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Review: Potential production of carotenoids from *Neurospora*

SRI PRIATNI
Research Center for Chemistry, Indonesian Institute of Sciences. Jl. Cisitu, Bandung 40135, West Java, Indonesia. Tel./Fax. +62-22-2503051/22-2503240 ♥email: sripriatni@yahoo.com

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Abstract. *Priatni S. 2014. Review: Potential production of carotenoids from* Neurospora*. Nusantara Bioscience 6: 63-68.* Carotenoids are abundant and widely distributed in plants, animals and microorganisms. Commercial use of carotenoids competes between microorganisms and synthetic manufacture. Carotenoids production can be increased by improving the efficiency of carotenoid synthesis in microbes. Some of the cultural and environmental stimulants are positively affecting the carotenoid content of carotenogenic strains such as *Neurospora*. *Neurospora* is a fungus that exhibits the formation of spores and conidia, the part of the cell for carotenoids biosynthesis. The Indonesian traditional fermented food, red peanut cake or oncom, especially in West Java, is produced from legume residues of *Neurospora* sp. This fungus has been isolated and identified as *Neurospora intermedia*. In order to apply this pigment for food and cosmetic colorants, encapsulation techniques of carotenoids have been developed to improve its solubility and stability.

Key words: Biosynthesis, encapsulation, *Neurospora*, pigments

INTRODUCTION

Carotenoids can not only be found as plant pigments in nature, but also in animal and microorganisms. Plants are able to synthesize carotenoids, by the presence of a little amount of biosynthetic precursors, together with derivatives of the main components in plants (Rodriguez and Kimura 2004). The roles of carotenoids in plants is to protect the photosynthetic system from excessive light, so it seems can to balance the absorption of sufficient light in this process (Cazzonelli 2011). The color of carotenoids plants are yellow, orange and red, which can be found in fruit, flowers, roots and seeds. However, carotenoids are not a sole producer of those colors, anthocyanins and quinine are also involved in it. Carotenoids content in animals are not from biosynthesize process, but usually from the accumulation of what they were eating. The color of egg yolk is due to carotenoids from the poultry feed that used intensively (Britton and Khachik 2009).

Carotenogenic species of fungi are categorized in some classes and their carotenoid synthesis can be easily found in nature. Carotenoids biosynthesis in several species depend on light. This indicates that carotenoids function in these organisms is for light protecting. Generally, carotenoids in fungi are the β-carotene, γ-carotene, torulene and their hydroxy and keto derivatives (Sandmann et al. 2008). Production of carotenoids from microorganisms commercially competes with synthetic manufactured by a chemical process. The efficiency of carotenoids biosynthesis could improve by microbial stimulation. Carotenoids biosynthesis are influenced by culture conditions, the level and activity of carotenoid biosynthetic enzymes and total carbon flux through synthesizing system (Bhosale 2004). In order to achieve simple and low cost of carotenoid production, *Neurospora* sp. is a potential microbial sources due to its carotenoids content and easy to cultivate on waste solid substrates. This review discussed the structural properties and biosynthesis of carotenoids. The information is used to evaluate the potential of carotenoids production from *Neurospora* sp. as foods and cosmetics colorant.

CAROTENOIDS STRUCTURE AND ITS PROPERTIES

Carotenoids are a member of isoprenoid compounds which was synthesized by tail-to-tail linkage of two C_{20} geranyl-geranyl diphosphate molecules (Britton 1995). Carotenoids are generally C_{40} tetraterpenoids that governed from eight C_5 isoprenoid units. The conjugated double-bond system in carotenoids structure is the chromophore for light-absorbing that gives these compounds an attractive color. In the metabolic system, the precursor phytoene converts into cyclic carotenes by a series of de-saturation and cyclization reaction. During the initiation process of conjugated double bonds, the chromophore will lengthen (Armstrong and Hearst 1996). The carotenoid structures can be modified by several processes such as of cyclization, hydrogenation, dehydrogenation, double bond migration, chain shortening or extension, rearrangement, isomerization, introduction of oxygen function, and combination of these processes (Rodriguez and Kimura 2004).

The long system of alternating double bonds and single bonds in carotenoid structure can form the central part of the molecule. The π-electrons which constitutes in a conjugated system are effectively delocalized over the

entire length of polyene chain. Basically, the double bond in carotenoids can exist in two configurations, that is *trans* and *cis*, it depends on the position of substituent groups which linkage in the central part (Britton 1995). The unsaturated carotenoid tends to isomerization and oxidation process. Isomerization of *trans* carotenoids to *cis* configuration is promoted by heat, acids, and exposure to light (Rodriguez and Kimura 2004).

The important property of carotenoids that will influence to HPLC analysis is the long conjugated polyene system that makes the all-trans isomers rigid, linear molecules. Practically, this property gives carotenoids the ability to absorb the light in visible region at 400-550 nm. According to this character, HPLC which is combined with photodiode array detector (PDAD) is suitable for identification of carotenoids. λ_{max} of each carotenoid component and the shape of spectrum can be monitored simultaneously in HPLC chromatograms (Khachik 2009). These properties are the characteristic of the carotenoids chromophore. Generally, carotenoids absorb at three wavelengths, resulting in three-peak spectra. The number of carotenoids structure is correlated with λ_{max} values. The greater number conjugated double bonds, the higher λ_{max} values (Rodriguez and Kimura 2004).

Carotenoids are sensitive to light, oxygen, heat, and acid degradation, due to the presence of many conjugated double bonds in the structure. Carotenoids are hydrophobic molecules with little or no solubility in water which was normally found in hydrophobic areas of cell, such as the inner core of membranes. They can access an aqueous environment when they are associated with protein or with other polar compounds. The polarity of carotenoids can be altered by interaction with a polar functional group and other molecules (Britton 1995).

Carotenoids have been attributed to an antioxidant property due to their ability to quench single oxygen and interact with free radicals (Rodriguez and Kimura 2004). Many studies of carotenoids concerning the relative antioxidant activity, they concluded that there are three types of carotenoids reaction with radical species, i.e. radical addition, electron transfer to the radical and allylic hydrogen abstraction (Yeum et al. 2009). Some studies suggest that radical forming can be influenced by the structure and level of carotenoids, and also the level of oxygen and the polarity of solvent. Carotenoids radical cation mediates the isomerization and modifications of carotenoids structure. A study showed that the oxidation of canthaxanthin and 8′-apo-betacaroten-8′-al resulting the formation of radical cation followed by formation of *cis*-isomers (Boon et al. 2010).

CAROTENOIDS PRODUCTION BY MICROORGANISMS

Commercial production of carotenoids by non-photosynthetic microorganisms is becoming an attractive prospect in the future. Production of ß-carotene from the fungus *Blakeslea trispora* was developed in Europe through fermentation process. At present, this product has been used in food industry for several purposes. Carotenoids was produced by fermentation process is competing with carotenoids from synthetic chemicals process or with extraction of some natural sources (Dufossé 2009). Under certain growth conditions such as limitation of nitrogen or high salt concentration and exposed to high light intensity, the green algae *Dunaliella salina* can accumulate high amounts of carotenoids (up to 13% w/w). This alga accumulates β-carotene, astaxanthin, zeaxanthin, lutein and cryptoxanthin (El-Baky et al. 2007). Microalgae such as of *Arthrospira*, *Chlorella*, *Dunaliella, Spirulina* and *Aphanizomenon* have been used as functional foods due to carotenoids content. Commercially, the application of these microalgae has been increased that can be found in the form of pills, tablets and capsules. Carotenoids primarily used for dietary supplements, fortified foods, food color, animal feed and pharmaceuticals and cosmetics (Vílchez et al. 2011).

By using carotenogenic microbe stimulants at certain external conditions of the cultures, hyper-production of carotenoids can be achieved with an effective cost. Several stimulators have been studied to increase of β-carotene production in *Blakeslea trispora* and *Phycomyces blakesleeanus*, which was grown under normal fermentation conditions. Irradiation of algae, fungi, and bacteria by white-light positively affect the accumulation of carotenoids. However, the intensity and protocol of illumination may depend on the microorganism properties (Bhosale 2004). Stimulation by light, temperature, chemical compound, metal ion and salt, and solvent to carotenogenesis process of *Rhodotorula* strains has been studied. Moreover, optimization of medium component and improvement of *Rhodotorula* strains are efforts to increase the carotenoids production (Heriyanto and Limantara 2009). El-Banna et al. (2012) reported that the isolation and identification of carotenoid-producing strains of *Rhodotorula glutinis* isolated from pin cushion flower. This strain produced 7 g/L dry biomass, 266 μg/g cellular carotenoids, 1.6 μg/L volumetric carotenoids, and 12.4% lipids, after fermented in yeast malt broth with shaking (100 rpm) at 30°C for 4 days.

Carotenoid production by microorganisms can be increased efficiently by two strategies, i.e. by enhancement of biomass production and biosynthesis carotenoids. Biosynthesis of carotenoid in the cell is depending on the activity of enzymes involved in the process. This activity can be altered by using the recombinant DNA technique. Hyper-production of carotenoid in the cell can be reached by the application of recombinant DNA technique (Rodney 1997). For example, gene crtS from *Xanthophyllomyces dendrorhous* has been cloned simultaneously; which is responsible in the conversion of β-carotene to astaxanthin. Astaxanthin production used two kinds of enzymes i.e. α-carotene hydroxylase and α-ketolase that work on each intermediates (Martín et al. 2008). Loto et al. (2012) reported that carotenoid production was increased by disrupting the C_{22}-sterol desaturase gene (CYP61) in *X. dendrorhous*. CYP61 gene encoded cytochrom P450 enzyme which is involved in mevalonate pathway. The study suggested that in *X. dendrorhous*, ergosterol

regulates the carotenoid biosynthesis by a negative feedback mechanism.

Carotenoids production in *Escherichia coli* or other bacteria cannot be synthesize naturally. Introducing of carotenogenic genes to *E. coli* has been studied to achieve the carotenoid biosynthesis *de novo.* In these bacteria, other isoprenoid compounds such as dolichols and quinines are also synthesized. The important carbon flux for biosynthesis of isoprenoid compounds have been directed by the introduction of the carotenogenic genes. Plasmids containing *crt* genes have been constructed and expressed in *E. coli* in purpose to synthesize the lycopene, ß-carotene and zeaxanthin (Dufossé 2009). General terpenoid pathway of carotenoids biosynthesis in *E. coli* involves the geranyl-geranyl diphosphate (GGDP) synthase (*crtB*) and phytoene synthase (*crtE*) gene, resulting C_{40} carotenoid phytoene. Desaturation and modification of phytoene desaturase (*crtI*) catalyzed by e.g. cyclases, hydroxylases, and ketolases, resulting many kinds of carotenoids. Acyclic and cyclic carotenoids synthesis determined by Phytoene desaturase (*crtI*) and lycopene cyclase (*crtY*) which is located at important branchpoints of the carotenoid biosynthetic pathway (Dannert et al. 2000).

CAROTENOID BIOSYNTHESIS BY *Neurospora* sp.

Neurospora, the ascomycetes fungi is grown in tropical or subtropical countries. *Neurospora* species are grown and sporulates quickly on the surface of fire scorched vegetation. Five species of *Neurospora* have been identified in Europe are *N. crassa, N. discreta, N. intermedia, N. sitophila* and *N. tetrasperma* (Jacobson et al. 2006). *Neurospora crassa* has been used as a model organism to study the responses of light in eukaryotic cells. Several processes such as induction of carotenoid production, protoperithecial formation, phototropism of perithecial beaks, perithecial polarity, and circadian rhythm are controlled by blue light (Belozerskaya et al. 2012). The characteristics of these strains have been identified to have four heterothallic species with eight-spore asci and pseudo homothallic species with four spores asci. Distribution of each species has a specific pattern; however they have similarity with each other. They are similar in morphology, its conidia color are orange or yellow-orange (Perkins and Turner 1988). *Neurospora* produced huge number of macro conidia and are easy to recognize in nature. This fungus is grown in various substrates such as bread, burned woods, corn cobs and waste of sugarcane industries (Pandit and Maheshwari 1996).

Neurospora population has been studied by collecting the strains from many regions in the world. Population of the genus was found everywhere, but the species from each region are different. Some species have similarity on the basis of vegetative morphology. The similarity of *N. crassa* and *N. intermedia* was identified when crossing with each other. The perithecia of these species have normal appearance, but *N. intermedia* has larger conidia than *N. crassa*. *N. intermedia* can grow on non-burned substrates (Turner et al. 2001). Accumulation of carotenoids in *Neurospora* sp. show has a correlation with latitude of sampling regions. *Neurospora* strains isolated from lower latitudes accumulate more carotenoids than strains which is isolated from higher latitudes. This is happened because low latitude regions receive more UV radiation than high latitude regions (Luque et al. 2012).

Neurospora species has been studied by biological species recognition (BSR) and phylogenetic species recognition (PSR) methods. By BSR method, *Neurospora* has been well characterized and used as model organism because its sexual cycle can manipulated easily (Dettman et al. 2003). Genetic and molecular analyses were carried out to *N. intermedia* strain from Maddur in Southern India (Souza 2005). Molecular identification of *Neurospora* N-1 which isolated from Indonesian red fermented cake or oncom, was carried out based on the genetic analysis partially on ribosomal DNA included in the sub-unit of 28S rDNA (*D1/D2* region) and internal transcribed spacer (ITS). The isolate was 100% homologeneity by amplification on D1/D2 region and 99% by amplification on ITS region to *N. intermedia*. Based on phylogenetic analysis using D1/D2 region and ITS methods, *Neurospora intermedia* N-1 has a close relation to *N. crassa* (Priatni et al. 2010).

A biological phenomenon of *N. crassa* has been investigated to study the biosynthesis of carotenoids pathway and its regulation. Identification of the albino strains *al-1* to *al-3* and characterization of the responsible genes was carried out to investigate the carotenoid biosynthetic pathway in *N. crassa*. Condensation of two GGPP molecules produced colorless carotene-phytoene which is catalyzed by *al-2* enzyme. Introduction of up to five conjugated double bonds into phytoene mediated by a*l-1*, resulting 3,4-didehydrolycopene via phytofluene, ζ-carotene, neurosporene and lycopene. These reactions were identified by using a PDA detector that monitored the changes of λ_{max}. The data showed that the absorption shifts toward longer wavelengths, and visually the color of desaturation products changes from yellow to red colors (Sandmann et al. 2008; Estrada et al. 2008). *Al-2* is bifunctional gene which contain cyclase and phytoene synthase, as found for homologous genes from *X. dendrorhous, Phycomyces blakesleeanus* and *Mucor circinelloides* (Arrach et al. 2002). The activation of two carotenogenic genes (*al-1* and *al-2*) by light did not correlate with accumulation of carotenoids in *Neurospora* sp. (Olmedo et al. 2013). Accumulation of neurosporaxanthin and some carotenoid precursors give characteristic orange of conidia and mycelia in *N. crassa*. Ca^{2+} signaling pathway indicates could be involved in regulation of carotenoid production in this fungus (Deka and Tamuli 2013). Carotenoids biosynthesis pathway in *Neurospora* was presented in Figure 1.

Identification of carotenoids in *N. intermedia* N-1 isolated from oncom has been carried out by using the HPLC equipped with a *photodiode array* (PDA) detector. Analysis of pigment extract shown that at least five carotenoid compounds were identified in spores of *N. intermedia* N-1 i.e. lycopene, neurosporen, γ-carotene, β-carotene and phytoene. This study suggested that

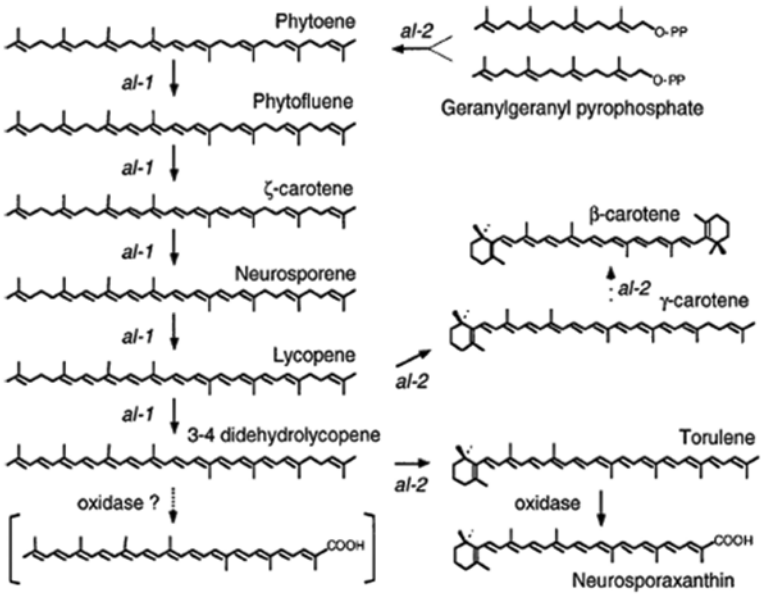

Figure 1. Biosynthesis of carotenoids in *Neurospora* sp. (Arrach N et al. 2002).

biosynthesis pathway in *N. intermedia* is similar with the biosynthesis in *N. crassa* (Priatni et al. 2010).

CAROTENOIDS PRODUCTION FOR FOOD AND COSMETICS COLORANTS

Carotenoids show several beneficial functions to human life such as anti-carcinogen, antioxidant, anti-inflammatory and chemo preventive agent for some cancer diseases. This pigment has been used also for food, beverages and cosmetic colorants. Nowadays, food and cosmetic industries were more interested to substitute synthetic colorants by natural pigments (Britton 1995; El-Baky et al. 2007). Most of foods and beverages which are marketed in Indonesia are colored with synthetic colorants which is not food grade and of course dangerous to health. Natural colorants will be a good alternative for synthetic colorants as long as its quality and safety are guaranteed. The potential carotenoid source in Indonesia has been explored to the palm oil which contains high amount of β-carotene (400-700 ppm). The technology separation of β-carotene in palm oil including extraction, adsorption and trans-esterification process, should be applicable and low processing cost (Darnoko 2008). *Neurospora intermedia* N-1 isolated from oncom has been studied for carotenoids production as an alternative for food and beverages colorant. This study recommended that the solid waste from tofu production is the best substrate fermentation due to high yield of spores production and concentration of the total carotenoids (Priatni et al. 2008). The fermented product of *N. intermedia* N-1 on solid waste tofu production was shown on Figure 2. The fermentation of *N. crassa* on tapioca by product and waste tofu has been carried also to produce the alternative poultry feed with high content of β-carotene (Nuraini et al. 2009).

In order to achieve optimum physical and chemical stability, and bioavailability of carotenoids, innovative processes for their production with modern methods of encapsulation technology have been developed and investigated (Ribeiro et al. 2010). Encapsulation of carotenoids has been studied by some researcher to improve its stability and solubility. Nanoparticle formation and encapsulation of β-carotene using copolymer casein-g-dextran was studied by hydrophobic interaction. The particle core was forming by interaction between β-carotene and hydrophobic segments of casein and the hydrophilic dextran shell makes the nanoparticle stable and dispersible in pH range 2-12 (Pan et al. 2007). The common techniques such as spray drying and inclusion complexation have been studied for carotenoids encapsulation. Lycopene powder was obtained by encapsulation through spray-drying technique with β-cyclodextrin (β-CD). The efficiency of encapsulation was between 94 to 96% and the average EY (encapsulation yields) was 51 ±1%. These complexes (lycopene-β-CD) were formed at a molar ratio of 1:4 (Nunes and Mercadante 2007).

Figure 2. Fermented product of *N. intermedia* N-1 on solid waste tofu (Priatni et al. 2008).

The encapsulation process of lycopene in oil has been carried out using coacervation technique. Complex coacervation is a spontaneous separation between two or more polymers forming an insoluble complex through the electrostatic interactions. On this study, the retention of lycopene was higher in the microcapsules compared to the free material. The potential of microencapsulation indicates has greater protection to carotenoid degradation (Rocha-Selmi et al. 2013). The other method for encapsulating this

pigment is by using supercritical technology. Some methods of supercritical technology have been developed. One interesting method is supercritical anti-solvent (SAS) method. The principle of this method is based on a rapid decrease of a solvent solubility (first solvent) by adding the second solvent as anti-solvent. The supercritical fluid or anti-solvent will saturate the first solvent through mixing and extraction process (Diego et al. 2010).

Microencapsulation of carotene extracts from *Neurospora* sp. Spores has been carried out by using protein base as shell material. Encapsulation carotenoid extract with sodium caseinate as shell material gives the highest microencapsulation efficiency, total carotenes, and carotenes retention values, compared to soy protein isolate and milk protein isolate (Pahlevi 2008). Another encapsulation process of carotenoids is from *N. intermedia* N-1 by using copolymer of gelatin and maltodextrin. In this study, the average EY of carotenoids powder obtained by using spray dryer was 48%. The stability of carotenoids powder can be maintained at low humidity and dark storage. Encapsulated carotenoids from *N. intermedia* N-1 were stable when they are stored in brown glass at RH between 20-30% (Gusdinar et al. 2011).

CONCLUSIONS

Production of carotenoids from microorganisms is an alternative for chemical process. Production of carotenoids by microorganisms will become an attractive prospect in the future. Biosynthesis of this pigment is influenced by culture condition, level and activity of carotenoid biosynthetic enzymes. Stimulation at certain external conditions to the growth of carotenogenic microbes can enhance carotenoids production. Some stimulants such as light, temperature, chemical compound, metal ion and salt, and solvent, have been used for hyper-production of carotenoids in microbes. Moreover, optimization of medium component and improvement of strains can be away to commercialize carotenoids production. *Neurospora,* the ascomycetes fungi are easy to grow in tropical country such as Indonesia. The carotenoids biosynthesis and its regulation of this fungus have been investigated. *Neurospora intermedia* which are isolated from red oncom have been studied for carotenoids production. Fermentation of *N. intermedia* on waste solid tofu production can produce high yield of carotenoids extract. In *N. intermedia* spores, at least five carotenoid compounds were identified i.e. Lycopene, neurosporen, γ-carotene, β-carotene and phytoene. However, these compounds are hydrophobic molecules with little or no soluble in water, they can access an aqueous environment when they are associated with protein or with other polar compounds. Encapsulation of carotenoids from *Neurospora* by using suitable copolymer can increase its solubility and stability. According to some studies, the application of carotenoids from *Neurospora* sp. for food and cosmetic colorants is very potential in the future.

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