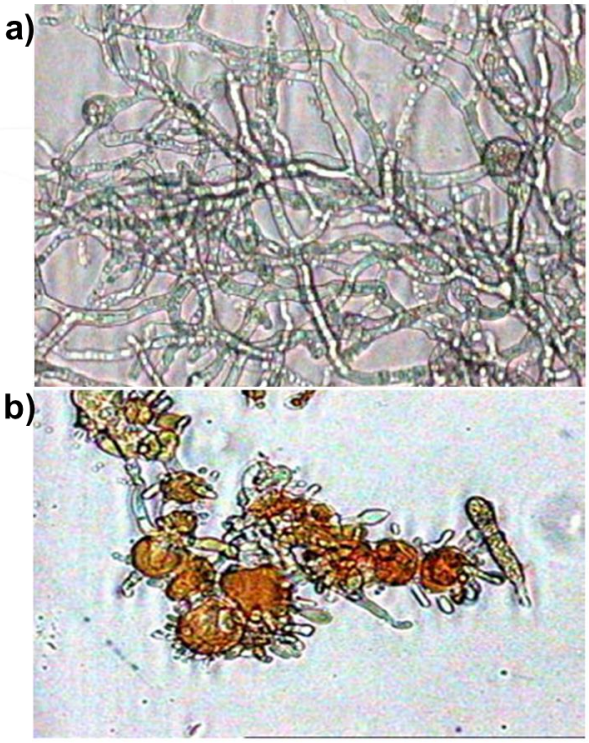

Figure 4. Microphotographs of *B. trispora* mycelia (a) without carotene and (b) containing high amount of carotene[12].

Industrial production of β-carotene and lycopene by *B. trispora*

The flow diagram of production of carotenoids by *B. trispora* is shown in **Figure 6**. Separate seed cultures for the two sexual mating types (+) and (-) strains are prepared and used to inoculate the culture medium. The bioprocess is performed in aerobic submerged batch fermentation under culture conditions which can yield fungal biomass with high β-carotene content. Among the readily assimilable carbohydrates (glucose, fructose, sucrose, lactose, maltose), glucose has been reported to be an excellent carbon source for fungal growth. However, rapid fungal growth due to the fast metabolism of high level of glucose results in low carotenoid yields (only up to 100 mg/L of culture medium)[12,19].

Cultivation of *B. trispora* in media containing two major carbon sources, one for the initial growth phase such as glucose and the second for the production phase such as a slowly metabolized nutrient (e.g. soybean oil, cottonseed oil, individual fatty acids), and a relatively high carbon-to-nitrogen ratio (C/N) triggers carotenoid production[12,20]. Previous investigation highlighted the interdependence of carotenoid pattern with the type and amount of extracellular oils and fatty acid composition of the intracellular TAGs[14]. For example, in cells grown on oleic acid (C18:1)-rich oil substrate, lycopene cyclization to β-carotene and desaturation of C18:1 aliphatic chains

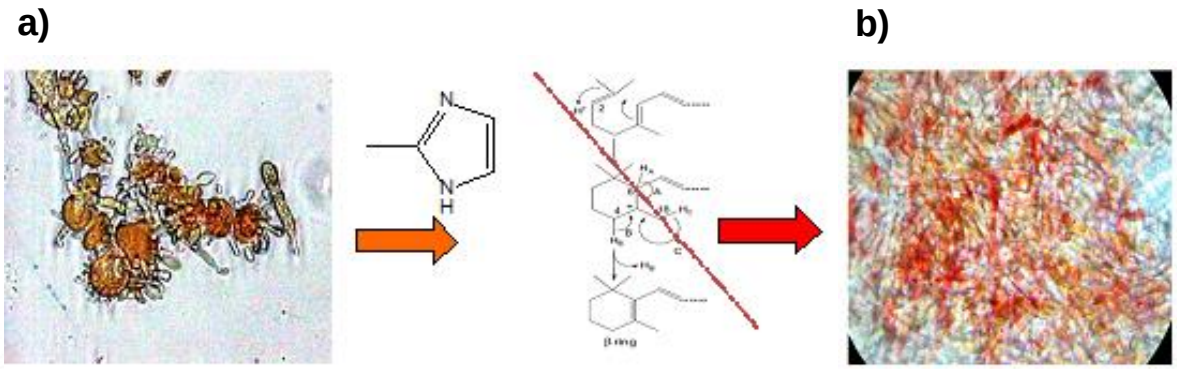

Figure 5. Morphological characteristics of *B. trispora* grown under optimum conditions for maximum yield of (a) β-carotene (in the absence of lycopene cyclase inhibitor) and (b) lycopene (2-methyl imidazole at 29.5 mg/L)[12,16].

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as part of intracellular TAG synthesis was suggested to compete for the available NADPH. In contrast, in mycelium grown on linoleic acid (C18:2)-rich oil substrate the *de novo* synthesis of fatty acids and their biotransformation was found to be insignificant resulting in higher flux of available acetyl-CoA, NADPH and ATP toward the synthesis of β-carotene. Carotenoid production has been found to be negatively affected by nitrogenous compounds derived from inorganic nitrogen sources (e.g. ammonium salts). On the other hand, the use of complex nitrogen sources such as corn-steep liquor has been common practice for carotenoid production by *B. trispora*[19]. Other chemical compounds can increase carotenogenesis in *B. trispora* such as α- and β-ionones and some natural terpenoids that do not affect growth, but directly induce key enzyme activities[21], and various antioxidants that act against the oxidation of carotenoids during fermentation[12].

The production of carotenoids by *B. trispora* has in recent years gained considerable interest due to the capacity of the fungus to convert a variety of economical substrate constituents or even industrial waste products into valuable end products[18,20]. The acid-hydrolyzed whole ground soy, the acid-hydrolyzed hexane extracted soybean oil meal and the unhydrolyzed corn were found to stimulate carotene production (350-400 mg/L of culture medium). Moreover, low-cost agricultural byproducts like citrus oils, citrus pulp, or citrus molasses were used as good sources of β-ionone, a stimulatory compound of carotenogenesis in *B. trispora*. Carotene yield was found to be greater in those treatments than in treatment supplied with pure β-ionone (up to 129 mg vs 98 mg/100 mL of culture medium, respectively). Whey and molasses were not found to support cell growth and β-carotene production of *B. trispora*, however the published information is limited. The main disadvantage of molasses and whey is the sucrose and lactose content, respectively. Crude glycerol derived from biodiesel production and soap manufacture has been also evaluated as a supplementary carbon source to glucose for β-carotene production by *B. trispora*. The results showed satisfactory β-carotene yield and high specificity (8.0 mg/g of dry biomass and 89 % of total carotenoid content, respectively) compared with those obtained using only glucose (1.0 mg/g of dry biomass and 35% of total carotenoid content). In culture media containing crude olive pomace oil or crude soybean oil in mixtures with glucose, more than 10-fold increase in carotenoid production, mainly that of β-carotene, by *B. trispora* was measured compared to that observed in medium with glucose as a sole carbon source[18,20].

In the commercial process, imidazole or pyridine are regulators that are used for the production of lycopene from *B. trispora* currently available as a food additive in the EU[6]. Considering toxicological aspects of the effective chemical inhibitors employed in the process for fungal lycopene, there are many candidates (e.g. 2-isopropylimidazole, 2-methylimidazole and 6-methyl-2-aminopyridine) that can replace imidazole or pyridine. For example, 2-methylimidazole with LD_{50} values similar to that of imidazole can be effective in terms of yield and selectivity of the biotechnological process for the production of lycopene (~ 400 mg/L, 94% of total carotenoids) at 16-fold lower level of addition compared with the dose of imidazole to achieve the same effect[15,22]. Noticeable, the purity of the final product is higher than that of lycopene extracted from tomatoes (not less than 5% of total colouring matters)[23]. Also, EFSA Panel considered lycopene from *B. trispora* to be nutritionally equivalent to natural dietary lycopene, but further safety trials are necessary[24].

In the cultivation systems, mechanical stirring in stirred tank reactor causes high shear stress, which has been marked as the main limitation on the efficiency of the bioprocess for lycopene production by *B. trispora*. To confront this, low impeller speed and high airflow rate has been proposed to achieve maximum production of β-carotene during cultivation of the fungus[25]. Also, the application of bubble column reactor is favourable for carotenoid production by *B. trispora* due to its advantages (lower shear levels compared to that in stirred-tank reactors, sufficient heat and mass transfer properties, compactness, and low operating and maintenance costs)[16].

In the recovery stage, biomass is harvested and β-carotene is extracted with the aid of food grade solvents (e.g. ethyl acetate, edible oils), concentrated and crystallized (**Figure 6**). The final product is either crystalline β-carotene (purity > 96.0 %) or it is formulated as a 30 % suspension in vegetable oil. Analytical evidence (i.e. HPLC analysis, stability tests and microbiological tests) has confirmed that the final crystalline product conforms to all the specifications set out in Directive 95/45/EC for a coloring agent in foodstuffs[26]. For lycopene, its crystals contain at least 95 % total lycopene (of which at least 90 % is all-trans-lycopene) and up to 5 % other carotenoids. Less than 0.1 % and 1 % of isopropanol and isobutyl acetate, respectively, and less than 0.0001 % of imidazole residue may be present in the final product. As its crystals are susceptible to oxidative degradation,

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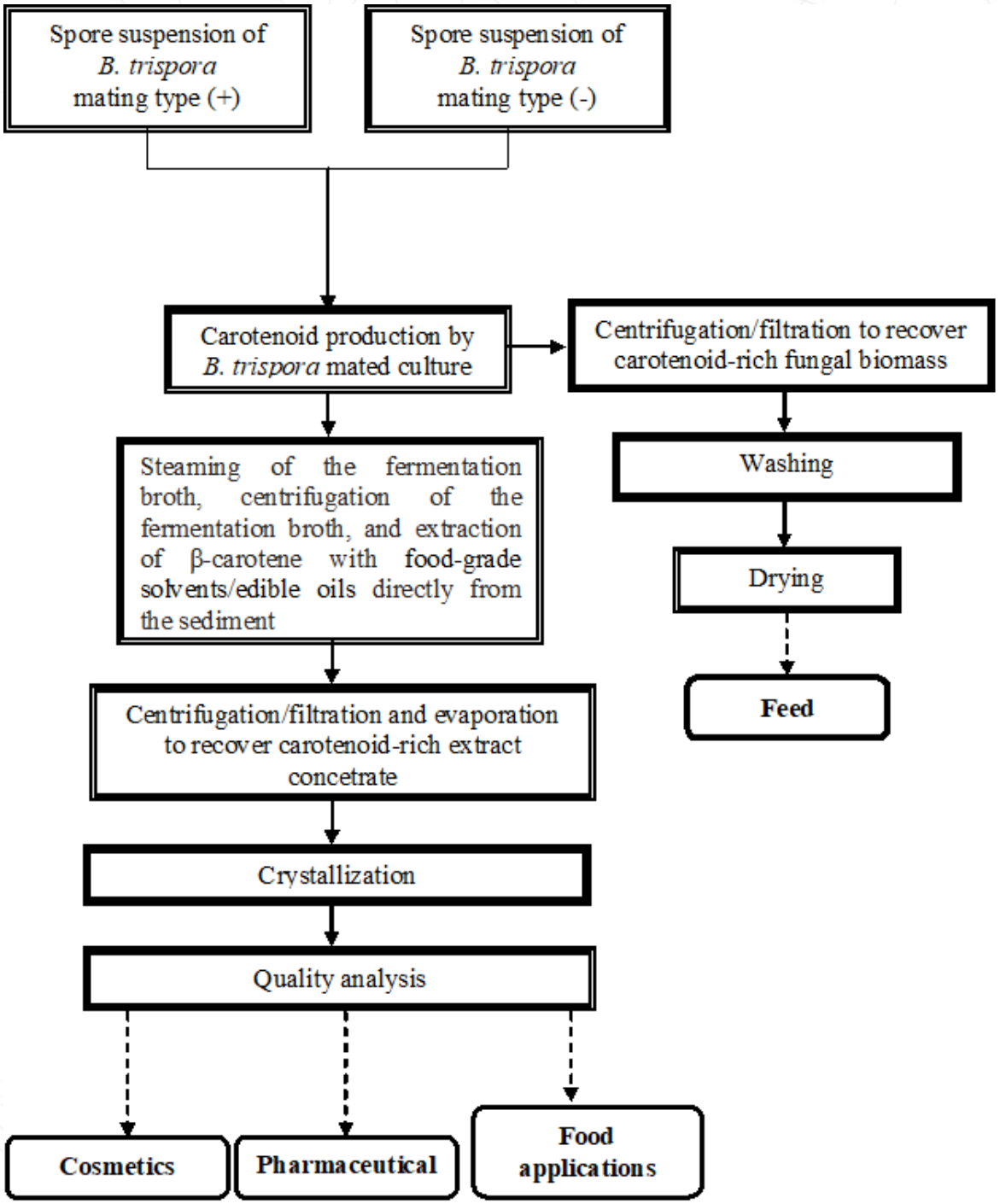

Figure 6. Flow diagram of production of carotenoids by *B. trispora*[18].

lycopene is mainly formulated as suspensions in edible oils or as water dispersible powders stabilized with antioxidants for use in food[23].

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