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Review

Biotechnological potential of natural food grade biocolorants

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Color becomes the most sensitive part of any commodity not only for its appeal but also it enhances consumer acceptability. In addition, the color of a food substance is important to indicate its freshness and safety that are also indices of good aesthetic and sensorial values. For natural color and additives, adherence to the norms of biosafety protocol, are limited. The demand for natural source of such compounds is increasing day by day because of awareness of positive health benefit out of natural compounds. It therefore, necessitates looking into natural sources of food grade colorants and their use potentials. It is found more justified to use the term biocolorant instead of biopigment. Since pigments are mostly water insoluble with exceptions of certain pigments of biological origin. This article includes the advancements of process development and other biotechnological aspects of natural food grade colorants.

Key words: Biocolorants, natural food colorants, carotenoids, anthocyanins, betalains, biotechnology.

INTRODUCTION

Color is a molecule that absorbs certain wavelengths of visible light and transmits or reflects others whereas colorant is the activity of that molecule (Hari et al., 1994). Colorant shows activity when applied to a substance. Since the early civilization, products are made to give attractive presentation by addition of natural colorants. Colorants become the most sensitive part of any commodity not only for its appeal but also, it enhances consumer acceptability (Clydesdale, 1993). Although, Aristotle and other ancient super scientists had attempted to understand the nature of light and color vision, it was not until Sir Newton that light was identified as the source of color sensation and defined as a class of spectra that reciprocates to the same color sensation. Such classes vary widely among different species, and to a lesser extent among individuals within the same species. Color is the result of complex surface property, transmission property, and emission property of an object (Nassau et al., 1985). All such factors contribute to the mixture of wavelengths of light that leave the surface of an object for the acceptance by human eye. A color is conditioned by the nature of the ambient illumination, and also by the color properties of nearby objects, via perceiving ability of eye through brain (Stich et al., 2002).

The color from bright green spinach, ruby red strawberry, and deep orange pumpkin that add visual delight, are due to naturally occurring color compound. Many studies have emphasized the relation of color with the flavor detection threshold, with the sweetness or salinity sensations, also with the susceptibility for preference (Newsome, 1986; Frick et al., 1988; Clydesdale, 1993). Possible reasons for use of colorants in food substances are enumerated (FNB, 1971), as:

1) to maintain the original food appearance even after processing and during storage;
2) to assure the color uniformity for avoiding seasonal variations in color tone;
3) to intensify normal color of food and thus to maintain its quality;
4) to protect the flavor and light susceptible vitamins making a light – screen support; and
5) to increase acceptability of food as an appetizing item.

This article prefers to use the term biocolorant instead of

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using biopigments. Pigments are water insoluble substances that are used to color articles like ink, paper, textiles and many more. But certain biopigments like anthocyanin is water soluble. Moreover, there are some biocolorants which are not pigment in any sense (structural colors, light emitting luciferin). Hence, biocolorants are coloring agent obtained from biological sources. The term natural biocolorants indicates that the source of the biocolor is natural or nature identical (biotechnological) whereas the term artificial biocolorants indicate that though the colorant is synthesized biologically yet it can be produced chemically. Also, natural biocolorants are different from artificial biocolorants not only for its source, but also in their composition and physiological activity. Generally, impurities are much more in natural biocolorants. Yet bioactivity of natural biocolorants is much more than their chemical counterparts.

Use of natural colorant in food industry appears to have multidimensional potential (Dufosse, 2004). For example, in addition to coloring property, β-carotene may be used in food as an essential vitamin source or betalains as source of essential amino acid or anthocyanins as quality control marker of food stuffs. Flavonoids are colorants with high pharmacological promise. Sometimes, diets with carotenoid mixtures are recommended instead of having just one particular carotenoid, because great variability of radicals and microenvironments take place *in vivo* (Quintanan-Hernandez et al., 1996).

Process development and use of new and available technologies for production optimization are the important area of research on natural food grade colors. Therefore, it requires a thorough review of the biotechnological potentials of ‘natural food grade biocolorants’.

HISTORY OF BIOCOLORANTS

Man has always been interested in colors. The art of dyeing is of long past and many of the dyes go back into prehistory. In Europe, it was practiced during the Bronze Age. The earliest written record of the use of natural dyes was found in China dated 2600 BC. In Indian subcontinent, dyeing was known as early as in the Indus Valley period (2500 BC) and has been substantiated by findings of colored garments of cloth and traces of madder dye in the ruins of Mohenjodaro and Harappa civilization (3500 BC). In Egypt, mummies have been found wrapped in colored cloth. Chemical tests of red fabrics found in the tomb of King Tutankhamen in Egypt showed the presence of alizarin, a pigment extracted from madder. The cochineal dye was used by the people of Aztec and Maya culture period of Central and North America. By the 4th century AD, dyes such as woad, madder, weld, Brazilwood, indigo and a dark reddish-purple were known. Brazil was named after the woad found there (Gulrajani, 1992). Henna was used even before 2500 BC, while saffron is mentioned in the Bible (Gulrajani, 2001). Use of natural biocolarants in food is known from Japan in the shosoin text of the Nara period (8th century) contains references to colored soybean and adzuki-bean cakes, so it appears that colored processed foods had been taken at least by people of some sections. Study of color intensified, since late 19^{th} century, to understand:

1. the phenomenon for survival of animals and plants,
2. the relation between color and evolution theories; and
3. the role imparting in comparative physiology.

Thus, studies on biocolorant are greatly impulsed by their multiple functions (Newsome, 1986). The art of coloring spread widely with the advancement of civilization (Krishnamurthy et al., 2002).

SOURCE OF BIOCOLORANTS

Plants, animals and microorganisms are the sources of natural biocolorants, but few of them are available in sufficient quantities for commercial use as food colorant and mostly are plant origin. For biotechnological production of such colorants, plants and microorganisms are more suitable due to their understanding of proper cultural techniques and processing. Natural colorants obtained from plant origin are pepper, red beet, grapes, saffron (FDA/IFIC, 1993; Bridle and Timberlake, 1997). Nowadays, fermentative production of food grade pigments are available in the market , for example; color from *Monascus* sp., astaxanthin from *Xanthophyllomyces dendrorhous*, Arpink red color from *Penicillium oxalicum*, riboflavin from *Ashbya gossypii*, and β-carotene from *Blakeslea trispora*. Also a number of microorganisms produce biocolors in good amount that includes *Serratia* and *Streptomyces* (Kim et al., 1997).

BIOCOLORANTS IN FOOD INDUSTRY

To consider the natural biocolors as the colorant, stability, yield and price are mostly the constrains. Most of them are sensitive to pH, heat, and sunlight (Wissgot et al., 1996). Inspite of such factors, the natural biocolorants are gaining importance because of health and hygiene, nutrition, pharmaceutical activities, fashion and environmental consciousness, indicates relative dependency on natural products. Colors derived from minerals (lead chromate, copper sulphate) may cause serious health problems (FDA/IFIC, 1993). However, in last few decades, synthetic additives are severely criticized, and consumers show inhibition toward these products, consequently they prefer to use the natural colorants (Freund et al., 1988; Francis, 1989). In the 1960s in US, the environmental activists demonstrated against the use of such food additives, and this attitude was spread out widely. Activists campaigned for the natural colorants highlighting their nutritional characteristics as a sales tool. A strategy that has failed earlier, later converted it into a total success, resulting from changes in social attitude. Thus, a world wide tendency to use natural colorants is generated. Currently, people interpret the content of synthetic products as contaminant and the tendency has been reinforced (Francis, 1995). There are number of advantages of using natural biocolorants, over synthetic colorants, further boosted because of their pharmacological properties. Products are of good market value if they are colored with natural compounds, for example; annatto in Cheddar cheese (Freund et al., 1988).

REGULATION AND ASSESSMENT OF BIOCOLORANTS

The principal markets of food grade biocolorants are at US, EU and Japan and emerging markets are in China, India and South Korea. Food colorants are tested for biosafety before its promotion and are controlled by various regulatory bodies around the world and regulation varies in different countries (Hallagan et al., 1995). In US, FD&C (Food, Drug and Cosmetic) numbers are given to synthetic food colorants approved by FDA that do not exist in nature, while in EU, E numbers are used for all additives of food applications. Thus, the approved list of food colors varies along the countries, it means each country has its own approved list, including limit of maximum daily intake. Out of them, some other regulatory agencies are there like PMDA (Pharmaceuticals and Medicinal Devices Agency) in Japan, SFDA (State Food and Drug Administration) in China, CDSCO (The organization and function of the Medicines Agency) in India and KFDA (Korea Food and Drug Administration) in South Korea etc. Yet, most of the food grade biocolorants approved by FDA or EU are also approved by other agencies. For example, in India, Rule 26 of The Prevention of Food Adulteration Rules (PFAR) permits 11 colors for food use: Lactoflavin, Caramel, Annato, Saffron, Curcumin etc., also approved by EU and FDA.

Under FDA regulations (FDA/IFIC, 1993), a colorant added to a food product cannot be considered natural, no matter what the source is. Unless the colorant is natural to the food product itself, for example; strawberry juice or red beet color is used to make the ice cream a pink hue for strawberry ice cream, it would not be considered as naturally colored, because the colorant from strawberry or beet are not a natural component of ice cream.

FDA considered only few colorants as food additives (FDA/IFIC, 1993). In 1958 additives were redefined and classified with three categories, appeared as:

1) substances approved by the FDA or the USDA (United States Department of Agriculture) during 1938 to 1958;
2) GRAS (Generally Recognized as Safe) substances do not require FDA evaluation and
3) all other substances used in food are evaluated to fit with the recommendations of FDA before commercialization (Clydesdale, 1993; Wissgot et al., 1996; Wodicka, 1996). The natural colors under section 205.606 of FDA list are only allowed in organic foods.

FDA uses the term indirect additives to group those additives which are used in the coloration for animal feeds and in course the animals are used as human food (Freund et al., 1988). It may happen that, some of the natural colorants of non biological origin are decertified (Red Dye #2, amaranth). All these evidences suggest that, the food grade substances have to follow strict regulations.

Further among the food additives, color additives are in no condition be considered as GRAS substances. But the accepted natural pigments, or colors (from red beet, carrot, fruits, pepper etc.) are grouped under ‘exempt of certification’ category (FNB, 1971).

COLOR APPEARANCE

It is important to note that a single coloring agent may not give the desired effect, the background color and of neighboring colored substances make a large input in the color look (Martin et al., 2007). Product concepts, requiring blue or green, limit the choice from certified colors only. Bluish purple can be achieved with carmine, but it does not create a true blue (Stich et al., 2002). Annatto or turmeric tends to represent a cheese color or have an eggy tone compared with the bright color produced with the FD&C-yellow. Now-a-days, fluorescent colors are also getting importance in food industry as consumer favor foods to glow under conditions (Martin et al., 2007). Turmeric is highly fluorescent thus, are commonly used in food. Normally, the physical and chemical properties of food product limit the choice of a colorant. A list of available shades for food grade biocolorant is presented in Table 1.

FOOD GRADE NATURAL BIOCOLORANTS

Natural biocolorants are under ‘exempt from certification’ category of FDA and EU for food use and are: annatto extract, dehydrated red beet, canthaxanthin, β-carotene, *Dactylopuis coccus* extract, cotton seed meal, grape skin extract, fruit and vegetable juices, tagetes extract, carrot oil, oil of corn endosperm, paprika and paprika oleoresin, riboflavin, saffron, turmeric and turmeric oleoresin and xanthophylls (flavoxanthins, rubiaxanthins, zeaxanthin). Chlorophylls, in spite of its large intake amount per day,

Table 1. Different shades available from a wide range of food grade biocolorants.

Color	Wavelength interval	Biocolorants	Colorant status	Colorant amount	Shades
Red	~630 - 700 nm	Red Beet Juice (Betanine)	Powder	0.3% to 0.45	
		Paprika	Emulsion (water soluble)	40,000 IU	
		Paprika	Powder	40,000 IU.	
Orange	~590 - 630 nm	Kesar Orange / Mango	Powder	1.5%	
		Annatto (Bixin)	Emulsion (oil soluble)	1.5%	
		Annatto (Norbixin)	Emulsion (water soluble)	1.5%	
Yellow	~560 - 590 nm	Turmeric	Powder	5%	
Green	~490 - 560 nm	Chlorophyll	Emulsion (oil soluble)	-	
		Chlorophyll	Emulsion (water soluble)	-	
Blue-violet	~400 - 490 nm	Anthocyanin	Powder	-	

FDA disallow it as food additives, but EU allows it. Chemical natures of different food grade biocolorants are listed in Table 2.

Yellow to orange color of Annatto comes from the outer layer of seeds of the tropical tree *Bixa orellana*. The carotenoids, bixin, and norbixin, are responsible for appearance of yellow to orange color. The pH and solubility affect the color hue; the greater the solubility in oil, the brighter is the color. Water soluble, oil soluble, and oil/water dispersible forms of annatto are available. Since it precipitates at low pH, is also available as emulsion, an acid proof state (Gloria et al., 1995). Annatto has been used for over two centuries as a food color especially in cheese and in various other food products (Haila et al., 1996).

Red beet (*Beta vulgaris*) extract shows variety of colors, depending on their content of yellow compound and may have a good flavor. Also a bluish-red color produced by a compound known as betanin and is stable at higher pH range than red cabbage extract (Im et al., 1990). There is no limit on its upper usage level. It has wide application in different food commodity from beverages to candy and from dairy to cattle products (Counsell et al., 1979).

β-Carotene is orange-yellow in color, oil soluble but can be made into a water dispersible emulsion. It is listed as GRAS compound with no restriction of usage level. Carrot (*Daucus carota*) is a good source of β-carotene (Barth et al., 1995). But most β-carotene for commercial use is now derived from algae. In *Dunaliella salina* and *D. bardawil*, higher amount of β-carotene accumulation has been observed in response to a combination of high light intensity, hypersalinity, and under nutrient stress (Phillips et al., 1995).

Canthaxanthin, a carotenoid, is commercially produced from the algae *Haematococcus lacustris.* Canthaxanthin is known mainly as the natural pigment of the orange-yellow chanterelle mushroom. Canthaxanthin has various physiological functions and can be converted into vitamin A under stress. Canthaxanthin is used in poultry for the appearance of color shade of the yolk, also in cosmetics and foods, particularly in dairy products (cheese), confectionary (soft and hard candy), fish and meat products, fruit products, beverages, snacks, beer, and wine. It is more stable in photo degradation than that of β-carotene (Miller et al., 1996). However, canthaxanthin is not considered as food additive under EU regulation.

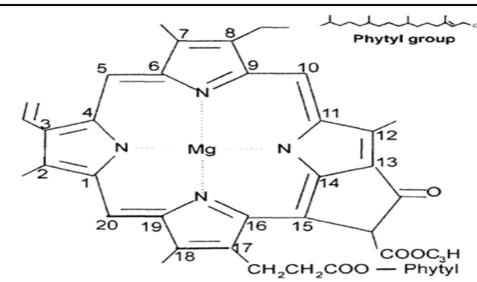
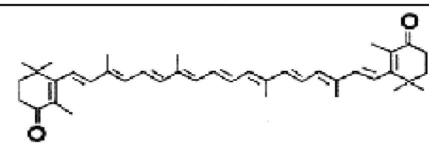
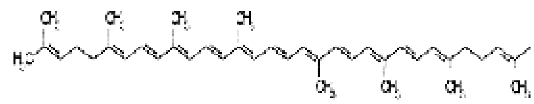
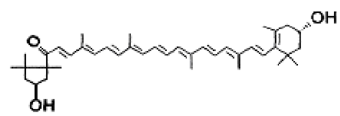
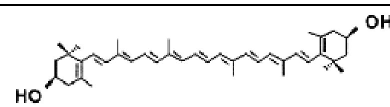
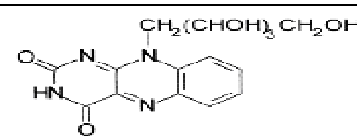
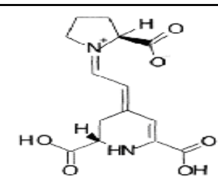
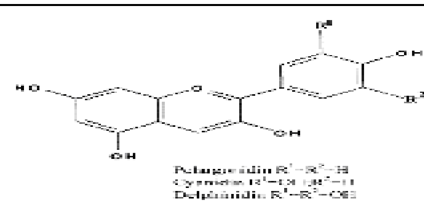
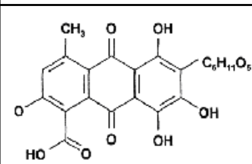
Cochineal insect (*Dactylopius coccus*), best cultivated on *Opuntia ficusindica*, extract is known to have carmine or carminic acid, which appears as magenta-red upon application. Water insoluble forms of carmine exhibit a color range from pink to purple. It is resistant to light, heat and chemical oxidation, often it is more stable than synthetic food colorants but unstable at low pH. The water soluble form is used in alcoholic drinks with calcium carmine; but water insoluble form is used in a variety of products. Together with ammonium, carmine is used in meat, sausages, processed poultry products, alcoholic drinks, beverages, bakery and dairy products, including desserts and sweets. On an average, people consume one or two drops of carminic acid annually with food (Ramirez-Cunz et al., 2008).

Cotton seed meal is the by-product after oil extraction from cotton seed. Gossypol is a fat soluble yellow pigment that occurs in cotton seed in bound and free forms. The bound form of gossypol is combined with free amino acids which constitute a medium quality protein feed (Conkerton et al., 1995).

Grape skin extract (enocianina) imparts a reddish purple color to beverages (Chenier et al., 1994). How-ever, the FDA restricts its use in alcoholic beverages or in beverage bases, also in carbonated drinks.

Orange, apricot, mango, and peach contributed significantly in increasing β-cryptoxanthin and β-carotene

Table 2. Chemical category of different food grade biocolorants.

Biochemical origin	Generic name		Example	Structure
Tetrapyrrole			Chlorophyll	
Isoprenoid	Carotenoid	Carotene	Lutine	
			Lycopene	
		Xanthophyll	Canthxanthin	
			Zeaxanthin	
	Irodoid		Crocin (Saffron)	
N-Heterocyclic Compounds (other than Tetrapyrroles)	Flavin		Riboflavin	
	Betalain		Betaine	
Benzopyran	Anthocyanins		Pelargonidin Cyanidin Delphinidin	
Quinone			Carminic acid	

concentrations of foods (Koes et al., 1994). Some fruits contain a single type of anthocyanin (cyanidin in apple, cherry, fig, etc), some contain two major types (cyanidin and peonidin in ascherry, canberry); or some with several anthocyanins (grape). As colorant, grape juice is available in a variety of colors: red (shades of cherry, raspberry or strawberry), purple and yellow. It gives a strawberry-red shade in application. It is used to color a number of non-beverage foods, including gelatin desserts, fruit fillings and in certain confectionaries. Anthocyanins from banana bract and *Oxalis triangularis* are found as potential source of food colorants (Alexandra et al., 2001). Acylated anthocyanins from different edible sources (black carrot cultivars) are also used in food industry (Giusti et al., 2003).

Vegetable juice is actually fermentable liquid product, either unfermented or lactic acid fermented, obtained from the edible part of one or more vegetables for direct consumption and preserved exclusively by physical means. The juice shall be free from skins, seeds and other coarse parts of the source vegetables. Tomato juice and blends based on tomato have long been popular and account for over 90% of the non-fruit juice trade. Lycopene is the principal compound derived from tomato, enlisted under US system. Under EU legislation, lycopene (E 160d) is considered as food additives. It is highly stable under a wide range of temperature and pH, hence used as common food colorant. It is available in liquid form or as cold-water dispersible powder. Carrot has long been a component of tomato blends (Chen et al., 1995). Red-cabbage juice produces a bright pink to red color to a product with a pH less than 4.0 and is soluble in water, but not in oil.

Use of Marigold (*Tagetes erecta*) flowers as source of food colorants is known from Aztec civilization. Marigold flowers are by far the most abundant natural source for commercial lutein (Vernon-Carter et al., 1996). The antimutagenic activity of carotenoids of Aztec marigold was evaluated.

The biocolorants and nutrients of carrot seed oil are: α-pinene (up to 13%), β-pinene, carotol (up to 18%), daucol, limonene, β-bisabolene, β-elemene, cis-β-bergamotene, γ-decalactone, β-farnesene, geraniol, geranyl acetate (up to 10%), caryophyllene, caryophyllene oxide, methyl eugenol, nerolidol, eugenol, trans-asarone, vanillin, asarone, α-terpineol, terpinene-4-ol, γ-decanolactone, coumarin, β-selinene, palmitic acid, butyric acid and other constituents (Vogelien et al., 1990). The presence of some essential amino and fatty acid increases the nutritional quality of carrot seed oil.

Corn endosperm oil is a reddish-brown liquid composed chiefly of glycerides, fatty acids, sitosterols, and carotenoid pigments obtained by isopropyl alcohol and hexane extraction from the gluten fraction of yellow corn grain (Bosevska et al., 1993). It is used in chicken feed as color additive.

Paprika (*Capsicum annuum)* is the pioneer and widely used carotenoid as food colorant. In paprika, red carotenoids are dominated by canthaxanthin, capsorubin where as yellow xanthophylls includes β-cryptoxanthin, zeaxanthin, antheraxanthin and β-carotene (Perez-Galvz et al., 2003). Paprika oleoresin is mainly extracted from the pods. It contains three main naturally occurring pigments: capsanthin, capsorubin and β-carotene. This combination produces a bright orange to red-orange in food products. The oleoresin is oil soluble, when emulsified becomes water dispersible (Vinha et al., 1997).

Riboflavin (vitamin B_2) has a variety of applications as a yellow food colorant. Its use is permitted in most countries. Applications include dressings, sherbet, beverages, instant desserts, ice creams, tablets and other products. Riboflavin has a special affinity for cereal-based products, but its use in these applications is somewhat limited due to its slight odour and naturally bitter taste. There are numerous microorganisms that produce riboflavin fermentatively (Stahmann et al., 2000). Riboflavin fermentation could be classified into three categories: weak overproducers (100 mg/L or less, *e.g. Clostridium acetobutylicum*), moderate overproducers (up to 600 mg/L, *e.g.* yeasts such as *Candida guilliermondii* or *Debaryomyces subglobosus*), and strong overproducers (over 1 g/L, e.g. the fungi like *Eremothecium ashbyii* and *Ashbya gossypi*).

Saffron (*Crocus sativa*) is considered the most expensive colorant as well as spice (Raina et al., 1996). Crocin, the main carotenoid in saffron, is water soluble (Tsimidou et al., 1993). The flower is light purple with thread-like red stigma, is the valued material. It takes in excess of 7×10^4 flowers to yield just 1 lb (0.45 kg) of saffron color. The odor of saffron is sometimes described as sea air to express its color shade and fragrance. The color appears as a powerful yellow in applications such as saffron rice (Winterhalter et al., 2000).

Turmeric is a bright yellow colorant made from the roots of the herb *Curcuma longa*. The pigments responsible for the color are known as curcuminoids: curcumin and related compounds. Solubility of turmeric compound depends on the processing medium. Turmeric oleoresin is water soluble; but oil extract can be added to fat based foods. At high pH, the extract turns orange. There is no usage restriction as long as the level conforms to Good Manufacturing Practices (GMP).

Chlorophylls constitute the most important subgroup of pigments under tetrapyrrole derivatives. Chlorophyll ‘a’ and ‘b’ are found in most of the plant groups, except algae and bacteria (Counsell et al., 1979; Lichtenhaler, 1987; Rudiger et al., 1988; Britton, 1991). It is used in jam, jelly, candy, ice cream and in several other products.

OTHER APPLICATION IN FOOD INDUSTRY

Food preservatives

Several natural biocolorants, including anthocyanin, additionally show antagonistic activity to certain bacteria, viruses and fungi thus to protect food from microbial spoilage (Bridle and Timberlake, 1997). Some are also active against protozoa (*Leishmania brasiliensis*), and insects (*Calliphora erythrrocephala*). Sometimes, carotenoids can act as sun screen to maintain the quality of food by protecting from intense light. It has been reported that corn carotenoids inhibit the synthesis of aflatoxin by *Aspergillus flavus* (90%) and by most of the *A. parasiticus* (30%) strains (Norton, 1997).

Quality control markers

Commonly for maintenance of GMP, level of anthocynin is used as an indicator to evaluate the quality of colored food (Boyles and Wrolstad, 1993). Anthocyanin profiles have been used to determine the quality of fruit jams. From anthocyanin profile, it can be easily detected that labeled black cherry jam which is prepared from common red cherries in reality or not (from less expensive fruit). In addition, adulteration of blackberry jam with strawberries can also be detected efficiently by the analysis of pelargonidin and cyanidin-3-glucoside content (Garcia-Viguera et al., 1997).

Nutritional supplements

Carotenoids are also used as vitamin supplements (Haliwell, 1996), since β-carotene is the precursor of vitamin A. In under developed countries, the diet is primarily of rice, there is every possibility of inadequate supply of vitamin A, which leads to night blindness and in extreme cases to xerophthalmia. Riboflavin is another example of natural food grade biocolorant which is an essential vitamin source and available in milk and in several leafy vegetables, meat, and fish (Counsell et al., 1979; Hari et al., 1994).

Yellow β-xanthins, in addition to their potential role as food colorant, may be used as a means of introducing essential dietary amino acids into foodstuffs. Cotton seed oil is not only a colorant but also enriched with essential amino and fatty acids. Gossypol is a fat-soluble yellow pigment that occurs, in bound and free forms, in cotton seed meal. But the bound form of gossypol is particularly used to combine with free amino acids (Conkerton et al., 1995). Cochineal colorant supplemented diet is recommended for hyperactive children.

Therapeutic properties

In addition to several other properties, β-carotene shows antioxidant properties particularly with carotenoids in paprika and annatto. Betacyanin is also with antioxidant and radical scavenging properties. Since betanin exerts a good bioavailability, red beet products may provide protection against certain stress related disorders. Owing to their outstanding antioxidant properties, xanthophylls particularly of lutein, zeaxanthin, and astaxanthin are lucrative as health care substance, and considered as protective agent against aging, mascular degeneration, and senile cataracts (Taylor-Mayne, 1996). It has been established that flavonoids present in different plant products (grape, soybean, peanut, wine, tea) show good antioxidant activity, sometimes better than the commercially available antioxidants (Frankel, 1993). Allomelanins (free of proteins) from plants, black bean, soybean, and sesame are found to suppress growth of tumorigenic cells of mammals. Lycopene, is thought to provide health promotion along with its function as a colorant. It is also supposed to act as an antioxidant. Grape seed extract is the primary commercial source of a group of powerful antioxidants known as oligomeric proanthocyanidins (OPCs), also generically called pycnogenol, a class of flavonoids. In addition to its coloring property, canthaxanthin also shows antioxidant property (Haila et al., 1996). Epidemiological studies have shown correlation between the consumption of chlorophylls and decreased risk of colon cancer (Fernandes et al., 2007).

STRATEGIC APPLICATION OF BIOTECHNOLOGY

Process developed with bacterial cultures

Among carotenoids under investigation for coloring and for biological properties, quite a meager number of compounds are available from natural extracts or by chemical synthesis. The list is rather short compared to the long list of 700 entries in the Carotenoids Handbook (Britton et al., 2004). Biotechnology could be a solution for providing more number of coloring compounds which are difficult to synthesize by chemical means.

Isorenieratene and hydroxyl derivatives were produced by *Brevibacterium linens*, *Streptomyces mediolani* or *Mycobacterium aurum.* Among these, the food grade *B. linens* (new name: *Brevibacterium aurantiacum* sp. nov.) is of particular interest as this microorganism is found in soft cheese. Its pigments have therefore been consumed by human for a long time.

A *Bradyrhizobium* sp. strain was described as a canthaxanthin (4, 4'-diketo-β-carotene) producer (Lorquin et al., 1997) and the carotenoid gene cluster was fully sequenced (Hannibal et al., 2000). This keto-carotenoid was also found in another microorganism, an extremely halophilic bacterium isolated from a salt farm, belonging to the genus *Halobacterium* (Asker and Ohta., 1999).

Culture of *Flavobacterium* sp. (Shepherd et al., 1976) in a nutrient medium containing glucose or sucrose, sulphur-containing amino acids such as methionine, cystine or cysteine, pyridoxine and bivalent metal ions was able to produce zeaxanthin 190 mg/L, with a cell concentration of 16 mg/g dried cellular mass.

Process developed with algal cultures

The alga *Haematococcus lacustris* contains large amounts of astaxanthin esters and has been considered as a potential source of astaxanthin and is commercially produced using bioreactor (Yuan et al., 1997). Also, echineone and canthaxanthin are identified in *Haematococcus* cultures. Other studies, *in vivo* (Yamane et al., 1997) and *in vitro* (Chumpolkulwong et al., 1997a) have shown that high astaxanthin production required high level of oxygen (aerobic conditions) and high C/N ratio but cell growth requires low C/N ratio. Also, it is suggested that the addition of ethanol during the second stage enhanced the production of astaxanthin 2.2 times whereas compactin resistant mutants of *H. pluvialis* (compactin inhibits HMGR that strongly blocks cholesterol formation) showed 2 times enhanced yield (Chumpolkulwong et al., 1997b). Several species of marine micro algae such as *Dunaliella bardawil* and *Dunaliella salina* produce β-carotene as their main carotenoid (Phillips et al., 1995).

Process development with fungal cultures

The fungus *Blakeslea trispora* is a well known β-carotene producer. The cell growth and β-carotene production are enhanced in medium containing surfactants such as Span or Triton, except Triton X-100 (Kim et al., 1997). HPLC analysis, stability tests and microbiological tests have shown that the β-carotene obtained by fermentation of *Blakeslea trispora* complies with the EC specification, and is free of mycotoxins or other toxic metabolites.

Another interesting model for β-carotene production is of *Phycomyces blakesleeanus* (Ootaki et al., 1996). The carotene content of the wild type grown under standard conditions is modest, about 0.05 mg/g dry mass; however, certain mutants accumulate up to 10 mg (Murillo et al., 1978). As for *Blakeslea trispora*, sexual stimulation of carotene biosynthesis remains essential to increase yield up to 35 mg/g (Mehta et al., 1997). However, it is found more potential while produced through fermenter (Cerdá-Olmedo, 2001).

Several strains of *Monascus* are exploited for commercial production of red and/or yellow pigments (Fabre et al., 1993). Solid state fermentation of *Monascus* using rice as substrate is well known in China and Japan. The red yeast, *Xanthophyllomyces dendrorhous* (formally *Phaffia rhodozyma*) synthesizes astaxanthin and zeaxanthin as its main carotenoids (Andrews et al., 1976, Roy et al., 2008). Commercial production of carotenoids using microorganism has been achieved in case of astaxanthin, by red yeast fermentation. Yeasts of the genus *Rhodotorula* synthesize carotenoids. Studies in this regard are done with *R. glutinis* but other important species are *R. gracilis, R. rubra,* and *R. graminis* (Sakaki et al., 2000; Simova et al., 2004; Tinoi et al., 2005).

Ascolor Biotech of Czech Republic is awarded patents of compounds from new fungal strains that produce a red colorant which can be applied in the food and cosmetic industries. The strain *Penicillium oxalicum* var. Armeniaca CCM 8242, obtained from soil, produces a chromophore of the anthraquinone type. After evaluation, the red colorant Arpink Red was recommended as 100 mg/kg in meat products are in non-alcoholic drinks, 200 mg/kg in alcoholic drinks, 150 mg/kg in milk products including ice creams and 300 mg/kg in confectionery items (Sardaryan et al., 2004).

Wood hydrolyzates are potential substrates for carotenoid production with *X. dendrorhous* (Yamane et al., 1997). Pine (*Pinus pinaster*) wood meals are used in the production of carotenoids. To achieve this, pine meals are treated with cellulases from *T. reesei* (Celluclast) and cellobiase from *A. niger* (Novozym). The enzyme hydrolyzed broth can be used for growth of *X. dendrorhous*, resulting in high growth rate (up to 0.07 /h) and high cell density (0.47 g/g of glucose, dry wt) and carotenoid yield up to 1.8 mg/l (Parajo et al., 1997).

Process development with plant cell culture

Plant cell and tissue culture are common practice for production of anthocyanins. Since culture ensures uniform quality and continuous production, anthocyanin production by cell cultures has been evaluated from *Vitis*, *Perilla*, *Aralia*, *Oxalis*, *Euphorbia milli*, *Fragaria*, grapes, carrot and from *Ajuga reptans* (Callebaut et al., 1997).

In cultured cells (*Vitis vinifera, Aralia cordata, Fragaria anansa*), high sucrose and low nitrate are found desirable for higher anthocyanin production. Though sucrose is an essential nutrient, it also acts as an osmotic agent when used at high concentrations. In cell suspension cultures of *Vitis*, phenylalanine supplementation inhibits cell division but enhances anthocyanin biosynthesis. Interestingly, it is observed that high ammonium concentration (24 mM) promotes anthocyanin acylation, which is important for food industry, since it improves anthocyanin activity by imparting stability (Kakegawa et al., 1995).

Another factor analyzed in plant cell pigment production is the pH, in relation to color stability. For anthocyanins, the optimum pH is toward acidic (pH < 7.0). Maximal growth of strawberry cells in suspension cultures is found at 30ºC, but increased level of anthocyanin production is at 20ºC and at pH 8.7 (Zhang et al., 1997). A two stage process (first stage for cell growth and second one for anthocyanin production) and model for batch and semicontinuous anthocyanin production are developed and found to promote anthocyanin accumulation to six fold in conditioned medium (Suvarnalatha et al., 1994).

Cultured cells of *Perilla frutescens* need light irradiation for 7 days for optimum anthocyanin yield (Zhong et al., 1991). In callus cultures of *O. linearis*, anthocyanin production is promoted by cytokinins but repressed by auxins such as NAA and 2,4-D. In darkness, cell cultures showed an intense pigmentation after illumination, although cultured cells of *Aralia cordata* have shown higher anthocyanin accumulation in total darkness. Riboflavin supplementation in *Fragaria anansa* cell cultures, promoted 3.2 times enhanced anthocyanin production, but only under light conditions.

In carrot, clonal variability has been observed to obtain less and high colored cells. Interestingly, the high colored cells have an intermediate limitation reflected by increment in pigment production under supplementation with dihydroquercetin, naringenin, and 4-coumarate (Vogelien et al., 1990). In carrot, suspension culture, fructose favors growth, while anthocyanin production is better with glucose, suggesting quantitative differences in the metabolism (Zwayyed et al., 1991). Culture filtrates and cell extracts of *Bacillus cereus*, *Staphylococcus aureus* and *Escherichia coli* (among others) are used as elicitors for anthocyanin production in carrot cell cultures, to get enhanced production up to 77%.

Possibilities to regenerate saffron plantlets, an *in vitro* corm development through somatic embryogenesis are understood. Evaluation of *in vitro* corms under field conditions revealed that, development and survival of corm are directly proportional to initial corm weight. *In vitro* survival percentage of corms having average corm weight less than 1 g is only 26%, whereas it is above 88% in corms above 1 g.

Siva et al. (2005) studied *Bixa orellana*, a food colorant yielding plant, for understanding the relationship between degree of genetic diversity (using isozymes) of various populations and their pigment content.

Betalain production has been detected in cell cultures of plant species belonging to five families of Caryophyllales. Red beet cell cultures (Leathers et al., 1992) and hairy root cultures (Hempel et al., 1997) are developed for betacyanin accumulation. Plant cell cultures are generally deep red or purple; indicate dominance of betacyanins. Plant hairy roots have become of interest as an alternative for cell culture, as well as their ability to accumulate betalains at comparable levels (Taya et al., 1992). Extra cellular production of betalains is observed in hairy root cultures under oxygen starvation (Kino-Oka et al., 1996). L-amino acids (total 9) to hairy root cultures of *B. vulgaris* are administered (Hempel et al., 1997). It causes *de novo* synthesis of two betaxanthins, portulaxanthin-II and vulgaxanthin-I, with minor quantities of muscaauri-VII, dopaxanthin, and indicaxanthin. These results give substantive support to establish the possibility of betaxanthin production at commercial level since, red beet is one of the best known betalain production models.

Production management by GM organisms

With the isolation of plant biosynthetic genes, it has been possible to study their regulation in response to environmental and developmental factors. Carotenoid overproducing plants have been generated with the aid of recombinant DNA technology.

Biotechnological approach has been used to engineer yeast strains for carotenoid overproduction. *Phycomyces blakesleeanus* carS mutant accumulates up to 100 times β-carotene than the wild type (2 to 5 mg/g dry wt) (Hempel et al., 1997). The mutant *P. blakesleeanus* S276 produce 9.2 µg - 1.6 mg/g dry wt. Astaxanthin overproducing *X. dendrorhous* JB2 shows production increment of 2.3 times (1.54 µg/mg dry wt) in relation to the wild and is commercially utilized for astaxanthin production from corn by-products (Bon et al., 1997). Jones et al. (2004) genetically modified the fungus, *Fusarium sporotrichioides* to produce the colorant and antioxidant, lycopene from the cheap corn fibre material, the leftovers of ethanol production. Using a novel, general method for the sequential, directional cloning of multiple DNA sequences, the isoprenoid pathway of the fungus was redirected toward the synthesis of carotenoids. Strong promoter and terminator sequences are added to carotenoid biosynthetic genes from the bacterium *Erwinia uredovora*, and the chimeric genes are introduced in the fungus and expressed at levels comparable to those observed for endogenous biosynthetic genes.

The heterogeneity found in the natural saffron population for morphological, developmental and yield component, are primarily due to genetic and environmental factors (Raina et al., 1996). Development of high yielding genotypes using the existing gene pool of saffron applying the biological tools are found potential for improving the productivity. Ten elite clones with distinct yield superiority are selected. Due to the absence of sexuality, mutation breeding is another approach for creation of genetic variability in saffron (Xuabin, 1992).

Golden rice is one of the major break–through in this regard. The β-Carotene biosynthesis pathway is introduced into rice endosperm by genetic engineering to tide over vitamin A deficiency (Beyer et al., 2002). It has been found as health promoting effect from a case study in Philippines (Zimmermann et al., 2004). Nutritional value of golden rice is improved through pro-vitamin A content (Paine et al., 2005).

Production management by ecofarming and ICM

For residual free production of food materials, there is no alternative of ecofarming. Economically viable natural colorants of plant origin may be cultivated in farm land and saffron and cochineal are the two most valuable of such plant products.

Commercial saffron is produced from dried stigmas of *Crocus sativus*, a member of the large family *Iridaceae*, and is cultivated in Azerbaijan, France, Greece, India, Iran, Italy, Spain, China, Israel, Morocco, Turkey, Egypt, and Mexico (Xue, 1982; Xuabin, 1992; Negbi, 1999). To understand the factors for better saffron yield, a series of studies are carried out during 2001 and 2002 from four main saffron producing areas of Iran. Generally, 4 kg/ha yield is common but several farms produce over 7 kg/ha. It is a short statured plant growing slowly. So, dense weed growth at any stage of crop production will have an adverse effect not only on its yield but on quality of the product as well. In terms of integrated weed management (IWM) more attention is paid to non-chemical weed control methods particularly physical, mechanical and crop-based approaches. Saffron is produced worldwide at an annual rate of 50 tons with a commercial cost of about $50 million (Negbi, 1999).

Global demand of carminic acid (*D. coccus*) as a basic colorant in food, drink, and cosmetic products has increased notably as a result of the prohibition of the use of synthetics (Baranyovits, 1978). The few countries that produce commercial carmine are Peru, Mexico, the Canary Islands and, more recently, Chile and Bolivia (Vigueras et al., 2001). Only at Peru, the commercial production of cochineal is 200 ton/year, whereas at Canary Islands only about 20 ton/year. France is believed to be the world's largest importer of cochineal, but Japan and Italy come next, along with Chile and Mexico. As much as 85% of the world demand is still gathered from the wild prickly pear cactus ('tuna' - as known locally) that grows thickly on the mountain sides in central Peru. Instituto de Investigacion Tecnologica Industrial y de Normas Tecnicas (IITINTEC) and Simon Fraser University of Peru have worked together to improve the carmine dye extraction process, providing a 23% yield of 62% pure carmine (commonly 20-23% yield of 52% pure carmine). Approximately 130,000 insects or 2 Kg dry insects are required to produce 1 Kg cochineal color and approximately 200 Kg of dried insects are produced weekly at the largest cochineal farm (Aldama-Aguilera et al., 2005). It is suggested that the reproductive tract of *D. coccus* is the possible site of carminic acid synthesis.

Annatto shrubs generally bear fruit after 2 years (Neal, 1965). Under ICM, plants develop fruit within 1 year of planting. An Indian plantation yields 529 kg/ha at the 2nd year and 2,483 kg/ha at 3rd year (Kanjilal et al., 1995).

CONCLUSION - CHALLENGES AND SCOPE

The number of approved colorants for food industry is limited, with certain exception in China and Japan. Therefore, it is important to look into traditionally used food colorants of different countries, to use as alternative source for food grade natural colorants. The source material for traditionally known food colorants may also be exploited in a different way. In China and Japan *Monascus* pigments, obtained as fermented products of rice and bread, have been used as food colorants and as therapeutics (Fabre, 1993). The red yeast *Rhodotorula glutinis* produces carotenoids which are used as natural food grade biocolorant and in fish diets (Park et al., 2007). However, it is not supported by the legislation of FDA and EU.

Some approved food colorants are known by their chemical name (canthaxanthin) while others are known by source (fruit juice or vegetable juice). The biocolorants identified by their chemical name can be synthesized easily by cheaper biotechnological sources. Coffee husks and *Eugenia myrtifolia* fruits are example of future alternative source for anthocyanins (Prata et al., 2007). Indian black plum, *Syngium cumini* has been found as a potential source of natural food grade biocolorant (Veigas et al., 2007). Biotechnology may play a crucial role for large fermentation of natural biocolorants. Even when the biocolorant is known by the source, organic farming and integrated crop management is necessary to yield food grade product. Strategic applications of biotechnology for production of natural food grade biocolorants are presented in Table 3.

It is also necessary to explore multidimensional application of natural food grade biocolorants. Red beet is the most common source of betalains. Although, structurally related to alkaloids, betalains does not cause toxic effects in the human system, and present considerably in high amount in certain foodstuffs, such as red beet, prickly pear fruits, and *Amaranthus* seeds. Moreover, there is no upper limit for recommended daily intake of betalains. Interestingly, certain foods are prepared in a way so that they can generate light (*biolume* products) in which betalains could play an important role, suggesting how exciting the area of natural colorant becomes. To mind the improvement of stability factor of color of strawberry beverage by fortification with polyphenolic copigments of rose petals is already done (Plamen et al., 2007).

Technological limitations are the major bottleneck for the commercial exploitation of the source materials. Designing of a proper bioreactor would help to ease out the situation. Commercial production of colorant by plant cell tissue culture could be achieved only when a fully automated predictable process with a more advanced technology is to be established by the joint effort of biotechnology and bioprocess engineering. Though mutant varieties with acquired character of food ripening and carotenoid accumulation are identified, but most of the regulatory genes are yet to be isolated (Bartley et al.,

Table 3. Strategic application of biotechnology in biocolorant production.

Name of food grade biocolorants	Original source	Biotechnological source	Large scale production using biotechnology
Monascorubramine	*Monascus purpurious*		Fermentation and bioprocess engineering
Astaxanthin	Plants	Fungus: *Xanthophyllomyces dendrorhous* Algae: *Haematococcus lacustris*, *H. pluvialis* compactin resistant mutant	Fermentation and bioprocess engineering
Arpink red		Fungus: *Penicillium oxalicum* var. armeniaca CCM 8242	Fermentation and bioprocess engineering
β-Carotene	*Daccus carota*	Fungus: *Blakeslea trispora, Phycomyces blakesleeanus car S* mutant	Fermentation and bioprocess engineering
		Algae: *Dunaliella salina, D. bardwil* GM plant: Golden Rice	Organic farming and ICM
Riboflavin	Milk	Moulds: *Ashbye gossypii, Eremothecium ashbyii, Ashbya gossypi*	Fermentation and bioprocess engineering
		Yeast: *Candida gulliermndii, Debaryomyces subglbosus* Bacteria: *Clostridium acetobutylicum*	
Bixin and Norbixin	*Bixa orella*		Organic farming and ICM
Betanin	*Beta vulgaris*	Higher yielding plant generated through somaclonal variation Hairy root culture	Organic farming and ICM Fermentation and bioprocess engineering
Canthaxanthin		Algae: *Haematococcus lacustris* Bacteria: *Bradyrhizobium* sp.	Fermentation and bioprocess engineering
Carminic acid	*Dactylopius coccus*		Organic farming and ICM
Cyanidin and Peonidin	Ascherry, Canberry	Higher yielding plant generated through somaclonal variation Cell culture	Organic farming and ICM Fermentation and bioprocess engineering
Acylated anthocyanins	Black Carrot		Organic farming and ICM
Lycopene	Tomato	GM fungus: *Fusarium sporotrichioides* GM bacteria: *Erwnia uredovors*	Fermentation and bioprocess engineering
Lutain	*Tagetes erecta*		Organic farming and ICM
Cepsorubin	*Capsicum annuum*		Organic farming and ICM
Zeaxanthin	Corn	Bacteria: *Flavobacterium* sp.	Fermentation and bioprocess engineering
Crocin	*Crocus sativus*		Organic farming and ICM
Curcumin	*Curcuma longa*		Organic farming and ICM
Chlorophyll	Spinach		Organic farming and ICM
Isorenieratene		Bacteria: *Brevibacterium aurartiacum*	Fermentation and bioprocess engineaing

1995; Chappell, 1995). There is scope to develop new microbial strains or transgenic plants for over production of coloring compound.

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