MICROBES AS POTENTIAL SOURCE OF BIOCOLOURS

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Summary

Microorganisms have been generally used as source of antibiotics, enzymes, vitamins, organic acids, food texturizing agents and so on. There has been a growing interest in the use of natural ingredients particularly in the food industry. Ingredients like colours can also be derived from the natural sources. Microbes can serve as potential candidates to fulfill the said purpose. Microorganisms produce various pigments like carotenoids, melanins, flavins, quinones, prodigiosins and more specifically monascins, violacein or indigo. This review article highlights the role of microbes as a potential source of natural colours and their applications.

Keywords: Biocolours, pigmented microbes, food grade, extraction

Introduction

The production of the synthetic colourants is economically efficient and technically advanced but many artificial synthetic colorants, usually used in foodstuff, dyestuff, cosmetics and pharmaceutical manufacturing processes, causes various hazardous effects like toxic diseases such as cancer and other behavioral problems in children. To counteract the ill effects of synthetic colourants, there is worldwide interest in process development for the production of pigments and colours from natural sources [1].

In recent years, there has been an increased awareness about ecofriendly colours. Due to a consumer demand, food manufacturers worldwide are moving to produce more natural colours, in an effort to replace potentially harmful artificial colourings now used in many foods and beverages. Food colouring now represents a $1.2 billion global market, with natural colours capturing 31% of the food market, but growing at a rate of 5%. However, these natural colours are largely plant extracts that have the disadvantage of variability and seasonal supply.

It is a well known practice to extract the natural colours from the plant sources but the yield is very low and they have low eco-efficiency [2].

Extraction of colours from the microbial source is an upcoming field. Various types of microorganisms like bacteria, fungi, yeasts and algae are coloured. Natural colours can be

extracted from these sources using simple and effective protocols. Before extracting the colours from microbes, they are looked for their safety and efficacy. Microbial colours should be non-toxic as they play a significant role as food colourants. Fungal cell production offers reliable scalable technology.
The various advantages of producing pigments from microorganisms include independence from weather conditions, easy and fast growth and colours of different shades can be obtained by growing on cheap substrates.

Sources of natural colorants

Bio-colourants are eco-friendly as they come from variety of natural sources such as:
1. Plants (Flowers, fruits, seeds, roots etc.)
2. Animals (Cochineal, lac etc.)
3. Microorganisms (*Monascus, Rhodotorulla, Bacillus, Achromobacter, Phaffia* etc.)

But only few of them are available in sufficient quantities for commercial use as food colorants. They are mostly of plant origin. For their biotechnological production, plants and microorganisms are more suitable due to greater understanding of their proper cultural and processing techniques.
One major advantage of using microbes as source of natural colours is that because of their high growth rate they can be mass multiplied. The major pigments produced by microbes are red, yellow and blue. Most research has been focused on yellow and red pigment production, such as monascue produced by *Monascus* sp., carotenoid from *Phaffia rhodozyma*, *Micrococcus roseus*, *Brevibacterium linens* and *Bradyrhizobium* sp., and xanthomonadin from *Xanthomonas campestris* pv.
However, study of blue bacterial pigments is limited, because many bacteria are not capable of producing blue pigment. Actinohodine-related blue pigments are produced by *Streptomyces coelicolor* A3(2), mixture of violacein and deoxybiolacein by *Chromobacterium violaceum* and *Janthinobacterium lividum*.
In addition to its application in dyeing fabrics, violacein also exhibits cytotoxic activity in human colon cancer cells, anti-leishmanial, anti-ulcerogenic, antiviral, antibiotic, anti-tumoral and anti-*Trypanosoma cruzi* activities.
The major natural biocolours of microbial origin are:
A) Molds- Fungal carotenoids have also been recently approved as future food colourants by the European Union for the production of polyketide azaphilone pigments. The main advantages of using colourants from fungal source is that it makes the manufacturer independent of the seasonal supply of raw materials, thus minimizing batch to batch variations. Non-toxigenic fungal strain like *Penicillium* and *Epicoccum* sp. can be used as food colourants [3].
i) *Monascus*: *Monascus* species produce monascus pigments that are used in production of traditional East Asian foods, such as red rice wine, red bean curd. "Ang-Khak" a traditional fermentation product in China, produced by fermenting rice with *Monascus purpureus* (also known as ang khak rice mold), is grinded and its powder form is used as food colourant or as spice in cooking. The different colors of monascus pigments are classified into three types- yellow, orange and red.
The pigments responsible for colouration in *Monascus* are ankaflavine, monascine and monascoavin (yellow), rubropunctatine and monascorubrin (orange), rubropunctamine

and monascorubramine (purple) [4]. These pigments are secondary metabolites of *Monascus* fermentation and produced mainly in cell bound state. The variation in colour is influenced by the culture conditions, in particular pH and the phosphorus and nitrogen source in the substrate. It is used in processed meats products, marine products, tomato ketchup etc.

ii) *Blakeslea trispora:* This fungus thrives in symbiosis with tropical plants and many of its strains can produce high level of carotene. The production of carotene from this mold includes two steps, in first step, the glucose, corn steep liquor or whey are used as substrates for aerobic submerged fermentation to produce the biomass while in second stage, biomass is isolated and transformed into a form suitable for the isolation of carotene. It is then extracted using ethyl acetate and subsequently purified and concentrated. Carotene from *B. trispora* is mainly *trans*- β -carotene with approximately 3 % other carotenoids.

iii) *Ashbya gossypi, Candida sp, Bacillus sp*.: These microbes produce riboflavin (vitamin B_2). It possesses yellow or yellow-orange colour and is being used as a food colorant and as a nutrient supplement in food products. In food industry, it is used in baby foods, breakfast cereals, pasta, sauces and processed cheese etc. Various biotechnological processes have been developed for industrial scale production of riboflavin. The riboflavin fermentation could be classified into three categories;

- Weak over producers: 100mg/L or less e.g. bacterium *Clostridium acetobutylicum*
- Moderate over producers: up to 600mg/L e.g. yeast *Candida gulliiermundii*
- Strong over producers: over 1g/L e.g. fungi *Ashbya gossypi*

B) Yeast

i) *Xanthophyllomyces dendrorhous (Phaffia rhodozyma*): This yeast is known for the production of an astaxanthin pigment. It is widely distributed in nature and is a principle pigment in crustaceans and salmonids. These carotenoid pigments impart orange-red colour to farm animal species when supplemented in their feeds. It is necessary to disrupt the cell wall of yeast (chemical, physical or enzymatic) for proper absorption of astaxanthin pigment by the animals in intestine prior to its addition in feed.

ii) *Rhodotorula*: The carotenoid pigments produced by this yeast are torulene, torularhodin & carotene. *Rhodotorula* is a common environmental inhabitant and can be cultured from soil, water and air samples.

C) Bacteria: A multifaceted secondary metabolites are produced by *Serratia marcescens*, *Pseudomonas magneslorubra*, *Vibrio psychroerythrous, S. rubidaea*, *Vibrio gazogenes*, *Alteromonas rubra*, *Rugamonas rubra* and Gram positive actinomycetes, such as *Streptoverticillium rubrireticuli* and *Streptomyces longisporus*. The actinomycete *Streptomyces coelicolor* A3 produces a closely related linear tripyrrole, undecyl-prodigiosin, and a cyclic derivative, butylmeta- cycloheptyl-prodiginine in a 2:1 ratio [5]. The red pigment of *S. marcescens* was isolated and named "prodigiosine". The best known prodigiosin, is a non-diffusible red pigment attached to the inner membrane [6]. Prodigiosin is a multifaceted secondary metabolite produced by *S. marcescens, Vibrio psychroerythrous, S. rubidaea, V. gazogenes, Alteromonas rubra, Lugomonas rubra* and Gram positive Actinomycetes such as *Streptoverticillium rubrireticuli* and *Streptomyces* *longisporus* [6]. This promising pigment possesses anti-fungal immunosuppressive, anti-proliferative and anticancer activity [7-8].

The microbial production of carotenoids when compared with extraction from vegetables or chemical synthesis seems to be a better option because of the problems of seasonal and geographic variability in the production and marketing of several of the colourants of plant origin. Moreover, microbial processes use natural low cost substrates as carbohydrate source.

Microbial colours are used in fish industry to enhance the pink colour of farmed Salmon. Some natural colourants are also used as antioxidants. Microorganisms produce various pigments like carotenoids, melanins, flavins, quinones, prodigiosins and more specifically monascins, violacin or indigo [9].

Table 1: Highlights of some pigment producing Microorganisms [9-10]

S. No.	Organism	Pigment	Color
1.	*Serratia marcescens*	Prodigiosin	Red
2.	*Corynebacterium insidiosum*	Indigoidine	Blue
3.	*Monascus roseus* *Monascus* spp.	Canthaxanthin Ankaflavin, Monascorubramin, Rubropunctatin	Orange, Pink Yellow, Red, Orange
4.	*Staphylococcus aureus*	Zeaxanthin	Yellow
5.	*Rugamonas rubra*	Prodigiosin like pigment	Red
6.	*Streptoverticillium rubrireticuli*	Prodigiosin like pigment	Red
7.	*Pseudomonas aeruginosa*	Pyocyanin Blue	Green
8.	*Haematococcus pluvialis*	Astaxanthin	Red
9.	*Dunaliella salina*	ß carotene	Orange
10.	*Bradyrhizobium sp.*	Canthaxanthin	Orange/ Dark red
11.	*Xanthomonas oryzae*	Xanthomonadin	Yellow
12.	*Phaffia rhodozyma*	Astaxanthin	Red
13.	*Serratia rubidaea*	Prodigiosin like pigment	Red
14.	*Vibrio gaogenes*	Prodigiosin like pigment	Red

15.	*Alteromonas rubra*	Prodigiosin like pigment	Red
16.	*Janthinobacterium lividum*	Violacein	Purple
17.	*Pacilomyces farinosus* *Paecilomyces sinclairii*	Anthraquinone	Red Under Research project
18.	*Penicillium oxalicum* *P. purpurogenum*	Anthraquinone Unknown (under developmental stage)	Red Red
19.	*Xanthophyllomyces dendrorhous*	Astaxanthin	Pink-red
20.	*Blakeslea trispora*	Lycopene β-carotene	Red, Yellow-orange
21.	*Saccharomyces neoformans*	Melanin	Black
22.	*Cordyceps unilateralis*	Naphtoquinone	Deep blood red
23.	*Ashbya gossypi*	Riboflavin	Yellow
24.	*Streptomyces echinoruber*	Rubrolone	Red
25.	*Rhodotorula* spp.	Torularhodin	Orange-red
26.	*Flavobacterium* spp.	Zeaxanthin	Yellow
27.	*F. sporotrichioides*		Under Research project
28.	*Mucor circinelloides*		Development stage
29.	*Neurospora crassa*		Under Research project
30.	*Phycomyces blakesleeanus*		Under Research project

According to a recent study, apple pomace can be utilized for the production of microbial colours using solid state fermentation (SSF). About 10-50g/L of apple pomace was incorporated in the basic medium for the production of *Rhodotorula* (pink colour), *Sarcina* sp., (dark yellow), *Chromobacter* sp., (dark red) & *Micrococcus* sp., (light yellow) [11].

The production of pigment in apple pomace based medium using SSF gives better yield of biomass & carotenoids. *Rhodotorula* sp. grew best at 30°C with an incubation period of 72hrs at pH 5.5 in the apple pomace based medium where as *Chromobacter* sp. producing dark red colour, grew best at 35°C with pH of 6.0 for 48hrs of incubation period.

Some other examples of microorganisms along with their optimum incubation temperature, incubation time and pH for the production of different pigments are mentioned below in the table.

Table 2: Incubation temperature, time and pH for different pigment producing microorganisms

S. No.	Microorganisms	Temperature (°C)	Time (Days)	pH
1.	*Rhodotorula*	30±1	3	4.5
2.	*Monascus purpureus*	28-35	4-14	5.5-6.5
3.	*Yarrowia lipolytica*	25	3	5.5
4.	*Phaffia rhodozyma*	20-22	3	5.0
5.	*Micrococcus sp.*	35	4	6.0
6.	*Sarcina sp.*	35	3	5.5
7.	*Chromobacter sp.*	35	2	6.0

However, at present none of the microbial pigments can replace synthetic pigment. The microorganisms such as *Monascus, Rhodotorula, Bacillus, Achromobacter, Yarrowia* and *Phaffia* produce a large number of pigments. An ideal pigment-producing microorganism should be capable of using a wide range of C and N sources, have tolerance to pH, temperature and minerals, and give reasonable colour yield. Non-toxic and nonpathogenic nature of pigment-producing micro-organisms coupled with easy separation from the cell biomass is stressed. The various advantages of producing pigments from microorganisms include independence from weather conditions, easy and fast growth, colours of different shades and growth on cheap substances. Studies revealed unstable, largely degradable and sensitive to heat, light, acidity and water activity as characteristics of microbial colour. Improvement in stability, safety and solubility can certainly make widespread use of microbial pigments in the food industry [12].
Use of microbial pigments in processed food is an area of promise with large economic potential. However, microbial pigments offer challenges due to high cost, lower stability and variation in shades due to changes in pH.

Table 3: Important food grade bio-colorants [13]

S. No.	Name of food grade bio-colorants	Original source	Biotechnological source	Approaches for Large scale production
1.	Monascorubramine	*Monascus purpurious*	---	Fermentation and bioprocess engineering
2.	Astaxanthin	Plants	**Fungus**: *Xanthophyllomyces Dendrorhous* **Algae:** *Haematococcus lacustris*. *H. pluvialis* compactin resistant mutant	Fermentation and bioprocess engineering
3.	Arpink red		**Fungus**: *Penicillium oxalicum* var. armeniaca CCM 8242	Fermentation and bioprocess engineering
4.	ß-Carotene	*Daccus carota*	**Fungus:** *Blakeslea trispora, Phycomyces blakesleeanus car S* mutant **Algae:** *Dunaliella salina, D. bardwil* **GM plant**: Golden Rice	Fermentation and bioprocess engineering Organic farming and Integrated Crop Management (ICM)
5.	Riboflavin	Milk	**Moulds:** *Ashbye gossypii, Eremothecium ashbyii, Ashbya gossypi* **Yeast:** *Candida gulliermndii, Debaryomyces subglbosus* **Bacteria:** *Clostridium acetobutylicum*	Fermentation and bioprocess engineering
6.	Bixin and Norbixin	*Bixa orella*		Organic farming and ICM
7.	Betanin	*Beta vulgaris*	Higher yielding plant generated through	Organic farming and ICM

			somaclonal variation Hairy root culture	Fermentation and bioprocess engineering
8.	Canthaxanthin		**Algae:** *Haematococcus lacustris* **Bacteria:** *Bradyrhizobium* sp.	Fermentation and bioprocess engineering
9.	Carminic acid	*Dactylopius coccus*		Organic farming and ICM
10.	Cyanidin and Peonidin	Ascherry, Canberry	Higher yielding plant generated through somaclonal variation Cell culture	Organic farming and ICM Fermentation and bioprocess engineering
11.	Acylated anthocyanins	Black Carrot		Organic farming and ICM
12.	Lycopene	Tomato	**GM fungus**: *Fusarium Sporotrichioides* **GM bacteria**: *Erwnia uredovors*	Fermentation and bioprocess engineering
13.	Lutain	*Tagetes erecta*		Organic farming and ICM
14.	Cepsorubin	*Capsicum annuum*		Organic farming and ICM
15.	Zeaxanthin	Corn	Bacteria: *Flavobacterium* sp.	Fermentation and bioprocess engineering
16.	Curcumin	*Curcuma longa*		Organic farming and ICM
17.	Chlorophyll	Spinach		Organic farming and ICM

Production of colours by fermentation has a number of advantages: cheaper production, easier extraction, higher yields, abundant raw materials and no seasonal variations. Fermented colors are already used today: *D. salina*, *B. trispora*, *Spirulina*, and *Monascus*. It is not unlikely that new, fermented colors such as lycopene from *B. trispora* will become allowed in the near future.

A giant leap forward in color production could be achieved by combining genetic manipulation and fermentation.

Microorganisms could be made to produce colorants in high yield by inserting genes coding for the colorant— even colorants not naturally produced by microorganisms (e.g., turmeric) could be made in this way.

Methods of Pigment extraction from bacterial cells*:*

Pigment extraction from the bacterial cells can be carried out by first centrifuging the cells at 8000×*g* for 5 min, and discarding the supernatant. The cell pellets are then rinsed with deionized water, followed by centrifugation (8000×*g* for 5 min) to recover the cells by discharging the supernatant again. The recovered cells are then fully mixed with 2mL ethanol (with a purity of 99.7%). The mixture of the cells and ethanol is treated by ultra-sonication until the cells are completely bleached. The ethanol extract is then separated from the cells by centrifugation at 10,000×*g* for 5 min. [14].

Conclusion: In the near future, the product with natural colours may have an increased demand, not only for the safety of health and environment but also for their beauty and novelty. Increased awareness for eco-friendly products in the developed countries has opened up a new channel for the export of hand printed fabrics printed with natural dyes. Natural colours should not be taken as a threat to synthetic colors. It may take decades to manufacture natural colours in a ready to use form if all it is possible. A very long and consistent effort is required, since we have just begun our search for natural colour source. It is estimated that world wide up to 70% of all plants have not been investigated fully and that only 0.5% has been exhaustively studied.

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