Production of Natural Food Colours Using Biotechnology

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Abstract

Biotechnology is a science which deals with the genetic manipulations of microorganisms, cells, tissues or their components in order to increase the production of the desired product significantly or improve the particular quality attribute of desired product or both. Different biotechnological approaches such as genetic engineering of microorganisms and plant tissue culture are being exploited world-wide for production of flavor and aroma products, biocolors, natural sweeteners, flavor potentiators etc. Biotechnology as an integrated multi-disciplinary science has profound impact on every industrial sector and the field of natural food colors is no exception to this. Research is being carried out to resolve the color crisis in the food industry through the biotechnology. In our work, an effort is made in solving the problems of natural food colors, which include: (1) improving the traditional methods for extraction of pigments, (2) microbial production of pigments, (3) *in vivo* pigment production by plant tissue culture and (4) genetic modification for pigment production.

Keywords: Natural food colors, Microbial production of pigments, Enzymatic pigment extraction, *Pseudomonas* species

1. Introduction

Food additives are those ingredients which are incorporated in the food to improve its texture, appearance and shelf life. Such additives comprise of a wide range of ingredients such as colors, flavors,, aroma compounds, sweeteners, emulsifiers, stabilizers, preservatives etc (See Table I - Ref. 7).

Sr. No.	FOOD ADDITIVES	KIND OF FOOD ADDITIVE	MICROORGANISM/ PLANTS (Tissue culture) INVOLVED
1.	Vanillin	Aromatic compound.	*Pseudomonas* species
2.	Eugenol	Aromatic compound.	*Corneybacterium, Pseudomonas*
3.	Ferulic acid (ref. 9.1.4)	Aromatic compound.	*E. coli, Pseudomonas*
4.	Glutamic acid (ref. 9.1.2)	Flavor	*Brevibacterium, Corneybacterium, Symphytum officinale*
5.	Citric acid	Flavoring agent	*Aspergillus niger*
6.	Cinnamic acid	Flavoring compound	*Nicotinia tabacum*
7.	Beta-carotene	Coloring compound	*Dunaleilla, Euglena*
8.	*Monascus* pigments	Color / pigments	*Monascus*
9.	Citronellol	Fragrance compound	*Ceratocysis variospora*
10.	Methyl benzoate	Fragrance compound	*Phellinus* species
11.	Monosodium glutamate	Flavor potentiator	*Corneybacterium*[7], *Bacillus subtilis*
12.	Thaumatin	Sweetener	*E. coli*
13.	Aspartame	Sweetener	*E. coli, Bacillus subtilis*[1]

Table I. Important food additives produced through Biotechnology (ref. 7)

Color is the most important characteristics of food, since common consumers usually judge the quality of the food from its color. An overall objective of addition of color to food is to make it appealing and recognizable. Moreover, there are several technical reasons for addition of color to food. In decade of sixty, synthetic colors such as azo dyes became highly popular as found colors owing to their low cost and easy availability. However, subsequently toxicological evidences and adverse physiological effects of many such synthetic colors have been deleted from permitted list for food uses and even some more are likely to be banned in near future. Consequently, the attention of food industry shifted towards suitable natural alternatives. Though the natural colors enjoy advantage of being relatively safe, they do suffer from several drawbacks such as their seasonal production, variation in quality and purity from source to source, available only in limited color shades, low concentration in source material, difficulties in extraction from the source, instability during storage and use, high cost etc. An attempt has been made to collate the information on the above written aspects (See Table 2- Ref. 1).

Microbes	Compounds	Solubility	Colour	Use
Bacteria				
Mycobacterium lacticola	Astaxanthin	Fat	Orange	Fish feed
Brevibacterium				
Flavobacterium	Canthaxanthin	Fat	Orange	Poultry feed
Yeasts	Zeaxanthin	Fat	Orange	Poultry feed
Phaffia rhdozyma				
		Fat	Orange	Fish feed
Rhodotorula	Astaxanthin			
Rhodosporidium		Fat	Orange to Yellow	
Sporobolomyces	β Carotene Torulene Torularhodine			Fish feed

Table 2 Colors Produced by Microorganisms (ref. 1)

2. Improving Traditional Methods for Extraction of Pigments

The traditional methods for extraction of coloring agents usually involve disruption of the source material followed by acidified aqueous extraction for anthocyanins, beet-red, cochineal etc. or by solvent extraction for chlorophyll, carotenoids, annatto etc. But, these natural coloring extracts, especially those obtained through aqueous extraction or direct pressing of plant materials; contain required pigments along with high amount of unwanted dissolved solids like salts, organic acids, phenolics, sugars etc. Such high solid extracts on concentration produces hygroscopic material which has several drawbacks. Various efforts are being made to eliminate the above discussed problems encountered in extraction and preparation of the natural coloring materials by use of enzymes and/ or microbes aided extraction from the conventional sources.

2.1 Enzymes Aided Extraction (Ref. 1)

Enzymes are used to facilitate the extraction, increased pigment yield and improve the stability and quality of the result and color preparation.

For commercial extraction of astaxanthin (See Figure 1- Ref. 5) from *Phaffia rhodozyma*, extra cellular enzymes produced by *Bacillus circulans* are used (Ref. 5).

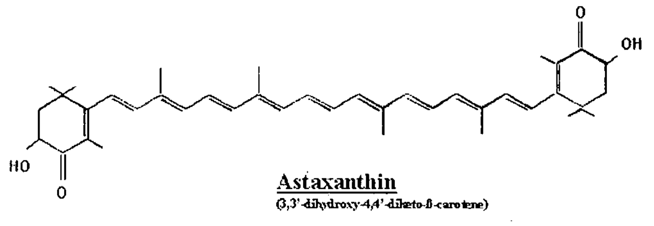

Fig. 1 Astaxanthin Structure (Ref. 5)

These enzymes digest the yeast cell-wall and allow easy extraction of pigment by acetone or ethanol, endopoly (-galacturonase) from Aspergillus species is used for extraction of beta-carotene from tissues and juices of various vegetables such as spinach, sorrel, lettuce, squash, green pepper, carrot etc.

Glycosidic and proteolytic enzymes such as beta-galactosidase and bromeilain are used in extracting saffron from gardenia fruits. In the case of algal biliproteins, proteins, protease is used to release the protein bound pigments.

Advantages over Traditional Methods

1. Proteinase treatment of cochineal extract produces better quality cochineal.
2. Similarly, treatment of pulped grape gives satisfactory extraction of anthocyanins and fermentation time is reduced considerably.
3. Addition of invertase to pectinase treated beet-juice increases betalains content by three fold when the juice is purified and concentrated by membrane processing. Use of these enzymes in extraction of anthocyanins from strawberries increases the pigment content up to 80%. The maximum juice of carrot can be achieved by cellulase + pectinase treatment of carrot, which gives 25% enhancement of carotenoids concentration in suspension.
4. When comminuted orange-red are pretreated with crude microbial enzymes obtained from yeast (*kluyveromyces* species) or moulds (*aspergillus* species), the carotenoids pigment extraction increases by 46-65%.
5. Pretreatment of annatto seeds with enzyme papain, cellulase or pectinase enhances the recovery of pigment during extraction in edible oil preparation of oil-soluble annatto-color. When all these enzymes are used in combination, the recovery of pigment is further enhanced.

Year	Development
1884	Monascus sp. was traditionally cultivated and utilized in the Orient for making red rice wine, red shaohsing wine and red Chinese rice
1954	The first carotenoid pigment from Cryptococcus was marketed
1963	Production of carotenoid pigments from Rhodotorula sp. started
Early 1970's	Astaxanthin was isolated from Phaffia rhodozyma, (in honor of Prof. Herman Jan Phaff) grown on exudates of deciduous trees in Japan and Alaska
Late 1970's and early 80's	Production of ß – carotene from Dunaliella salina took place
1985	Betatene Limited Corporation was established for cultivation of D. salina on large scale for producing natural ß – carotene products

Table 3 History of Production of Microbial Colors (Ref. 1)

2.2 Microorganisms-Aided Extraction (Ref. 3)

As discussed earlier, traditional methods (See table 3- Ref. 1) of extraction have several drawbacks, for e.g.: powdered beet-root extract has a characteristic odor and high nitrate-nitrite content, which limits its application in foods. Similarly, sugars in anthocyanins extract produce furfural during concentration and drying extract, which leads to pigment degradation. Moreover, the presence of such co-extracted materials limits concentration of the pigment concentrate prepared from the extract. The remedy for such problems is application of fermentation using appropriate microorganisms during and/ or after extraction.

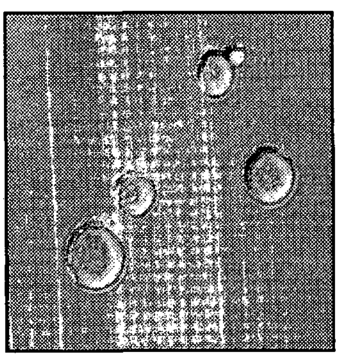

Fig. 2 *Saccharomyces cerevisiae* (Ref. 10)

Approximately, 80% of beet-root juice solids are carbohydrates and nitrogenous compounds; fermentation of the juice (beet-root) using yeasts (*Saccharomyces* species) and moulds (*aspergillus* species) produces the solids substantially, giving 5-7 fold increase in pigment content on a dry weight basis.

The dried products become free from beet-root odor and have reduced nitrite-nitrate content. For the production of food coloring matter from the source, the extracted juice is fermented using pure culture of wine-yeast. Similarly, for the production of color concentrate with minimum sugar and acid content and maximum purity with elder berries, involves fermentation process using *Saccharomyces* species (See figure 2- Ref. 10).

Advantage

In application of such fermentation process, yeast cells and ethanol are important byproducts obtained during the process. These yeast cells may be harvested and can be used as a source of biomass for animal food supplement. Ethanol's economic and industrial values are well known.

3. Microbial Production of Color Compounds

Production of materials by culturing microorganisms has several advantages. The rapid growth of microbes cuts the production time to matter of days and the process leads itself to continuous operation compared to plant or animal sources. Moreover, production is flexible and can be controlled easily.

3.1 Bacteria

Carotenoids are very well-known and highly popular food colorants. They are produced on a large scale by alkaliphillic yeast pigmented strains of *Bacillus* and *Dunaliella salina* (a type of halophile pink micro-algae especially found in sea salt fields, known for its anti-oxidant activity. Few organisms can survive in such highly saline conditions as salt evaporation ponds. To survive, these organisms have high concentrations of β-carotene to protect against the intense light and high concentrations of glycerol to provide protection against osmotic pressure. This offers an opportunity for commercial biological production of these substances). The commercial cultivation of Dunaliella for the production of β-carotene throughout the world is now one of the success stories of halophile biotechnology.

3.2 Yeast

The carotenoid, astaxanthin, produced by yeast *Phallus xhodoxyma*, is considered as the important source of the natural pigment for coloring foods. Efforts have been made by research workers for optimization of astaxanthin production by yeast.

The studies include effect of temperature, pH, and source of carotenoid, substrate concentration etc. The growth of *Phaffia rhodozyma* with 10% molasses gives 2-3 times more astaxanthin than with glucose or sugar blend. Use of grape juice may also be a useful raw-material, especially, where surplus of grape juice is available. Temperature is an important factor in the pigment production. At 200 C, mainly astaxanthin (80-90%) is produced. In addition to *Phaffia rhodozyma*, even food spoiling yeast, *Xerophyte*, produces highly pigmented colonies, which may be likely to contribute in the natural colorings for food in near future.

3.3 Moulds

The flingus *Monascus* produces at least six major related pigments which could be categorized as: -

Orange pigments: rubropunctatin ($C_{21}H_{22}O_5$) and monascorubin ($C_{23}H_{26}O_5$).

Yellow pigments: monascin ($C_{21}H_{26}O_5$) and ankaflavin ($C_{23}H_{30}O_5$).

Red pigments: rubropunctamine ($C_{21}H_{23}NO_4$) and monoscorubramine ($C_{23}H_{27}NO_4$).

Traditionally, *Monascus* has been cultured on rice and other cereals, by solid state fermentation. The red colored rice (Anka or Angkak) is used as a natural colorant for bean curd, meat and wine. *Monascus* pigments have been used extensively in Asia for centuries and are generally recognized as safe (GRAS) as food colorant. Nowadays, the purified *Monascus* red pigments are widely used as colorants in processed seafood, sausages and sauce in Asia. The pigment is extensively used in oriental countries like China, Indonesia, and Japan etc. as a color additive in wine and also as preservative in meat. In Chinese medicine, the red mould rice is purported to cure several illness such as indigestion, nausea, dysentery, muscle bruises etc. The applications of *Monascus* pigments are:-

1. Instant Noodles

A noodle making company evaluated the *Monascus* pigments as colorant for flavoring, ingredients of instant noodle, and found that it was acceptable and did not interfere with the flavor components.

2. Chinese Sausage and Dumpling (Bao)

3. Milk-Products and Candy

The crude pigment is a mixture of red, yellow and purple pigmented polyketides. It can be made water-soluble as well as oil-soluble, thus, useful for both aqueous-based and oil-based food products. The pigment is heat-stable and can autoclaved. These properties together with a color range from yellow to red make the pigment a good communicate for usage as food colorant. The details for production of pigmented by organisms of flingus *Monascus*[6] has been studied very extensively. Effects of various substrates, the substrate concentration, pH, aeration, oxidation, ferric ion etc. are investigated.

Algae

The development of micro-algae biotechnology has created a well published branch of industrial microbiology: Micro-algae constitute a reservoir of substances of commercial values. The production of valuable biochemicals from micro-algae is based on exploiting their highly efficient photosynthetic machinery. Red algae (*Rhodophyta*) (See Figure 3- Ref. 10) and blue green algae (*Cyanophyta*) produce group of light absorbing pigments based on bilin or tetra pyrrole skeleton.

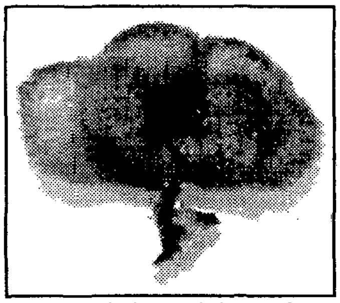

Fig. 3 *Rhodophyta* (Red algae) (ref. 10)

These phycobilin proteins have potential as natural colorants in food, cosmetic pharmaceuticals particularly substitutes for synthetic

Main pigments	Chlorophyll-a, alpha- and beta-Carotene, c-Phycoerythrin, Allophycocyanin, r-phycocyanin, other chlorophylls absent, though Chlorophyll-d has been reported once.
Nuclear material	Organized into membrane-bound nucleus
Food reserves	Floridean starch
Chloroplast features	Chloroplast present, membrane 2-layered, the thylakoids are unstacked and phycobilisomes present.
chloroplast endoplasmic reticulum	absent
Cell wall	Cellulose (or sometimes xylans), polysaccharides, calcification in some species.
Flagella	Absent

Table 4 Characteristics of Rhodophyta Algae (Ref. 9)

dyes (See table 4- Ref 9). The protein bound pigments are separated by proteinase treatment and extracted into dilute alkali. Pigment preparations either in water or alcohol are suggested for use in chewing gum, frozen confections, sherbets, confectioneries and ice-candies.

4. Plant Tissue Culture Pigment Production

Plant Tissue Culture (PTC) in fermentation has potential for production of secondary metabolites such as pigments, flavoring compounds, pharmaceutical etc. PTC technique offers several advantages over the conventional plant sources. These include:-

i) More reliable and more predictable, since it's independent of weather, season, plant variability etc.
ii) Continuous harvesting rather than once or twice in a year.
iii) Simple and easy extraction due to highly aqueous aggregates of cell instead of complex tissue of plant-organs.
iv) Reduction or even elimination of co-extracted material, thus minimizing or avoiding problems of co-extracted material.

Two most important steps in optimizing pigment production through PTC is:-

i) Continuous selection of high producer cells.
ii) Control of photo chromes level.

The other important parameters are: - (1) Light. (2) Aeration. (3) Nutrients and (4) Mutation of cells. To be economically viable, only those metabolites with a market value of around 480 US $ per kg could be considered for production through PTC. Attempts have been made and success has been obtained to produce anthocyanin, betanin, carotenoids, saffron and other pigments through PTC.

5. Genetic Modification for Pigment Production

In genetic modification, hereditary apparatus for animal, plant or bacteria cell is altered so as to produce more or different chemicals or perform new function. The alteration in hereditary apparatus may be bought about by:-

i) Appropriate mutation through physical and/ or chemical means.
ii) Genetic engineering through recombinant DNA technique.

Typically, pigment is produced intracellularly in the organism. However, by cloning of genes responsible for pigment production, it's possible to obtain hyper-pigment producers. These hyper-pigment producers excrete pigment into the growth medium, thus making the process more economical. Example of such technique is extra-cellular secretion of the pigment by the mutant of *Monascus* as discussed below: "A hyper pigment-producing mutant, R-10847, was derived from *Monascus kaoliang* F-2 (ATCC 26264) through a series of mutagenesis steps. The mutant produced a large quantity of *Monascus* pigment when grown in mantou (steamed bread) by solid culture. The mutant produced pigments extracellularly by extruding the pigments outside the cell in a lump together with some viscous substances. The productivity of pigment was about 100-fold greater than that of the wild type. The mutant lost the capability of spore formation, the growth rate decreased, and both the size and quantity of conidia were reduced. The color of the pigment produced by the mutant changed from orange to deep red according to the following reaction":

Monascus kaoliang (Orange) —NTG→ R-1 —UV→ R-108 —NTG→ R-1084 —S→ R-10847 (Deep-red)

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1. **NTG**: N-methyl-N'-nitro-nitroso guanidine.
2. **UV**: Ultraviolet radiation.
3. S: Spontaneously UV, UV light Irradiation.

The mutated *Monascus* (R-10847) has been widely used in the Orient for red wine brewing, red soybean cheese processing, food coloring, and medical therapy. Since the finding of carcinogens in coal tar dyes, a series of synthetic food colors was successively banned, and Monascus pigment has been considered as a natural pigment to replace the synthetics. Its pigments hydrolytic enzymes such as amylase and protease have been studied extensively.

6. Conclusion

The world market (See table 5- source ref. 9.2.4) for natural food colors is expanding at very rapid rate mainly due to pressure brought about by changes in legislations and consumers preference for food products containing only natural ingredients. However, limited availability of suitable natural alternative and difficulties in their extraction and preparation are the challenges to the manufacturers of natural food colors. Biotechnology can certainly play an important role in meeting the current and future requirements of natural colors for food.

Sr. No.	NAME OF THE ORGANISATION	PRODUCT
1.	Roche Products Pvt. Ltd., Australia	Carotenoids
2.	Roche Vitamins Fine Chemicals, Canada	Beta-carotene
3.	Gist Brocades, The Netherlands	Beta-carotene
4.	Bush Boake Allan Co., U.S.A.	Natural colors and wide variety of Bioflavors
5.	Overseal Color Inc., U.S.A.	Curcumin and Anthocyanins
6.	BASF, Germany	Beta-carotene and Eugenol
7.	Chr. Hansen, Denmark	Natural Colors

Table 5. Organizations involved in Commercial Production of Food Additives from Biological Sources (ref. 10).

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