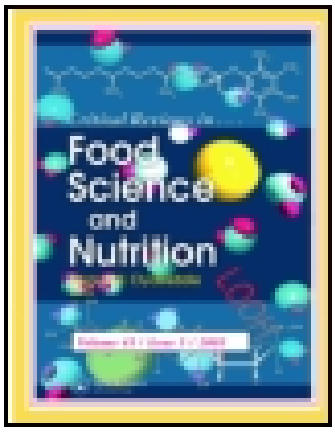

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Availability and Utilization of Pigments from Microalgae

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ABSTRACT

Microalgae are the major photosynthesizers on earth and produce important pigments which include chlorophyll a, b and c, β-carotene, astaxanthin, xanthophylls and phycobiliproteins. Presently, synthetic colourants are used in food, cosmetic, nutraceutical and pharmaceutical industries. However, due to problems associated with the harmful effects of synthetic colourants, exploitation of microalgal pigments as a source of natural colours becomes an attractive option. There are various factors such as nutrient availability, salinity, pH, temperature, light wavelength and light intensity which affect pigment production in microalgae. This paper reviews the availability and characteristics of microalgal pigments, factors affecting pigment production and the application of pigments produced from microalgae. The potential of microalgal pigments as a source of natural colours are enormous as an alternative to synthetic colouring agents which has limited application due to regulatory practice for health reasons.

Keywords: Microalgae, pigments, chlorophyll, carotenoid, phycobiliproteins, astaxanthin

INTRODUCTION

Microalgal pigments are extensively used in various industries including food, nutraceutical, pharmaceutical, aquaculture and cosmetic industry (Fig. 1). In addition, it has been used in clinical/research laboratories, which are effective as label for antibodies and receptors (Santiago-Santos et al., 2004). Antioxidants, anti-inflammatory, neuroprotective and hepatoprotective properties are also exhibited by phycobiliproteins (Spolaore et al., 2006). As microalgae culture is eco-friendly and renewable, there is increasing likeness to use microalgae in aquaculture such as live feed for larviculture industry, premix for feed formulation/supplement and bioremediation for improvement of water quality (Khatoon et al., 2007), production of high health organisms and enhancement of animal colour (astaxanthin). Some of the microalgae have been exploited for centuries for food and health care.

Microalgae are a diverse group of simple, plant like organisms which are unicellular or filamentous microorganisms and are able to harness solar energy which accounts for the large quantities of biomass accumulation through the photosynthesis mechanism (Matsunaga et al., 2005). They are classified according to their colours as chlorophyceae (green), rhodophyceae (red), cyanophyceae (blue-green) and phaeophycae (brown) (Graham and Wilcox, 2000). Chlorophyll, carotenoids and phycobiliproteins exhibit colours ranging from green, yellow and brown to red. Natural colourants from different microalgae like phycocyanin (blue pigment from *Spirulina*), ɓ-carotene (yellow pigment from *Dunaliella*) and astaxanthin (yellow to red pigment from *Haematococcus*) are gaining importance over synthetic as they are nontoxic and non-carcinogenic (Dufoss et al., 2005).

Microalgae and Pigments

Microalgae are a heterogeneous group of cryptogamic plants comprising 13 large phyla and several smaller groups still incompletely studied (Reynolds, 2006). They range in form from unicellular, through colonial, filamentous and siphonaceous. Among the different phyla of microalgae, cyanobacteria are oxygenic photosynthetic prokaryotes showing large diversity in their morphology, physiology, ecology, biochemistry and other characteristics. Chlorophyta are unicellular, multicellular, filamentous, siphonous and thallus and primarily freshwater algae whereas, cryptophytes are unicellular and widely distributed both in fresh water and marine environments. Dinophytes are also unicellular with two dissimilar flagella and most are distributed in marine environment (Reynolds, 2006). Different phylum of microalgae contains different pigments as listed in Table 1.

CHARACTERISTICS OF MICROALGAL PIGMENTS

Chlorophylls, carotenoids (carotenes and xanthophylls) and phycobilins are three major classes of photosynthetic pigments in microalgae. Chlorophylls and carotenoids are generally fat soluble molecules whereas, phycobilins are water soluble.

There are three types of chlorophylls *a*, *b* and *c*. The skeleton of chlorophyll molecule is the porphyrin macrocycle consisting of tetrapyrrole rings (Humphrey, 2004; Scheer et al., 2004). Phorbin structure is formed by the attachment of a single isocyclic ring to one of the pyrrole

rings (Humphrey, 2004). Each pyrrole ring contains four carbon atoms and one nitrogen atom. A central hole consists of facing inward facing nitrogen atoms in which a Mg^{2+} metal ion can easily bind. The formyl group in ring II of chlorophyll *b* is exchanged by methyl group in chlorophyll *a* (Scheer et al., 2004). Due to these structural differences, chlorophyll *a* has blue/green pigment with maximum absorbance from 660 to 665 nm and chlorophyll *b* has green/yellow pigment with maximum absorbance from 642 to 652 nm (Humphrey, 1980). Numerous degradation products are formed due to exposure of chlorophyll molecules to weak acids, oxygen or light and consequently accelerate their oxidation (Cubas et al., 2008).

Carotenoids consist of terpenoid pigments which are derived from a 40-carbon polyene chain. It provides carotenoids with distinctive molecular structures and the associated chemical properties including light-absorption features that are essential for photosynthesis (Del Campo et al., 2007). Carotenoids may be complemented by cyclic groups and oxygen-containing functional groups. Therefore, hydrocarbon carotenoids are named as carotenes as a whole whereas, oxygenated derivatives are known particularly as xanthophylls with oxygen being there as hydroxyl groups (e.g., lutein), oxi-groups (e.g., cantaxanthin) or a combination of both (e.g., astaxanthin) (Del Campo et al., 2007). There are two types of carotenoids namely primary and secondary carotenoids. Structural and functional components of the cellular photosynthetic apparatus are the primary ones (*i.e.*, xanthophylls) whereas, the secondary carotenoids include those produced by microalgae to large levels after exposure to specific environmental stimuli (Eonseon et al., 2003). Xanthophylls are relatively hydrophobic molecules therefore they are typically linked with membranes or involved in non-covalent binding to specific proteins and are usually localized in the thylakoid membrane. Secondary carotenoids are found in lipid vesicles

(Grossman et al., 1995). Carotenoids can be extracted by using the organic solvents such as acetone, methanol or dimethyl sulfoxide (DMSO).

Phycobiliproteins are light harvesting pigments commonly present in cyanophyceae and cryptophyceae (Glazer, 1994). According to their amino acid sequences and spectroscopic properties, they can generally be divided into three classes consisting of red-coloured phycoerythrin, blue-coloured phycocyanin and allophycocyanin (Bermejo et al., 2003). They are assembled in phycobilisomes and are attached to the surface of the thylakoids for photosynthesis.

Phycobiliproteins are oligomeric proteins, made up from chromophore-bearing polypeptides belonging to two families (Ŭ and ɓ) possibly originating from a common ancestor (Glazer, 1984). In addition, phycobiliproteins of the blue-green microalgae *Synechococcus* sp. and *Aphanocapsa* sp. were characterized with respect to homogeneity, isoelectric point and subunit composition (Glazer, 1971). Phycocyanin, allophycocyanin and phycoerythrin consist of two different noncovalently associated subunits with molecular weights of about 16,000-20,000 dalton, 15,500-17,500 dalton and 20,000-22,000 dalton, respectively (Glazer, 1971).

Phycobiliproteins have various spectral properties because of the bilins which have individual absorption spectra (Reis, 1998). According to Bryant et al. (1979), there are four main classes of phycobiliproteins namely allophycocyanin (APC, bluish green), phycocyanin (PC, blue), phycoerythrin (PE, red) and phycoerythrocyanin (PEC, orange) in cyanobacteria and red microalgae. The absorbance maximum of each class is as follows: allophycocyanin λ_{Amax} 650~655 nm, phycocyanins λ_{Amax} 615~640 nm, phycoerythrin λ_{Amax} 565~575 nm and phycoerythrocyanin 577 nm whereas they emit light at 660nm, 637nm, 577nm and 607 nm respectively. Arad and Yaron (1992) also found that the microalgal extract of *Pseudomonas*

aeruginosa was blue with maximum absorbance at a wavelength of 620 nm and a red fluorescence with maximum emission at 642 nm. In most cyanobacteria, the main phycobiliprotein is C-phycocyanin. Protein of C-phycocyanin isolated from wild-type cells of the blue-green microalgae *Oscillatoria agardhii* consist of two polypeptide chains with methionine (Glazer, 1994; Peter et al., 1992). The chains are joined with a disulfide bridge and both contained at least one chromophore group per chain. The amino acid composition, peptide maps and amino-terminal sequence of the two chains discovered are similar in structure.

FACTORS AFFECTING THE MICROALGAL PIGMENT PRODUCTION

Different environmental parameters such as temperature, irradiances, coloured wavelength, photoperiods, pH, nutrient limitation, nitrogen supplements, salinity, pesticides and heavy metals stress can have an effect on the production of microalgal pigments (Hemlata and Fatma, 2009). According to Richmond (1986), a variety of environmental and nutritional factors can affect the productivity and cell composition in microalgal cultures. Grossman et al. (1993) also reported that the phycobiliprotein composition of microalgal cells can be changed by some of these environmental conditions. Prassana et al. (2004) reported that in response to environmental parameters like light intensity, light wavelength, temperature and nutrient availability, cyanobacteria can control their tetrapyrrole content and composition. According to Pisal and Lele (2005) different stress parameters such as cell division inhibition (vinblastine), nitrogen starvation, high salinity and high irradiation with high temperature can enhance the carotenoid content of microalgae whereas, vinblastine has very little favourable effect.

Light and Temperature

Light and temperature are the most important factors that affect the overall biomass production in phototrophic microalgal cultures (Carvalho and Malcata, 2003). According to Kagawa and Suetsugu (2007), different spectral proportion of light such as red:far red, blue:red, green:red and blue:green has effect on the relative pigment composition and likely act as photomorphogenic signals in microalgae. The productivity of microalgae is affected by the individual light regimen.

Variation in chlorophyll and phycobiliprotein levels is subjected to an adaptive response which allows proficient light harvesting. Cyanobacteria favours low light intensities and stimulate phycobiliproteins production (Grossman et al., 1993). Mur and Elema (1983) also reported that cyanobacteria prefer low light intensity because of their low specific maintenance energy rate and pigment composition. There have been several reports suggesting that at low irradiance more accessory pigment is synthesized (Grossman et al., 1993; Goedheer, 1976; MacColl and Guard-Friar, 1987; Wyman and Fay, 1987). The amount of light availability is modified by the cell density and the consequence of mutual shading of cells eventually affecting the pigment content of microalgal cells (Ramos et al., 1987; Richmond, 1988).

The effect of light intensity on pigment production at different experimental irradiance showed that 25 µmolphotons/m^2/sec as the best intensity for phycobiliproteins production for blue-green microalgae (*S. maxima, Anabaena* NCCU-9, *Synechococcus* NKBG) whereas, 60 µmolphotons/m^2/sec was suitable for other blue-green microalgae such as *Nostoc* UAM 206 (Table 2). However, according to Madhyastha and Vatsala (2007), the production of phycocyanin and phycoerythrin in *Spirulina* was enhanced by higher light intensity (135 µmolphotons/m^2/sec).

Light intensity plays an important role in controlling the pigment accumulation in microalgal cells. An inverse relationship was found in chlorophyll content per cell which showed that at low intensity chlorophyll production was high in green algae (*Dunaliella salina* 66.9 mg/g) whereas, with high irradiance of 2300 lux chlorophyll production was low (Table 2). Similarly Chauhan and Pathak (2010) reported that in the low light intensity (27 µmolphoton/m^2/sec) chlorophyll content was 14.7 mg/g whereas at 54 µmolphoton/m^2/sec it was 11.6 mg/g. Chlorophyll content in *S. platensis* biomass is influenced by the composition of the cultivation medium and cellular age. It has been shown that culturing of microalgae under high light intensity contains lower biomass chlorophyll content whereas culturing in low light intensity present higher biomass chlorophyll content. Therefore, there is an inverse relationship between the light intensity and chlorophyll content (Bogorad, 1962; Eloranta, 1986).

Light intensity also plays a vital role in controlling the ɓ-carotene accumulation in algal cells and subsequently enhanced production of carotenoids from *D. salina* (Pisal and Lele, 2005). ɓ-carotene content per cell increased sharply with light intensity. It indicates that ɓ-carotene can be greatly enhanced at higher irradiation (11.28 µmolphoton/m^2/sec) in blue-green microalgae.

Imamoglu et al. (2009) reported that under high light intensity (546 µmolphoton/m^2/sec) astaxanthin accumulation was high (30 mg/g) in green microalgae (*H. pluvialis*) (Table 2).

Salguero et al. (2003) reported that intense illumination induced oxidative stress resulting in an increase of astaxanthin synthesis. Therefore, active oxygen molecules are produced by excess photo oxidation (Ip and Chen 2005). Fábregas et al. (2001) and Kim et al. (2006) found that light quality is more imperative than quantity as flashing light enhanced the rate of astaxanthin production in *H. pluvialis* per photon by at least 4 fold when compared to culture carried out under continuous light sources. Wang et al. (2003) reported that the effect of irradiance influenced the culture density, cell maturity, medium nutrients profile and light path.

There is a relationship between the light colour and pigment production in different microalgal species. Lopez-Figueroa et al. (1990) suggested that there are three main photoreception systems like B/UV light photoreceptor (BLP), green light photoreceptor (GLP) and red/far red photoreceptors that are involved in diurnal changes of the photosynthetic pigment content. According to Schirmer et al. (1985), phycobiliproteins absorb light quanta and transfer the energy to the active chlorophyll molecules involved in photosystem. Hemlata and Fatma (2009) found that coloured light has no stimulatory effect on phycobiliproteins production in blue-green microalgae (*Anabaena* NCCU-9). However, various studies have reported that red light positively affect the phycobiliproteins production in other blue-green microalgae such as *Anacystis nidulans, Synechococcus* sp., *Calothrix* 7601, *Nostoc* UAM 206, *N. muscorum* whereas, blue light stimulate the phycobiliproteins production and enhances the chlorophyll production in *Spirulina* sp. (Table 2).

Rodriguez et al. (1991) reported that decrease in irradiance influenced the increase in phycobiliproteins production in nitrogen fixing cyanobacteria. Green light induced an increase in the C-phycoerythrin content whereas, C-phycocyanin content was enhanced when the cultures were irradiated by red light (Rodriguez et al., 1991). Madhyastha and Vatsala (2007) reported that white light intensity was preferable for chlorophyll synthesis and the highest accumulation of C-phycocyanin was 5.5 mg/ml whereas, other pigments like phycobiliproteins and carotenoid content roughly remained constant in *S. fussiformis*. In addition, they also found that green light has no positive effect on pigment production except for phycocyanin and red light treatment which did not show the positive effect on pigment production in *S. fussiformis.*

The most elementary factor of all living organisms is temperature as it affects metabolic processes and biochemical composition of cells. In addition, optimal growth temperature and tolerance to the extreme values generally differ for different microalgae strains (Hemlata and Fatma, 2009). However, an extreme change in temperature exerts stress in microalgae. High temperature favours accumulation of carotenoids (e.g., ɓ-carotene) in blue-green microalgae (García-González et al., 2005).

Optimum temperature for production of phycobiliproteins was at 30°C for *Anabaena* sp. (Hemlata and Fatma, 2009) and other researchers have reported 25°C, 35°C, 36°C as optimum for *S. platensis*, *Anaebaena* sp., *Nostoc* sp. (Moreno et al., 1995) and *Spirulina* sp. (Chaneva et al., 2007). In *Synechococcus* sp. phycobiliproteins production was optimum at 36°C. Chauhan and Pathak (2010) reported that higher amount of chlorophyll was obtained at 28°C since high temperature could have elicited changes in the osmotic pressure damaging the cells. Likewise optimum temperature for production of astaxanthin was at 28°C for green microalgae (*H.*

pluvialis (Domínguez-Bocanegra et al., 2004) whereas, other researchers have found 25°C and 30°C as optimum for *H. pluvialis* (Olaizola, 2000) and *Chlorella zofingiensis* (Ip and Chen 2005). In addition, researchers have also reported that 25°C and 30°C as optimum for ɓ carotene production for blue-green microalgae *D. salina* (García-González et al., 2005; Del Campo et al., 2001). Moreover, optimum temperature for production of carotenoids (lutein) was at 28°C for *Muriellopsis* sp. (Del Campo et al. 2000), *C. protothecoides* (Wei et al., 2008), *C. zofingiensis* (Wei et al., 2008), *Neospongiococcus gelatinosum* (Del Campo et al. 2000). However, Macías-Sánchez et al. (2009) and Mendes et al. (1995) reported that 55°C and 60°C was the extreme temperature for the production of total carotenoids for green microalgae such as *C. vulgaris* and *Nannochloropsis gaditana*, respectively (Table 3).

pH

The influence of pH on the photosynthetic pigments of cyanobacteria has received little attention (Hemlata and Fatma, 2009). The solubility and the bioavailability of nutrients are influenced by the alteration of pH. Poza-Carrión et al. (2001) analyzed the combined effect of pH, irradiance and inorganic carbon availability on the growth and pigment composition of the cyanobacterium *Nostoc* sp. strain UAM206, isolated from rice fields. Chlorophyll *a* content was not affected by the increase in pH whereas there was an increase in phycocyanin, phycoerythrin and allophycocyanin content.

The maximum phycobiliproteins production was obtained at pH 8 in case of blue-green microalgae such as *S. platensis* (Hemlata and Fatma, 2009). On the other hand, optimum

phycobiliprotein production for *Nostoc* sp. was found at pH 9. Similarly, pH 9 was also found to be optimum for the production of chlorophyll for *S. platensis* (Chauhan and Pathak 2010). In the blue-green microalgae *D. salina*, ɓ-carotene production was optimum at pH 7.5 ((García-González et al., 2005; Del Campo et al., 2001). pH 8 was the best for optimum production of carotenoid in green microalgae *Scenedesmus almeriensis,* whereas it was pH 7 for *Chlorococcum citriforme* and *Neospongiococcus gelatinosum* (Del Campo et al., 2000) (Table 4).

Salinity

Salinity has a great impact on pigment production in microalgae. It has been reported that the detachment of phycobilisomes from the thylakoid membrane cause rapid entry of sodium ions leading to a reduction in photosynthesis (Rafiqul et al., 2003) which influences pigment production. Other researchers have suggested that the detachment can also occur due to the energy transfer from phycobiliproteins to photosynthetic reaction centre (Schubert et al., 1993; Schubert, 2000) and uptake of other mineral nutrients (Hasegawa et al., 2005).

Osmosis plays a crucial role in pigment content since microalgae are cultivated in saline environment (Pisal and Lele, 2005). Higher concentration of the salt (NaCl) produces hypertonic solution in the external environment of the cell. Due to this condition, there is a net flux of water molecules leaving the cell which causes cell shrinkage and consequent damage of the cell and cell components.

Lowest concentration of NaCl (10 ppt) increased the phycobiliproteins production (135.73 mg/g) in blue-green microalgae *Anabaena* NCCU-9 (Hemlata and Fatma, 2009). However, other researchers have found that 15 ppt increased the phycobiliproteins production (66.7 mg/g) in *Oscillatoria* sp. (Table 5). The highest chlorophyll-*a* and total carotenoids were found at salinity 2 ppt for *Dunaliella viridis* (Ilkhur et al., 2008). In addition, Pisal and Lele (2005) have reported that optimum concentration of salinity for carotenoid production was at 3ppt.

Avron and Ben-Amotz (1992) reported that salinity has a great effect on chlorophyll and ɓ-carotene production. They reported that the chlorophyll content decreased with the increase in salinity whereas ɓ-carotene production was enhanced with the increase in salinity in blue green microalgae.

Other factors

Other factors such as pesticides, heavy metals and nutrient limitation also affect the pigment production in microalgae. The inhibitory effect of pesticide on phycobiliproteins is reported by many phycobiologists. Xia (2005) and Battah et al. (2001) reported that phycobiliproteins production decreased in blue-green microalgae such as *Nostoc sphaeroids* and *Anabaena variabilis* under thiobencarb pesticide stress. Prasad et al. (2005) suggested that more exposure of pesticide on intracellular thylakoid membrane of phycobiliproteins cause more damaging effect on phycobiliproteins resulting in their detachment.

Nitrogen is one of the principal nutrient requirements of growth media for any cell. The culture organisms may be in stress due to the absence of nitrogen or starvation. During nitrogen starvation, the microalgae cease to divide since nitrogen is the primary requirement for all the metabolic activities of the cell. Nitrogen starvation conditions can cause an excessive formation of free radicals on nitrogen starved cells and consequently ϐ-carotene content increase markedly to 7.05 pg/cell from 1.65 pg/cell (Møller et al., 2000). On the other hand, adverse effect was found on chlorophyll synthesis during nitrogen starvation because chlorophyll molecule contains four nitrogen atoms (Ben-Amotz et al., 1989). Hence, the cell organelles cannot actively synthesize chlorophyll under nitrogen stress. Furthermore, chlorophyll concentration in *S. platensis* biomass enhances with an increase of nitrogen concentration in the culture medium (Piorreck et al., 1984).

Richmond (1986) reported that cyanobacteria have special requirements to nitrogen source. Under nitrogen free environment, blue-green microalgae (*Anabaena* NCCU-9) produced highest amount of phycobiliproteins (Hemlata and Fatma, 2009). Loreto et al. (2003) also reported that in *Anabaena* 7120, the amount of phycobiliprotein was higher in nitrogen-free media than nitrate grown cultures whereas *Fischerella* sp. produced more phycobiliproteins under nitrate grown cells than nitrogen-free media (Soltani et al., 2007). According to Marin et al. (1998) there was no significant effect of nutrient concentration of chlorophyll *a* and carotenoid of blue-green microalgae. They found that low nitrate concentration negatively affected growth but increased the carotenoid content.

Astaxanthin synthesis was reported to increase by adding iron in media (Choi et al., 2002; Fábregas et al., 2003; Kang et al., 2006). The mechanism of iron electro valencies and counter

ions has an impact on cell growth and accumulation of astaxanthin (Cai et al., 2009). According to Cai et al (2009) by adding 18 εmol/L Fe^{2+}-EDTA induced synthesis of astaxanthin more effectively. On the other hand, ϐ-carotene content increased markedly when the culture media were supplied with 450 εM $FeSO_4$ and 67.5 mM acetate (Mojaat et al., 2008). Moreover, Fábregas et al. (2003) reported that photosynthetic fixation of carbon enhanced the astaxanthin synthesis and it is also produced more efficiently in outdoor condition when accurate nitrate dosage is supplied (García-Malea et al., 2009).

APPLICATIONS OF MICROALGAL PIGMENTS

Due to the toxic effects of several synthetic dyes there is an increasing preference to use dyes obtained from different natural sources (Sinha et al., 2012). According to Dufosse et al. (2005) and Sekar and Chandramohan (2008), microalgal pigments are being used as natural colours. Among microalgae, efficient production of pigments, such as carotenoids from *Dunaliella*, astaxanthin from *Haematococcus*, phycobiliproteins or phycocyanin from *Spirulina*, red algae and cyanobacteria is being utilized in food, pharmaceutical and cosmetic industries. Phycobiliproteins, chlorophylls, ϐ-carotene and astaxanthin are being commercially used in different fields and are being described below:

Phycobiliproteins

Phycobiliproteins are being used in the commercial sector as natural dyes. Thus, phycocyanin is widely used as food pigment to replace the current synthetic pigments. Native pigment prices of phycobiliproteins products are US$ 3 to US$ 25/mg and they can reach US$ 1500/mg for certain cross-linked pigments. Phycocyanin derived from *S. platensis* is used as a colourant in food items such as chewing gum, ice sherberts, popsicles, candies, soft drinks, dairy products and jellies. In addition, it is being used as colourant agent in lipstick and eyeliners (Santiago-Santos et al., 2004). C-phycocyanin is used as natural protein dye in the food industry (Sekar et al., 2008). Phycoerythrin derived from *Phorphyridium aerugineum* and *S. platensis* are also used in colour confectionary, gelatine deserts, fermented milk products, ice creams, sweet cake decoration, milk shakes and cosmetics. Besides colouring properties, phycoerythrin has yellow fluorescence properties and this fluorescent colour is used to make transparent lollipops originating from sugar solution, dry sugar-drop candies for cake decoration and soft drinks and alcoholic beverages (Dufoss et al., 2005).

Phycobiliproteins play a significant role in fluorescent based detection systems mainly for flow cytometry because of their spectral properties (Kronick and Grossman, 1983). Due to the absorbance spectrum properties, phycoerythrin has been used as a second colour in fluorescent labelling antibodies (Sekar et al., 2008). De Rosa et al. (2003) reported that phycoerythrin labelled with streptavidin can be used for the detection of DNA and protein probes. Low-molecular weight cryptomonad-derived phycobiliproteins are also used in flow cytometry both in extracellular and intracellular labelling applications (Telford et al., 2001).

Phycocyanin is used as a pharmaceutical agent because of their antioxidant, anti-inflammatory, neuroprotective and hepatoprotective properties (Sekar et al., 2008). Phycocyanin

derived from *Aphanizomenon flos-aquae* (AFA) is a strong antioxidant and applied *in vitro* against oxidative damage (Benedetti et al., 2004). In addition, C-phycocyanin derived from *S. platensis* actively influenced serum cholesterol concentrations and imparted a stronger hypocholesterolemic activity (Nagaoka et al., 2005). Moreover, phycocyanin also plays a role in hepatoprotective and anti-inflammatory effects in a human hepatitis animal model. Phycocyanin reduced the alanine amino transferase (ALT), aspartate amino transferase (AST) and malondialdehyde (MDA) in the serum (González et al., 2003). Furthermore, it has radical scavenging properties and inhibits microsomal lipid peroxidation. Phycocyanin also reduces oedema, histamine release myeloperoxide activity and the levels of prostaglandin and leukotrins in the inflamed tissues (Sekar et al., 2008). Phycocyanin have anti-cancerous effect by reducing the tumour necrosis factor (TNF-Ŭ) in the blood serum of mice treated with endotoxin and also neuroprotective effects in the rat cerebella granule cell cultures. Shih et al. (2003) reported that allophycocyanin inhibit entero virus 71- induced cytopathic effects, viral plaque formation and viral induced apoptosis. Phycocyanin derived from *S. platensis* was found to inhibit the growth of human leukaemia K562 cells (Liu et al., 2000). R-phycoerythrin subunits were used for improving the selectivity of photodynamic therapy and treatment for the mouse tumour cells S180 and human liver carcinoma cells SMC 7721 (Bei et al., 2002).

Chlorophylls

One or more types of chlorophyll are present in microalgae. The primary photosynthetic pigment, the chlorophyll *a* is abundant in cyanobacteria and rhodophyta. Chlorophyll *b* is present

in chlorophyta and euglenophyta which are similar to higher plants and marine microalga whereas chlorophylls *c*, *d* and *e* are present in fresh-water diatoms. Chlorophyll is an essential compound not only used as an additive in pharmaceutical but also used in cosmetic products. Chlorophyll *a* has been extensively used as a colouring agent because of its stability. This substance is usually obtained from higher plants in which other kind of chlorophyll is also synthesized. On the other hand, *S. platensis* has only chlorophyll *a*. The *Spirulina* sp. has the largest source of chlorophyll which is used for colorants as a substitute of artificial colour. Gross (Gross, 1991) reported that in Brazil approximately 0.06 mg/g chlorophyll from spinach is used as a natural green colorant while the *Spirulina* sp. biomass contains 1:15 mg/g of this pigment (Henrikson, 1989). Therefore, an attractive alternative source of chlorophyll pigment is the cyanobacterium (*S. platensis*) which is used as a natural colour in food, cosmetic and pharmaceutical products. Besides their application as food and pharmaceutical colourants, chlorophyll derivatives can exhibit health promoting activities. Ferruzi and Blakeslee (2007) suggested that chlorophyll compounds usually have medicinal application because of its wound healing and anti-inflammatory properties. Additionally, Balder et al. (2006) suggested that due to the consumption of chlorophyll there is a decrease in the risk of colorectal cancer.

β-carotene

There are more than 400 carotenoids available in nature and ϐ-carotene is perhaps the most important one. Some of these molecules are provitamin A and have a range of diverse biological functions and actions particularly in relation to human health (Pisal and Lele, 2005).

Researchers reported that ɓ-carotene exerts numerous benefits for human body, since the human body converts ɓ-carotene to vitamin A via the body tissue. Agarwal and Rao (2000) reported that vitamin A is necessary for the human body as it helps the immunity of the body and prevent cataracts, night blindness and skin diseases. In multivitamin preparations, ɓ-carotene is used as pro-vitamin A (retinol) and also used in the formulation of healthy foods (Spolaore et al., 2006; Krinsky and Johnson, 2005). ɓ-carotene from *Dunaliella* is used as food colourants to improve the appearance of margarine, cheese, fruit juices, baked goods, dairy products, canned foods and confectionary to attract the consumers. In addition, ɓ-carotene is also used as colourant and a precursor of vitamin A in pet foods (Cantrell et al., 2003).

ɓ-carotene has been associated with decreasing the hazard of several degenerative diseases including cancer (Ausich, 1997; Sandmann, 2001). It also has anticancer, anti-aging, immunemodulator properties (Rock, 1997). A few epidemiological researchers have found that ɓ-carotene from *Dunaliella* sp. contains 40 % 9-*cis* and 50% all-*trans* stereoisomers that plays a crucial role for lowering the incidence of several varieties of cancer and degenerative diseases (Ben-Amotz, 1999). In addition, Albanes et al. (1976) and Törnwall et al. (2004) investigated that the antioxidant properties of ɓ- carotene helps to mediate the harmful effects of free radicals for preventing the life threatening diseases such as arthritis, coronary heart diseases, premature aging and various forms of cancer. *Dunaliella* microalgae contain oxygenated carotenoids (Xanthophylls) which have better anti-cancerous activity and higher bioactivity (Roodenburg et al., 2000). Similarly, Mattson (2004) reported that ɓ-carotene can stimulate the immune system thus being potentially involved in more than 60 life-threatening diseases including various forms of cancer, coronary heart diseases, premature ageing and arthritis. In addition, ɓ-carotene also

decline the cognitive ability associated with Alzheimerôs disease caused by persistent oxidative stress in the brain (Mattson, 2004). Nakashima et al. (2009) found that cognitive impairment can be prevented by using transgenic mice fed with extracts from *Chlorella* sp. containing ß-carotene and lutein. Furthermore, colon cancer development can be inhibited by ß-carotene extracted from *C. ellipsoidea* and *C. vulgaris* (Plaza et al., 2009).

In 1986, ß-carotene produced from *Dunaliella salina* by Western Biotechnology (Hutt Lagoon, Australia) has been commercialized worldwide. Similarly, ß-carotene from other microalgae especially cyanobacteria is being produced in large scale in India. Recently, in markets there is a competition in between the microalgal carotenoids and the synthetic form of pigments. Microalgal carotenoids have the benefit of supplying natural isomers which is superior to the synthetic form (García-González et al., 2005). Some of the health benefits of ß-carotene studied in human and animal models have been tabulated in Table 6.

Astaxanthin

Astaxanthin has no provitamin activity like ß-carotene. *In vitro* studies by researchers have found astaxanthin to be effective for the prevention of oxidation of low density protein which can be applied to prevent arteriosclerosis, coronary heart disease and ischemic brain development (Miki et al., 1998). Dietary administration of astaxanthin has proven to inhibit

carcinogenesis in the mouse urinary bladder, rat oral cavity and rat colon (Tanaka et al., 1995). Moreover, astaxanthin has the ability to induce xenobiotic metabolizing enzymes in rat liver (Gradelet et al., 1996).

According to Jyonouchi et al. (1991), astaxanthin has the activity to enhance *in vitro* antibody production by mouse spleen cells stimulated with sheep red blood cells and human blood cells. Okai and Higashi-Okai (1996) have been suggesting that astaxanthin can modulate the humoral and non-humoral immune systems and enhances the release of interleukin-1 and the tumour necrosis factor Ŭ in mouse to a greater extent. In the presence of optimum amount of antigen, it has the ability to enhance the production of immunoglobulin A, M, G and on T-helper cell antibody production (Jyonouchi et al., 1994).

Astaxanthin act as a super vitamin E because of its stronger antioxidant activity which is 10 times higher than ß-carotene and more than 500 times more effective than Ŭ-tocoferol (Jyonouchi et al., 1994). Due to its stronger antioxidant activities, it has preventive effect against aflatoxin carcinogenicity (Gradelet et al., 1996) and inhibitory effect on lipid peroxidation mediated by active form of oxygen (Miki, 1991). In addition, antioxidant activity has been reported under both hydrophilic and hydrophobic conditions (Kobayashi et al., 1999) and also used as a photoprotectant against ultra violet irradiation (Savouré et al., 1995). According to Suzuki et al. (1996), astaxanthin containing preparations are used for prevention of light induced aging of skin. Alejung and Wadstroem (1998) developed an oral preparation for the treatment of *Helicobacter* infections of the mammalian gastrointestinal tract.

Astaxanthin can be used to prevent the neuronal damage associated with age related macular degeneration due to its powerful bioactive antioxidant properties (Snodderly, 1995). In

addition, astaxanthin can be helpful in treating Alzheimerôs disease, Parkinsonôs disease, ischemic reperfusion injury, spinal cord injuries and other types of central nervous system injuries due to its ability to cross the blood brain barrier and it does not form any crystal in the eye (Tso and Lam, 1996).

Synthetic astaxanthin is the predominant source of carotenoids for salmonids. Natural sources of astaxanthin for commercially raised salmonids can be utilized by processed crustacean waste from the krill, shrimp, crab and crawfish and another natural source *Phaffa rhodozyma*. Dietary astaxanthin can be used for the flesh pigmentation of Atlantic salmon and rainbow trout. The yearly worldwide aquaculture market of this pigment is expected at US$ 200 million with an average price of US$ 2500/kg (Hejazi and Wijffels, 2004). This pigment is used in contrast to synthetic form of the pigment produced by BASF (Ludwigshafen, Germany) and Hoffman-La Roche (Basel, Switzerland). Astaxanthin has been used to enhance the immunity of fish and shrimp for efficient growth and survival of fish. In addition, it also has an efficient role in aquaculture production and livestock feed market (Dufoss et al., 2005; Torrissen et al., 1989; Storebakken, 1988).

REGULATORY PRACTICE

Plant derived natural materials have been used to provide colour in food, drugs and cosmetics since time immemorial. However, synthetic organic dyes were developed to impart colouration since they were economical. Colour that is provided by means of synthetic pigments makes it necessary for the manufacturers to follow certain guidelines due to the toxic nature of the chemicals so that the product does not pose any risk to the worker and consumer as well as the environment. Food colour regulations vary from country to country. In the EU and the UK, all colour additives, whether artificial or natural, need to be approved for use in food and beverages. In the United States, all colour additives are regulated by the Food and Drug Administration (FDA) under authority granted by the Federal Food, Drug, and Cosmetic Act of 1938 and the 1960 Colour Additive Amendments to the Act. According to FDA, colour additives are classified into two classes (i) certified colour additives (synthetic), and, (ii) colour additives exempt from certification (natural). The act requires that both classes of colour additives should meet the same standards for safety, including compliance with the Delaney Clause which states that no colour additives shall be considered safe if it is found to induce cancer when ingested by man or animal. Therefore, there is a high consumer demand for natural colourants for use in different industries. These natural colour additives which are exempted from certification have a variety of uses, especially in foods (Freund et al., 1988), drugs, cosmetics and have few restrictions on their use at levels consistent with good manufacturing practice (GMP).

ɓ-Carotene found to be negative in genotoxicity tests (Bagdon *et al.,* 1960; Haveland-Smith, 1981; Heywood et al., 1985) is approved for use in the USA as a colour additive for foods, drugs and cosmetics. The FAO/WHO Expert Committee on Food Additives (JECFA, 1974) established an acceptable daily intake of 0-5 mg/kg body weight as a sum of carotenoids

used as colour additives. Astaxanthin is approved as food colouring for specific uses in animal and fish food by the FDA. It is approved as feed additive at EU level for salmon and trout at 100 mg kg^{-1} complete feed and is given the E number E161j. According to Stewart et al. (2008), regulation requires that the quantity of astaxanthin from *Haematococcus* algae meal in finished feed when used alone or in combination with other astaxanthin colour additive sources should not exceed 80 mg/kg of the finished feed. The FDA has recently amended the colour additive regulations to allow the safe use of astaxanthin dimethyl disuccinate for fish feeds designed to improve the pink/red colour of salmon and similar fish for human consumption. Chlorophyll (E140) and chlorophyllin (E141) are currently permitted food colourants. Phycobiliprotein products do not currently require pre-market clearances by the FDA but can be subject to GMP requirements.

CONCLUSIONS

Major pigments such as chlorophyll *a*, *b* and *c*, ɓ-carotene, astaxanthin, xanthophylls and phycobiliproteins have a wide range of promising applications in diagnostics, biomedical research, therapeutics, colorings in cosmetics, dairy products and other foods. They are gaining importance over synthetic ones since they are nontoxic and non-carcinogenic. The content of pigments depends on the species of microalgae and cultivation conditions. Temperature, salinity, irradiances, wavelength, photoperiods, pH, nutrient limitation, nitrogen supplements, pesticides and heavy metals affect the production of microalgal pigments. Hence, the factors above should

be taken into consideration for microalgal pigments production which can be used for different applications.

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Table 1 Pigments from different microalgae

Phylum	No. of genera/species	Common name	Pigments	References
Chlorophyta	Approximately 500/16,000	Green microalgae	Chlorophyll *a*, *b*, *β*-carotene, prasinoxanthin, siphonaxanthin, astaxanthin	Graham, 2000; Van den et al., 1995; Lee, 1999
Diatomophyceae /Diatoms	>200/100,000	Brown microalgae	Chlorophyll *a* and *c*, *β* -carotene, fucoxanthin, diadinoxanthin	Hasle and Syvertsen, 1997; Round et al., 1990; Canter-Lund and Lund, 1995
Cryptophytes	About 12-23/200	Cryptomonads	Chlorophyll *a* and *c*, carotenoids and phycobiliproteins	Graham, 2000; Van den et al., 1995; Lee, 1999
Cyanobacteria	Total 10/>2000	Blue-green microalgae	Chlorophyll *a*, xanthophyll and phycobiliproteins	Graham, 2000
Euglenophyta	About 40/900	Euglenoids	Chlorophyll *a* and *b*, diadinoxanthin, neoxanthin, and *β*-	Graham, 2000; Van den et al., 1995; Lee, 1999

			carotene	
Dinophyta	About 130 /220	Dinoflagellates	Chlorophyll *a*, *c*, carotenoid (β - carotene), peridinin	Graham, 2000; Van den et al., 1995; Lee, 1999

Table 2 Effects of light intensity and light colour on pigment production in different microalgal species

Light intensity (μmolphoton/m^2/sec; light colour)	Species	Pigments	Production (mg/g)	References
27; white	*Spirulina platensis*	Chlorophyll	14.7	Chauhan and Pathak, 2010
54; white	*Spirulina platensis*	Chlorophyll	11.6	Chauhan and Pathak, 2010
25; green	*Spirulina maxima*	Phycobiliproteins	96.0	Tomasseli et al., 1997
25; red	*Synechococcus* NKBG 042902	Phycocyanin	63.0	Takano et al., 1995
25; white	*Anabaena* NCCU-9	Phycobiliproteins	124.9	Hemlata and Fatma, 2009
25; white	*Synechocystis* sp. PCC 6701	Phycobiliproteins	25.9	Hong and Lee, 2008
60; red	*Nostoc* UAM 206	Phycocyanin	94.4	Poza-Carrión et al., 2001
				Poza-Carrión et al., 2001
		Phycoerythrin	17.7	Poza-Carrión et al., 2001
		Allophycocyanin	26.4	
0.97; blue	*Chondrus crispus*	Phycocyanin	0.3	Franklin et al., 2002
		Phycoerythrin	2.8	Franklin et al., 2002
546; fluorescent	*Haematococcus pluvialis*	Astaxanthin	30.0	Imamoglu et al., 2009

11.28; white	*Dunaliella salina*	Chlorophyll	66.9	Pisal and Lele, 2005
	Dunaliella salina	ɓ-carotene	9.8	Pisal and Lele, 2005
32.43; white light	*Dunaliella salina*	ɓ-carotene	17.7	Pisal and Lele, 2005
	Dunaliella salina	Chlorophyll	44.5	Pisal and Lele, 2005

Table 3 Optimum temperature for pigment production in different microalgal species

Temperature (°C)	Species	Pigments	Production (mg/l)	References
25	*Spirulina platensis*	Phycocyanin	0.0036	Silveira et al., 2007
	Dunaliella salina	ɓ-carotene	2.5	García-González et al., 2005
	Haematococcus pluvialis	Astaxanthin	13.0	Olaizola, 2000
28	*Spirulina platensis*	Chlorophyll	0.9	Chauhan and Pathak, 2010
	Muriellopsis sp.	Lutein	5500.0	Del Campo et al. 2000
	Chlorella protothecoides	Lutein	10.0	Wei et al., 2008
	Chlorella zofingiensis	Lutein	3.4	Wei et al., 2008
	Haematococcus pluvialis	Astaxanthin	98.0	Domínguez-Bocanegra et al., 2004
30	*Scenedesmus almeriensis*	Carotenoids	4.9	Sánchez et al., 2008
	Dunaliella salina	ɓ-carotene	13.5	Del Campo et al., 2001
	Chlorella zofingiensis	Astaxanthin	10.3	Ip and Chen 2005
35	*Anabaenopsis* sp., *Nostoc paludosum*	Phycocyanin	0.13, 0.167	Moreno et al., 1995
		Phycoerythrin	0.008, 0.006	
		Allophycocyanin	0.063, 0.101	
36	*Arthronema africanum*	Phycocyanin	0.23	Chaneva et al., 2007
		Allophycocyanin	0.12	
39	*Scenedesmus almeriensis*	Carotenoids	20.0	Macías-Sánchez et al., 2009
50	*Synechococcus* sp.	Carotenoids	1510.0	Macías-Sánchez et al., 2005

55	*Chlorella vulgaris*	Carotenoids	80.0	Mendes et al., 1995
60	*Nannochloropsis gaditana*	Carotenoids	25.0	Macías-Sánchez et al., 2009

Table 4 Effects of pH on pigment production in microalgae

pH	Species	Pigments	Production (mg/g)	References
6.5	*Chlorella zofingiensis*	Astaxanthin	0.01	Ip and Chen 2005
	Chlorella protothecoides	Carotenoid	0.01	Wei et al., 2008
	Muriellopsis sp.	Lutein	5.5	Del Campo et al., 2000
7.0	*Chlorococcum citriforme*	Lutein	7.2	Del Campo et al., 2000
	Neospongiococcus gelatinosum	Lutein	7.6	
7.5	*Dunaliella salina*	ϐ-carotene	0.002	García-González et al., 2005
8.0	*Scenedesmus almeriensis*	Carotenoid	0.004	Sánchez et al., 2008
	Anabaena sp.	Phycobiliproteins	102.2	Hemlata and Fatma, 2009
	Synechocystis sp.		25.9	Hong and Lee, 2008
9.0	*Nostoc* sp.	Phycocyanin	21.7	Poza-Carrión et al., 2001
		Phycoerythrin	36.2	
		Allophycocyanin	7.2	
	Spirulina platensis	Chlorophyll	0.9	Chauhan and Pathak, 2010

Table 5 Effects of salinity on pigment production in microalgae

Salinity (ppt)	Species	Pigments	Maximum Production (mg/g)	References
3	*Dunaliella salina*	ϐ-carotene	54.12	Pisal and Lele, 2005
10	*Anabaena* NCCU-9	Phycobiliproteins	135.73	Hemlata and Fatma, 2009
15	*Oscillatoria* sp.	Phycoerythrin	66.70	Chu et al., 2002

Table 6 Health benefits of ϐ-carotene studied in human and animal models

Pharmacological benefits	Studied in	Effects	References
Liver microsomes (antiperoxidase)	Rats	Stimulate microsomal lipid peroxidation by $FeSO_4$ and cysteine; subsequently induce a high level of lipid peroxidation in rat liver microsomes	Searle and Willson, 1983
Liver mitochondria (antiperoxidase)	Rats	Inhibitory activity against the action of free radicals on rat liver mitochondria	Miki, 1991
Seminal vesicle (antiperoxidase)	Bovine	Inhibitory effect on oxidation of Arachidonic acid in bovine seminal vesicle and inhibit the production of prostaglandin	Albanes et al., 1976
Cancer	Human volunteers	Reverse precursor lesions Prevent the regression of precursor lesion to overt malignancies Reduce incidence of malignancy Reduce cancer mortality	Schwartz et al., 1986
Oral cancer	Hamster	Carcinogenesis Protective 98% regression of tumours	Mathews-Roth, 1982
Skin cancer	Mouse	Protective decrease in tumour incidence by	Gao et al., 1994;

		39% (crystal) and 29% (beads)	Comstock et al., 1991
Esophagus cancer	Human volunteers	Strong inverse association	Peter et al., 1992
Colon cancer	Human volunteers	Strong inverse association	Graham et al., 1991
Breast cancer	Human volunteers	Strong inverse association	Comstock et al., 1991
Lung cancer	Human volunteers	Strong inverse association	Comstock et al., 1991

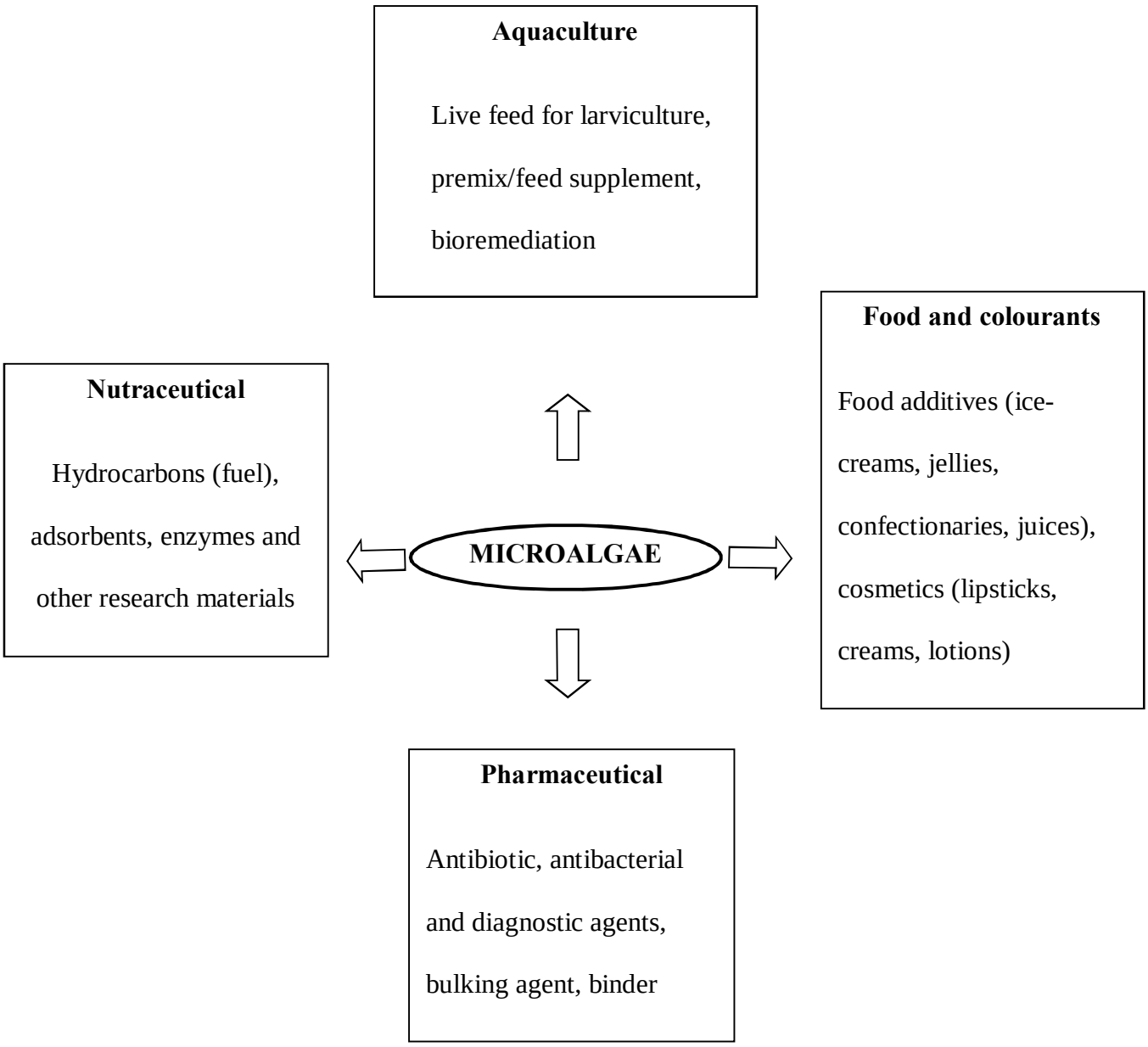

Figure 1 Commercial applications of microalgae in different fields