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Lactic acid fermentation for the production of pyranoanthocyanins as food colorants

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1. INTRODUCTION

Color plays an important role in life. It can carry important physiological and psychological links in humans, since color evokes some expectations e.g., triggering perception of taste, stimulating the appetite, sending signals for choosing a product (Clydesdale, 1991; Downham & Collins, 2000; Dufossé, 2006; Lakshmi, 2014). Color has always served as a quality indicator for food used to determine the acceptability of a product. A colorant may be of natural or synthetic origin, they are compounds capable of imparting color when they are added to food. Therefore, the development of attractive products through color is of great importance for the food industry (Dufossé, 2006; Aberoumand, 2011; Hisano, 2016b).

The use of food colorants is far from being a modern development. In fact, this practice goes back to ancient Egypt, where saffron was used to color food (Joshi et al., 2003; Aberoumand, 2011; Esteves Torres et al., 2016). Until the late nineteenth century, the use of pigments derived from animals, colored minerals, spices and plant extracts known as natural colorants, e.g. saffron, indigo, and cochineal, were the main sources for coloring foodstuffs (McKone, 1991; Downham & Collins, 2000; Harasym & Bogacz-Radomska, 2016). In 1856 with the development of Mauveine as the first synthetic colorant by the British chemist William Henry Perkin allowed the world of colorants to spread rapidly (Sousa et al., 2008; Cañamares & Lombardi, 2015; Hisano, 2016b). Following this achievement, some chemistry companies started producing colorants including those for the use in foodstuffs. Nevertheless, some of them were toxic e.g., copper sulphate, indigo or mercury sulphide (McKone, 1991; Downham & Collins, 2000; Hisano, 2016a; Esteves Torres et al., 2016). Regulations regarding the safety of food colorants did not exist during that time. It was in 1906 when the declaration of the use of colorants in food was placed under governmental supervision with the creation of the Pure Food and Drug Act against Food Adulteration in the US (Hisano, 2016a, 2016b). With the creation of this act, an extensive review of food additives began to assess their safety and avoid mislabeled products to ensure the protection of consumers. Moreover, in the 1960s, the E number system was introduced. This system permitted the use of those additives which had been previously permitted by the corresponding authorities. With the introduction of this system, consumers' concern regarding the safety of additives temporarily decreased (Saltmarsh, 2014). In the 1980s, a full declaration of ingredients and additives used in a food product became obligatory. Due to dissent from countries, no uniform regulation for colorants exists. Meanwhile, clean labeling has gained increasing interest and has become a trend in the food industry. This term defines labels where all ingredients are not from an artificial origin (Downham & Collins, 2000; Saltmarsh, 2014). Thus, interest in clean labeling is continuously growing in the food market, since consumers try to avoid products containing synthetic additives, particularly colorants and preservatives (Downham & Collins, 2000).

Today, within the European Union (EU), it is obligatory that all additives used in a product must be declared. The EU has authorized the use of 43 colorants (European Directive 94/36/EC) and this authorization ensures that adverse health effects and quantities of use are strictly regulated. Each colorant has been assigned an E number, whereby 17 are synthetic colorants and 26 are natural or naturally derived (Downham & Collins, 2000; Mortensen, 2006; Aberoumand, 2011). All of them are regulated without making any distinction among their source of origin, which is, synthetic (e.g. azo-dyes) or natural (e.g. anthocyanins).

In the food industry, colorants are added to processed food e.g., soft drinks, candies, margarine, cheese, jam, and confectionery. In 2016, the total colorant market size was estimated at $1.3 billion, while the demand for natural colorants in 2020 is expected to reach $1.7 billion. North-America and Europe are the largest markets for natural colorants (Harasym & Bogacz-Radomska, 2016).

Among the natural colorants, anthocyanins, betalains, caramel, carminic acid, carotenoids, chlorophylls, curcuminoids, and minerals can be named. Anthocyanins are Generally Recognized as Safe (GRAS) (Aberoumand, 2011; Harasym & Bogacz-Radomska, 2016). The interest in anthocyanins has been increasing since they have been reported to possess positive health effects. Antioxidant, anti-inflammatory, and anti-degenerative activities are some of the biological properties that have been associated with them. In the food industry, anthocyanins have been used as food colorants for a long time in many products, e.g., jams and beverages (Downham & Collins, 2000; Shipp & Abdel-Aal, 2010). Nevertheless, techno-economic factors limit their use, such as their low stability and the higher productions costs compared to artificial colorants.

The food industry is forced to find a way to substitute artificial colorants with natural counterparts (Aberoumand, 2011). The application of biotechnology brings an opportunity for the synthesis of natural colorants. The use of microorganisms in the food industry plays an important role due to their high potential in multiple applications, easy production and easy downstream processing (Wissgott & Bortlik, 1996; Dufossé, 2006; Mapari et al., 2010; Venil et al., 2013; Dufossé et al., 2014).

In 1996, Cameira-dos-Santos et al. found two novel anthocyanin derivatives. These compounds were products from the reaction between the major anthocyanins in wine, i.e., malvidin 3-monoglucoside and malvidin 3-(6-*p*-coumaroyl)monoglucoside with 4-vinylphenol (4-VP). In comparison with anthocyanins, these new pigments showed higher stability and a wider range of colors even at higher pH. These pigments are present in processed food and they occur as a product of the direct reaction between anthocyanins and small molecules such as pyruvic acid and hydroxycinnamic acids resulting in a new pyran ring. The properties displayed by these pigments known as pyranoanthocyanins make them promising candidates to be used as food colorants. However, the recovery of pyranoanthocyanins from natural sources is limited due to the low quantities in which they can be found (Vallverdú-Queralt et al., 2016). Pyranoanthocyanins are reaction products of anthocyanins and different compounds from microbial metabolism (Cameira-dos-Santos et al., 1996; Fulcrand et al., 1996; Hayasaka & Asenstorfer, 2002; Wang et al., 2003). Colorants required a previous extraction to be used. In contrast, a coloring foodstuff can be defined as a food extract or concentrate able to be used as food with coloring effects. Within the EU, a coloring foodstuff may be labeled as an ingredient appearing mainly as concentrates. Therefore, an E label is not required. The substitution of colorants can be accomplished by the use of coloring foodstuff, which, for the food industry, can be useful in trying to avoid the use of E additives and offering an eco-friendly alternative for the consumers. Among sources of natural colorants, black carrot juice (BCJ) is known for its application as coloring foodstuff (Downham & Collins, 2000; Wrolstad & Culver, 2012; Lakshmi, 2014). The phenolic composition in black carrots offer a good source of precursors for the synthesis of pyranoanthocyanins. In black carrots, hydroxycinnamic acids can be found as natural compounds (Kammerer et al., 2003). They can play an important role as copigments to improve anthocyanins‘ stability. Furthermore, they can act as precursors for the formation of more stable pyranoanthocyanins. So far, the development of protocols for the synthesis of pyranoanthocyanins in BCJ and their further use as food coloring is lacking.

2. BACKGROUND

2.1. Food colorants

The color presented by natural or processed products is an attribute that influences their consumer acceptance. Color is described as a property that consumers associate with taste, nutritional value and the overall quality of a product.

The use of colorants can be traced back to ancient times. There are reports of the Egyptians (400 BC) using natural pigments for food, drugs, and cosmetic purposes. The introduction of synthetic colorants was made in 1856 with “Mauve”, by the British chemist William Henry Perkin while experimenting to find a cure for malaria. Since the discovery of this colorant, many other synthetic dyes were used to substitute natural dyes in an uncontrolled way (Sousa et al., 2008).

Pigments or dyes are terms often used to describe a colored substance. The difference between both is their size and solubility. Pigments are insoluble in the medium, whereas a dye is soluble (Sousa et al., 2008; Cañamares & Lombardi, 2015; Esteves Torres et al., 2016).

Food colorants are mainly used to mitigate color losses occurring during processing, to bring color to uncolored products, to improve the natural color, or to standardize a product from batch-to-batch thus improving their acceptability and making them more appetizing (Aberoumand, 2011). The colorants used in foodstuffs may be classified into four main categories: (1) natural colorants, (2) natural identical colorants, (3) synthetic colorants, and (4) inorganic colorants (Mortensen, 2006; Mapari et al., 2010; Aberoumand, 2011).

As mentioned by Dabas et al. (2011), natural colorants can be defined as natural pigments or pigments made by living organisms. Sources of them can be plants, animals, or different microorganisms. Phenolic compounds like anthocyanins, as well as numerous other compounds are naturally occurring colorants. Among these natural colorants, carotenoids and anthocyanins are probably the most known and investigated (Mazza & Brouillard, 1987; Malien-Aubert et al., 2001; Schwarz & Winterhalter, 2003; Wu & Prior, 2005; Esteves Torres et al., 2016). Rich sources of anthocyanins like blackberries, grapes, raspberries, elderberries, red cabbage, and black carrots are already used by the food industry for extracting and isolating these natural pigments (Mortensen, 2006). However, the use of anthocyanins in food is limited due to their low stability affected by several factors, and difficulties in their extraction and purification (Baublis et al., 1994; Sarni-Manchado et al., 1996; Giusti & Wrolstad, 2003; Schwarz & Winterhalter, 2003; Ignat et al., 2011; Sigurdson et al., 2017). Colors resulting from modification on the material by living organisms may also be included in this category, i.e., biocolors that cannot be found in nature. Pyranoanthocyanins are a good example of biocolors, their formation occurs during wine ageing, were microbial products react with anthocyanins acting as precursors for these new pigments. The production of carotenoids, flavonoids and other compounds by microorganisms is well documented in literature (Dufossé, 2006; Chattopadhyay et al., 2008; Wrolstad & Culver, 2012; Heer & Sharma, 2017). As mentioned by Heer and Sharma (2017), the use of pigments produced by microorganism is a recent trend. The biosynthesis of these compounds can be easily scaled up since, compared to the cultivation of edible plants, the process is not weather dependent, and the production conditions can be standardized. Therefore, biotechnological production of such colorants represents an attractive alternative. Cochineal is maybe the most known insect origin pigment belonging to this group. With its origin in south and central America, the red pigment produced by insects has been applied for dying of textiles, food products and cosmetics (Madsen et al., 1993; Méndez-Gallegos et al., 2003; Takaichi et al., 2003; Borges et al., 2012; Velmurugan et al., 2013). Examples of microorganisms that are known to produce biocolors are *Staphylococcus aureus*, *Serratia marcescens* (Heer & Sharma, 2017), and

Vibrio gazogenes (Alihosseini et al., 2008), the algae *Dunaliella salina* (Pisal & Lele, 2005; Spolaore et al., 2006), the yeast *Rhodotorula* sp. *Rhodotorula glutinis* (Hernández-Almanza et al., 2014), and *Phaffia rohodozyma* (Johnson & Lewis, 1979; An et al., 1989), and the fungi *Monascus purpureus* (Fabre et al., 1993; Akihisa et al., 2005; Lakshmi, 2014).

Regarding natural identical colorants, these compounds are produced by chemical synthesis, replicating the molecular structure of naturally occurring compounds. Synthetic colors are compounds like azo-dyes that are not found in nature. After the discovery of mauvine in 1856, many others were synthetized revolutionizing the colorant industry and opening up new opportunities for processed food. In the late nineteenth century, natural colorants were almost completely replaced by synthetic colorants.

There are some factors affecting the stability of coloring agents, e.g., temperature, light, or other components found in the media like oxidizing and reducing agents (Joshi et al., 2003; Mortensen, 2006; Socaciu, 2008). Since their discovery, synthetic colorants have been used more extensively due to their properties, e.g., higher stability, color intensity, hue, and most probably their low production costs. However, there are just a few natural colorants that can be found in quantities abundant enough to make their application feasible. In addition, natural colorants have been shown to be less efficient compared to the artificial ones, suggesting that higher quantities of the natural colorants need to be added to reach the desired color. This, in turn, can directly affect the flavor, stability, and the cost of the product (Mortensen, 2006; Bechtold & Mussak, 2009; Wrolstad & Culver, 2012).

In recent years, consumer awareness has increased, as several studies have reported side effects of synthetic colorants e.g., toxicological effects, carcinogenic effects, allergic reactions, and the well-investigated attention deficit-hyperactivity disorder (ADHD) (Combes & Haveland-Smith, 1982; Sasaki et al., 2002; Joshi et al., 2003; Schab & Trinh, 2004; Bateman et al., 2004; Wang et al., 2006; McCann et al., 2007; Pan et al., 2009; Sharma et al., 2011; Elbanna et al., 2017). Probably, the most extensive study regarding artificial colorants was published by the University of Southampton. The study reported a detrimental effect of six artificial food colorants children's behavior. While the UK prohibited the application of these six colorants in food, the EU only requests that a label warning of potential adverse effects in children be added.

Therefore, the challenge for the food industry is the substitution of synthetic dyes with colorants obtained from natural sources. Moreover, and as suggested by the regulation of the European Commission 13333/2008 to avoid the use of food additives, the coloring food market will probably change toward more natural options like coloring foodstuffs. Actually, extracts from aronia, black carrot, blackcurrant, and hibiscus can be found in the food industrial market. Table 2.1 shows an example given by SMARTIES® which successfully avoids using artificial colorants through implementing coloring foodstuffs and clean label (Bobe & Michel, 2011).

Table 2.1. Colorants used for SMARTIES®

Colorants used in 2006	Colorants used in 2009
E104 Quinoline yellow	Lemon (yellow)
E110 sunset yellow	Safflower (yellow)
E122 Carmoisine	Radish (red)
E120 Carmine	Red cabbage (red)
E133 Brilliant blue	Hibiscus (red)
E124 Ponceau 4R	Spirulina (blue)
	Black carrot (purple)

Thus, the implementation of coloring foodstuffs can be simplified because no extraction techniques are required. In addition, using coloring food stuffs adds value to the food, thanks to their potential health benefits and their application can be standardized over the world.

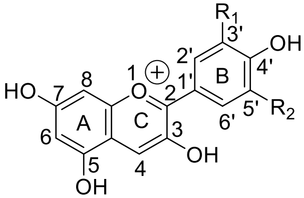
2.2. Anthocyanins

The word anthocyanin derives from two Greek words: *anthos*, flower and *kyanos*, blue. Anthocyanins belong to the family of flavonoids and are water-soluble pigments causing the red, pink, blue, or purple colors of many plants, i.e., flowers, fruits, and vegetables (Wu & Prior, 2005; Castañeda-Ovando et al., 2009; Motilva et al., 2013; Quina & Bastos, 2018). These compounds are located in vacuoles of plant cells acting as a defense against pathogens and predators, attracting animals for pollination and seed dispersal, providing plant pigmentation, acting as light screen against harmful radiation and as antioxidants (Stintzing & Carle, 2004). Due to the range of attractive colors varying from pink to purple hues, they are used as natural food colorants (Bakker & Timberlake, 1997; Stintzing & Carle, 2004; Bordignon-Luiz et al., 2007; Khoo et al., 2017; Quina & Bastos, 2018). Anthocyanins represent an alternative in the food industry to substitute synthetic colorants. Many edible plants are source of anthocyanins including, purple sweet potato, red grapes, black carrot, blackcurrant, and elderberry (Downham & Collins, 2000; Wrolstad & Culver, 2012; Lakshmi, 2014).

Anthocyanin extracts are used in the food industry as additives in beverages, jellies, confectioneries and jams. All anthocyanins are used under classification E 163 number of the EU legislation of food additives (Wrolstad & Culver, 2012). The application of anthocyanins in the EU is at *quantum satis* level, i.e., no maximum level is specified, with exception of fruit-flavored breakfast cereal where a maximum of 200 ppm is established (Hendry & Houghton, 1996; Frick, 2003; Bechtold & Mussak, 2009). Nevertheless, their application in the food industry is limited due to their poor stability shown at low acid conditions, occurring during processing and storage. However, anthocyanins are of scientific interest due to their potential health benefits, which include acting as antioxidant, anti-inflammatory, anti-tumor, neuroprotective, and chemopreventive agents

(Prior, 2003; Kong et al., 2003; Tarozzi et al., 2007; Yao et al., 2010; Bishayee et al., 2011; 2011b; Sun et al., 2012; Khoo et al., 2017).

The basic structure of anthocyanins are the anthocyanidins (2-phenylbenzopyrylium or flavylium salts), which consists of a C6-C3-C6 skeleton with an aromatic ring A bonded to a heterocyclic ring C containing oxygen and linked by a carbon-carbon bound to a second aromatic ring B (Figure 2.1). The principal differences between the anthocyanidins are the hydroxy or methoxy substituents at the A and B rings. More than 90% of all anthocyanins identified are based on six anthocyanidins, i.e., pelargonidin, cyanidin, peonidin, delphinidin, petunidin, and malvidin (Mazza & Miniati, 1993; Kong et al., 2003; Mercadante & Bobbio, 2008; Fernández-López et al., 2013; Quina & Bastos, 2018).



Anthocyanidin	R_1	R_2	Λ max [a]
Pelargonidin	H	H	494
Cyanidin	OH	H	506
Delphinidin	OH	OH	508
Peonidin	OCH_3	H	506
Petunidin	OCH_3	OH	508
Malvidin	OCH_3	OCH_3	510

[a] λ max given in nm

Figure 2.1. Structures of the most abundant anthocyanidins

In nature, anthocyanidins mainly occur as glycosides. The sugars that are commonly bound to the anthocyanidins are glucose, galactose, arabinose, rhamnose, and xylose. Glycosylation renders more stable anthocyanins compared to their respective aglycones. They can primarily