CHAPTER 2
LITERATURE REVIEWS

2.1 Astaxanthin

Astaxanthin (3,3'-dihydroxy-β,β-carotene-carotene,4,4'-dione) is a distinctive red pigment belonging to the family of the carotenoids, which is classified as xanthophylls (Figure 2.1). It is widespread in nature and found in plants, animals, and microorganisms. The majority are hydrocarbons of 40 carbon atoms whose molecular formula is $C_{40}H_{52}O_4$ with a molecular weight of 596.86 g/mol (Foppen, 1971). Astaxanthin has two asymmetric carbon atoms at the 3 and 3' position and can exist in three configurations, including identical enantiomers of [3S,3'S], [3R,3'R], and [3R,3'S] (Figure 2.1).

The presence of the hydroxyl and keto endings (Figure 2.1) on each ionone ring shows some unique features, such as high antioxidant activity and a more polar configuration than other carotenoids. Actually, free astaxanthin is particularly sensitive to oxidation. In nature, it could be found either conjugated to proteins, such as muscle or exoskeleton, or esterified with one or two fatty acids, which stabilize the molecule (Guerin *et al.*, 2003).

Since animal cannot synthesize astaxanthin *de novo*, thus it needs to be provided in diet to acquire coloration. Although mammals and most fish are unable to convert other dietary carotenoids into astaxanthin, however, crustaceans (such as shrimp and some fish species including koi carp) have a limited capacity to convert closely related dietary carotenoids into astaxanthin, and they benefit from being fed astaxanthin directly (Guerin *et al.*, 2003). US Food and Drug Administration (FDA) reviews a safety profile of astaxanthin and approves astaxanthin as a feed coloring agents (or color additive) for specific uses in animal and fish feeds (U.S. Food and Drug Administration, 2009). The European Union (aka European Commission) considers it as food dye with the E number system, E161j (Wikipedia, 2009a).

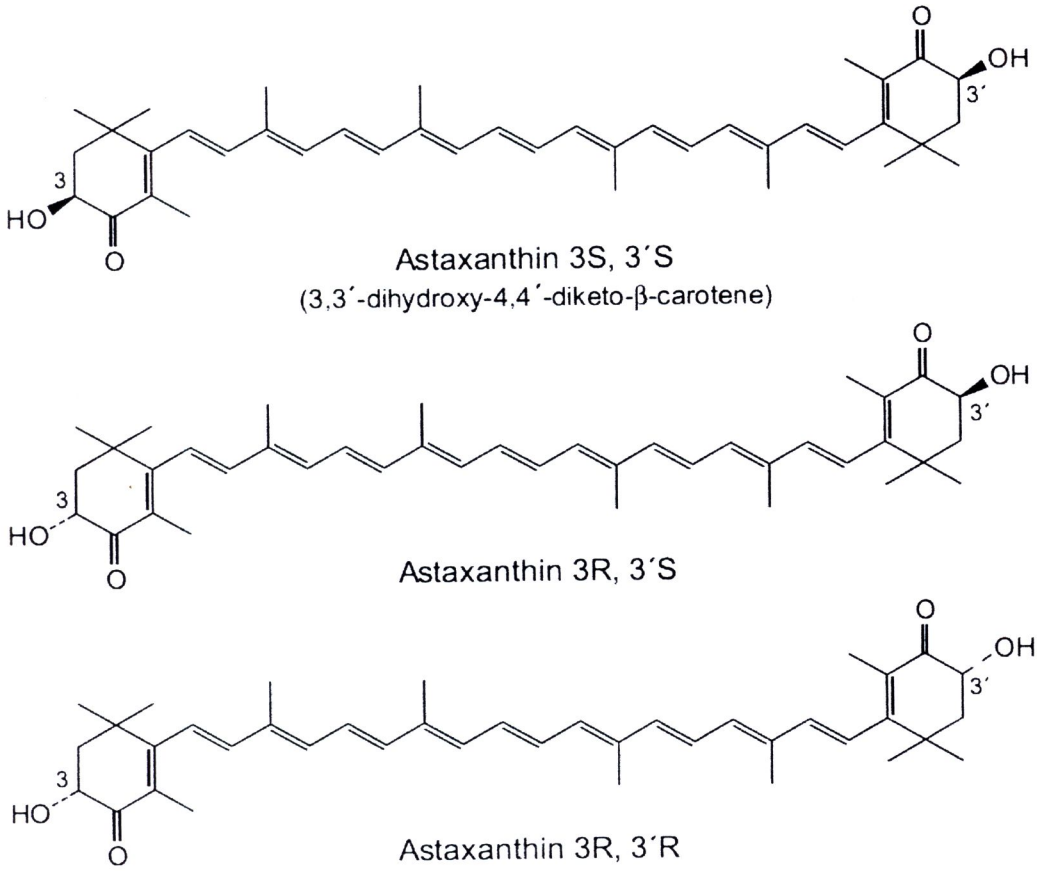


Figure 2.1 Structure of astaxanthin with several configurational isomers (Guerin *et al.*, 2003)

2.2 Properties of Astaxanthin

Astaxanthin is an important pigment widely used in aquatic farm animal and human health. This has stirred great interest in astaxanthin and prompted numerous research studies concerning its potential benefits to both humans and animals.

2.2.1 Antioxidant Property

Free radicals such as reactive oxygen species (ROS) or reactive nitrogen species (RNS) while present in excess will cause oxidative stress. It is important that, free radicals may cause cellular damage such as DNA fragmentation, lipid peroxidation, and protein oxidation. Moreover, they relate to several diseases such as cancer, cardiovascular disease, and inflammations (Hussein *et al.*, 2006; Pashkow *et al.*, 2008; Preuss *et al.*, 2009). Miki (1991) reported that astaxanthin shows ability antioxidant activity, as high as 10 times more than other carotenoids such as zeaxanthin, lutein, and β-carotene; and

100 times more than α-tocopherol (vitamin E) (Figure 2.2). Astaxanthin is the powerful antioxidant against singlet oxygen species ($^1O_2$) and peroxyl radical; a molecule having an unpaired electron which will react with another molecule in order to obtain this missing electron resulting in cellular damage. Astaxanthin protects against oxidative stress via two mechanisms: (1) quenching of singlet oxygen and releasing heat as final product and (2) scavenging of free radicals to terminate chain reactions leading to break down of its molecule (Schroeder and Johnson, 1995).

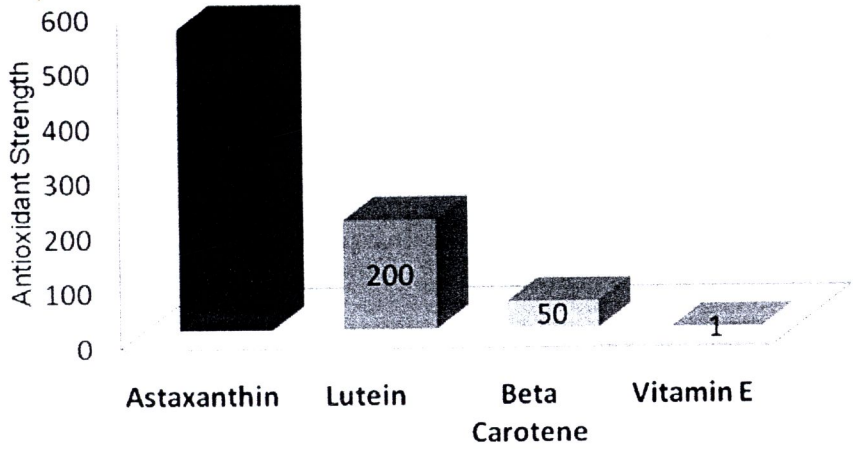


Figure 2.2 Singlet oxygen quenching rate of carotenoids and α-tocopherol (Vitamin E) (Miki, 1991)

2.2.2 Applications for Human Health

2.2.2.1 Anti-Cancer Properties

Several studies have demonstrated the anti-cancer activity of astaxanthin in mammals. The supplementation of astaxanthin on various cancer cell types showed that oral administration of astaxanthin inhibits carcinogenesis in mice urinary bladder (Tanaka *et al.*, 1994), oral cavity (Tanaka *et al.* 1995a), and rat colon (Tanaka *et al.*, 1995b). This effect has been partially attributed to suppression of cancer cell proliferation. Furthermore, dietary astaxanthin is also significant by effective in reducing growth of mice tumor (weight and size), fibrosarcoma cells (Jyonouchi *et al.*, 2000).

2.2.2.2 Prevention of Cardiovascular Diseases (CVD)

The risk of developing arteriosclerosis in humans correlates with the cholesterol content bound to Low Density Lipoprotein (LDL). Many studies have documented that high levels of LDL are related to prevalence of cardiovascular disease. Inhibition of oxidation of LDL has been supposed as a likely mechanism through which antioxidants could prevent the development of arteriosclerosis. Several studies have considered carotenoids as inhibitors of LDL oxidation. Astaxanthin could reduce LDL oxidation in humans, which correlated with coronary heart disease in contrast to other antioxidants (Pashkow *et al.*, 2008). Thus, astaxanthin could benefit heart health by modifying blood levels of LDL and HDL cholesterol.

2.2.3 Astaxanthin in Animal Feed Industries

Astaxanthin is mainly used for fish farm such as salmons and trouts, shrimp, and crab. Since animal cannot synthesize astaxanthin by itself, the coloration has to be provided to their feed to impart pigmentation. Moreover, it has many other important functions in fish related mainly to reproduction including acceleration of sexual maturity, increasing fertilization and egg survival, and a better embryo development (Putnam, 1991). The studies have demonstrated in crustaceans, mainly on shrimp, that astaxanthin increases tolerance to stress, improves the immune response, and enhances larvae growth and survival (Gabaudan, 1996; Darachai *et al.*, 1999). Furthermore, utilization of astaxanthin in the aquaculture is considered not only as provider of pigmentation to increase consumer acceptance but also as a necessary nutrient for adequate growth and reproduction of commercially available farmed animals.

2.3 Source of Astaxanthin

Astaxanthin available in the market is produced from either chemical or biological process.

2.3.1 Synthetic Astaxanthin

The most supplement employed in feed industries is astaxanthin. It sells about 2,500 $US per kilogram with an annual worldwide market estimated at 200 $US million, however, the fact is that more than 95% available was chemically synthesized (Lorenz and Cysewski, 2000). Synthetic astaxanthin is of identical configuration to that produced by microorganisms and it composes of isomers [3S, 3'S], [3R, 3'S], and [3R,

3'R] (Figure 2.1) in ratio 1:2:1 respectively. However, the growing demand for biological supplement in food, high cost of synthetic pigments, moreover and an increasingly strict regulation regarding safety concern of chemical supplemented as food additive (Tangeras *et al.*, 2003) has stimulated the search for natural sources of astaxanthin with potential for industry.

Then, production of astaxanthin from natural source, i.e., *X. dendrorhous* (yeast) and *Haematococcus pluvialis* (microalgae) are more interesting than synthetic astaxanthin.

2.3.2 Microalgae

The major market for astaxanthin demand is as a pigmentation source in aquaculture, primarily in salmon and trout. Although 95% of astaxanthin available in the market is chemically synthesized concern regarding regulation results in demand of biological products, which, in turn, provides an opportunity for the production of natural astaxanthin. Furthur, microalgae *Haematococcus* are the most interesting in production of astaxanthin commercially since it accumulates high astaxanthin (red cysts of *Haematococcus* contain astaxanthin accounting for 80% of the carotenoid fraction). The production system consists of two steps. Firstly, microalgae were cultivated large ponds under controlled conditions such as pH, carbon dioxide, and light and, secondly, break down of cell wall was accomplished to harvest astaxanthin (Lorenz and Cysewski, 2000). The biomass is finally dried to obtain a fine powder of reddish color.

In principle, the production of *Haematococcus* algae as a source of natural astaxanthin is relatively simple. However, *Haematococcus* growth in a neutral culture medium was contaminated by other strains of microalgae, amoeba, and protozoa (Lorenz and Cysewski, 2000), presenting problem in scale up.

2.3.3 Yeast

Xanthophyllomyces dendrorhous (formerly *Phaffia rhodozyma*) is the yeast commonly known as red yeast, which accumulates astaxanthin as a major carotenoid. *P. rhodozyma* is able to produce astaxanthin at concentrations of up to 80% of its total carotenoid content (Andrewes *et al.*, 1976) and is, therefore, considered as a potential source for astaxanthin production. It can ferment several of sugars including glucose, maltose, sucrose, raffinose, and cellobiose (Johnson and Lewis, 1979). This property differentiates it from other carotenoid-producing yeast. There are reports on the use of

inexpensive carbon sources for production of astaxanthin by *X. dendrorhous*, including, cane molasses (Haard, 1988); sugar cane juice (Fontana *et al.*, 1996); grape juice (Meyer and du Preez, 1994); hydrolyzed peat (Martin *et al.*, 1993); raw coconut milk (Domínguez-Bocanegra and Torres-Muñoz, 2004); and plant extracts (Kim *et al.*, 2007). The cultural aspect of *X. dendrorhous* simplifies manipulation and up-scale production, therefore, the yeast has been recognized as the major source for producing natural astaxanthin.

2.4 *Xanthophyllomyces dendrorhous*

The yeast *X. dendrorhous* (formerly *Phaffia rhodozyma)*, accumulating high astaxanthin content, is a possible candidate for commercial production of astaxanthin. *Phaffia rhodozyma* was first isolated during the 1960s by Herman Jan Phaff, and was initially designated as *Rhodozyma montanae* due to its color, and region found and eventually assigned as *Phaffia rhodozyma*. The yeast was recognized as basidiomycetes based on its morphology, cell wall properties, mode of bud formation, pigmentation, and metabolic properties. Despite many attempts have made several years to mate the various strains and observe a dikaryotic mycelium and teliophore formation, a sexual life cycle was not found. Later Golubev (1995) found the teleomorphic strain (sexual state) and named it as *Xanthophyllomyces dendrorhous* by depleting of yeast nitrogen base, while supplementing exogenous polyols, and especially ribitol. The yeast had several unusual characteristics and the remarkable property which include red-orange colonies due to the present of carotenoid pigments. It could grow within the temperature ranging from 0-27 °C. A major property that distinguishes *X. dendrorhous* from other genera of related yeast is the composition of carotenoids it produces. Astaxanthin has been identified as the major pigment in *X. dendrorhous*. Wild strains of *X. dendrorhous* contain up to 500 μg/g dry weight yeast of total carotenoid, of which 40-95% is astaxanthin (Johnson, 2003). Astaxanthin produced by *X. dendrorhous* has particular feature that is 3R,3'R-configuration which differs from other sources. This feature facilitates animal absorption (Johnson, 2003). This was the first example of a naturally occurring carotenoid biosynthesized in different optical forms, and it raised interesting questions regarding the properties of the enzymes involved in the apparently distinct R-astaxanthin biosynthetic pathways in *X. dendrorhous* compared to those of other organisms that contained astaxanthin with the S configuration (Andrewes and Starr,

1976). However, this configuration was approved by FDA as a food coloring agents (or color additive) for specific usage in animal feed and food industry (Johnson, 2003).

2.5 Factors Affecting Growth and Astaxanthin Production

Several studies available in literature have demonstrated that growth and carotenoids production by *X. dendrorhous* were greatly affected by a number of operating parameters such as carbon source, nitrogen source, C/N ratio, pH, temperature, and some chemical enhancers.

2.5.1 Carbon Source

Carbon source has affected growth and astaxanthin production because it is necessary in synthesizing carbon skeleton of cell and carotenoids. *X. dendrorhous* had the ability to vigorously ferment several of sugars such as glucose, maltose, sucrose, and raffinose including cellobiose. Fang and Cheng (1993) reported that fructose, glucose, and sucrose highly promoted cell mass production, while sucrose at the same concentration (10 g/L) could significantly enhance astaxanthin production since *X. dendrorhous* could produce invertase enzyme (Johnson and Lewis, 1979). Several attempts have been made to investigate several carbon sources of industrial potential for astaxanthin production by *X. dendrorhous*, including, cane molasses (Haard, 1988); sugar cane juice (Fontana *et al.*, 1996); grape juice (Meyer and du Preez, 1994); hydrolyzed peat (Martin *et al.*, 1993); raw coconut milk (Domínguez-Bocanegra and Torres-Muñoz, 2004); and plant extracts (Kim *et al.*, 2007).

Effect of carbon sources on both growth and astaxanthin production is summarized in Table 2.1.

2.5.2 Nitrogen Source

Yeast cells contain nitrogen around 10% of their dry weight. Thus, nitrogen source is one of the necessary components in the fermentation medium. Different nitrogen sources could greatly affect the yield of astaxanthin (Du *et al.*, 2005). Vustin *et al.* (2004) suggested that abundant nitrogen in the medium may enhance cell growth but suppress the enzymes involved in converting β-carotene to astaxanthin. Fang and Cheng (1993) have reported that peptone, nutrient broth, beef extract, and casein hydrolysate promoted pigment production providing that peptone, in particular, was considered the best nitrogen source yielding high astaxanthin production (Table 2.2). In addition, yeast

Table 2.1 Effects of carbon sources on growth and astaxanthin production by *X. dendrorhous*

Carbon source	Growth (g/L)	Total Astaxanthin (mg/L)	Astaxanthin Content (mg/g $_{yeast}$)	References
Glucose (10 g/L)	4.78	7.81	1.633	Fang and Cheng (1993)
Fructose (10 g/L)	4.90	7.15	1.451	
Sucrose (10 g/L)	4.60	7.75	1.689	
Maltose (10 g/L)	4.05	5.33	1.317	
Molasses (10 g/L)	3.81	3.97	1.039	
Coconut milk	9.20	-	0.850	Domínguez-Bocanegra and Torres-Muñoz, (2004)
Wood hydrolysates	10.00	-	2.14	Cruz and Parajo (1998)
Green onion	6.73	9.61	1.43	Kim *et al.* (2007)
Cabbage	6.57	8.76	1.33	
Sesame leaf	11.20	11.26	1.01	
Pineapple juice (RS 10 g/L)	5.48	-	0.493	Jirasripongpun *et al.* (2008)

extract led to high cell concentration. *X. dendrorhous* can utilize simple nitrogen sources such as ammonium salts. For example, ammonium sulfate ($(NH_4)_2SO_4$) is a commonly used nitrogen source in yeast growth media which has been shown to affect both growth and carotenoid production of several *X. dendrorhous* mutant strains (An *et al.*, 1989) since it also provides a source of assimilable sulfur. *X. dendrorhous* is incapable of utilizing nitrate, but could hydrolyze urea since it could produce urease enzyme (Johnson and An, 1991). However, no growth was observed in mutant NCHU-FS301 (Fang and Cheng, 1993). Ni *et al.* (2007) investigated effects of ammonium sulfate $(NH_4)_2SO_4$, potassium nitrate (KNO_3) and beef extract on astaxanthin production by *X. dendrorhous* using mixture design and found that proportion of three nitrogen sources was very important to astaxanthin production. Further, the nitrogen source of choice in the growth medium is yeast extract; however, high price hinders its use in large quantities. Therefore, nitrogen source with high efficiency is vigorously sought.

Table 2.2 Effects of nitrogen sources on growth and astaxanthin production by *X. dendrorhous*

Nitrogen source	Cell mass (g/L)	Total astaxanthin (mg/L)	Astaxanthin Content (mg/g $_{yeast}$)
Peptone	4. 373	7.206	1.648
Beef extract	5.165	8.046	1.558
Casein hydrolysate	5.450	8.379	1.538
Soytone	3.340	4.678	1.401
Nutrient broth	4.793	7.531	1.571
Yeast extract	6.657	8.638	1.298
Urea	0.105	Nd	Nd
Ammonium sulfate	1.725	2.300	1.334
Potassium nitrate	3.983	5.323	1.336
Ammonium nitrate	1.032	0.954	0.924

Source: Fang and Cheng (1993)

2.5.3 C/N Ratio

C/N ratio could significantly influence cell growth and carotenoid biosynthesis in *X. dendrorhous*. Cell growth is enhanced by maintaining low C/N ratio, while a high C/N ratio enhances astaxanthin production (Roy *et al.,* 2008). Most studies have suggested that high C/N ratio is more favorable for the biosynthesis of carotenoids in *X. dendrorhous*. Thus, a low initial C/N ratio of the medium supports cell growth but decreases astaxanthin production which is performed following a two-stage fed-batch culture system. Yamane *et al.* (1997b) proposed that high initial C/N ratio may decrease the NADPH consumption for primary metabolism such as protein synthesis, so as to drive more NADPH available for astaxanthin biosynthesis. Vustin *et al.* (2004) reported that a marked decrease in cell growth was concomitant with an increase in the astaxanthin content of cell as the C/N ratio of medium was increased in *P. rhodozyma* cultures, and rapid growth was coupled with lower activities of the key enzymes involved in astaxanthin synthesis. The authors then suggested that abundant nitrogen in the medium may enhance the cell growth but suppress the enzymes for β-carotene conversion to astaxanthin. In mycelia cultures of *Gibberella fujikuroi* fungus, while nitrogen feeding increased the cell growth, nitrogen limitation stimulated carotenoid

biosynthesis, perhaps by imposing C/N imbalance and driving most of assimilated carbon to the secondary metabolism pathways (Garbayo *et al.*, 2003).

2.5.4 pH

pH of growth medium is significant factor affecting the astaxanthin production in *X. dendrorhous* cultures reported by many previous studies. Several studies have identified pH 5.0 as optimum for cell growth and astaxanthin biosynthesis in the cultures of wild *X. dendrorhous* strains (Johnson and Lewis, 1979; Hu *et al.*, 2006) and their mutants (Meyer and Du Preez, 1994; Fang and Cheng, 1993). In addition, the optimum pH for the growth of *X. dendrorhous* is pH 6.0 whereas optimum pH for astaxanthin production is 5.0 (Hu *et al.*, 2006). Liu and Wu (2007) reported in their studies that the optimal medium pH for cell growth was 5.85 which was significantly higher than 5.0. Others have also reported much higher pH optima, such as pH 6.9 for maximum cell growth of *X. dendrorhous* ATCC 24202 in a continuous (chemostat) culture (Vázquez and Martin, 1998), and pH 6.0 for high volumetric astaxanthin yields with the same strain in a batch culture (Ramírez *et al.* (2001). The difference in the pH optima found in many studies may be attributed to the differences in the basic media and the yeast strains. Regardless of the deviations, a general trend observed from these studies is that a slightly acidic condition is favorable for astaxanthin biosynthesis (Liu and Wu, 2007).

2.5.5 Temperature

Temperature was the factor significantly influencing the astaxanthin production (Ramírez *et al.* (2001). Johnson and Lewis (1979) described the importance of temperature on growth and astaxanthin synthesis. The optimum temperature reported for wild type strains ranges between 20 and 22°C. In the case of mutant strains, the optimal temperature can vary. Fang and Cheng (1993) obtained a mutant strain, showing higher astaxanthin production at 15°C. On the other hand, Ramírez *et al.* (2001) reported that the synthesis of carotenoids by the mutant strain 25-2 at 16°C was lower than the production obtained at 22°C. The growth and pigment synthesis decreased considerably at temperatures under 18 and above 23°C. The authors report that the optimum temperature was 19.7°C; however, high yields were also obtained between 18 and 22°C. The previous results agreed with those reported by Johnson and Lewis (1979).

2.5.6 Oxygen Supply

2.5.6.1 Dissolve Oxygen

Oxygen supply to the culture broth may be a key factor for growth and astaxanthin production by *X. dendrorhous*. Yeast is unable to grow well in the absence of oxygen because it provides a substrate for respiratory enzyme during aerobic growth. In general, oxygen is required for certain growth-maintaining hydroxylation such as those involving the biosynthesis of sterols and unsaturated fatty acids; in case of *X. dendrorhous*, biosynthesis of carotenoids (Walker, 1998). Liu *et al.* (2006) have studied effects of oxygen transfer on the growth and carotenoid (astaxanthin) production of the red yeast *P. rhodozyma* in liquid cultures. Both cell growth and carotenoid production in shake-flasks increased with the decrease in the liquid volume from 50 to 20 mL and the increase in the shaker speed from 200-250 rpm. The direct correlation between carotenoid yield and oxygen uptake rate (OTR) suggests that carotenoid biosynthesis may be enhanced by the increase in the respiratory activity of *P. rhodozyma.* The equation below shows substrate required for astaxanthin production indicating that 4 moles of oxygen are needed for astaxanthin production of one mole (Yamane *et al.*, 1997b).

$$12C_6H_{12}O_6 + 4O_2 + 14NADPH + 48NAD^+ = 1C_{40}H_{52}O_4 + 32CO_2$$
$$+14NADP^+ + 48NADH + 12H_2O + 34H^+$$

(Equation 2.1)

Large amount of NADH is produced as a side product of the astaxanthin production supporting that astaxanthin production might also be repressed with an increase in NADH accumulation. Since oxygen can reoxidize NADH to NAD^+, therefore, it is considered that a sufficient supply of oxygen could enhance pigment production (Yamane *et al.*, 1997b). Moreover, aerobic fermentation in the presence of fully aerobic culture conditions and high levels of readily metabolizable sugar resulting in a reduced biomass yield and formation of fermentation products which is known as Crabtree effect.

2.5.6.2 Oxygen Vector

X. dendrorhous is an aerobic organism whose growth (primary metabolism) is dependent on the oxygen supply in the culture system (Liu *et al.*, 2006). Oxygen supply

plays a major role in liquid fermentations of aerobic microorganisms owing to poor solubility as well as low diffusion rate of oxygen into broth; thus, limiting growth of organisms being cultivated. Attempts have been made to overcome such limitation such as supply of pure oxygen to the system (Ho *et al.*, 1990; Lee and Kim, 2004; Wang, 2000; Liu and Wu, 2006). Oxygen vectors are the compounds that when added to growth media can enhance the oxygen transfer rate through microorganisms, resulting in a higher capacity for oxygen solubility about 15 to 20 times as compared with water (Ju and Ho, 1989; Wilhelm and Battino, 1986). Oxygen vectors are generally non-toxic to the cultivated microorganisms. Therefore, additions of some organic solvents with high oxygen solubility to the culture medium as oxygen vectors are being explored. The main oxygen vectors used in biotechnology are hydrocarbons; some organic solvents such as *n*-hexadecane (Ho *et al.*, 1990; Lee and Kim, 2004) and n-dodecane (Wang, 2000). These organic solvents usually have higher oxygen solubility than the liquid medium since oxygen vectors form small droplets when present in liquid medium which may be directly adsorb at the bubble surface within the culture medium, thus increasing the contact area for oxygen transfer (MacLean, 1977; Rols and Goma, 1989; Liu and Wu, 2006). da Silva *et al.* (2006) employed 1% (v/v) n-dodecane for enhancing *Crypthecodinium cohnii* growth and docosahexaenoic acid (DHA) production and reported that highest DHA content of biomass and DHA percentage of TFA of 6.14 and 51%, respectively, were obtained. The authors, then, concluded that higher products were obtained as a result of an increase in dissolved oxygen tension (DOT) in culture medium. In addition, Rols and Goma (1991) employed 19% (v/v) of soybean oil as oxygen vector in the cultivation of *Aerobacter aerogenes*, which enabled a 1.85-fold increase of the volumetric oxygen transfer (k_La) coefficient leading to high cell concentration. Moreover, Liu and Wu (2006) investigated effectiveness of five liquid hydrocarbons, i.e., *n*-hexane, toluene, *n*-decane, *n*-dodecane, and *n*-hexadecane as oxygen vectors on *X. dendrorhous* cultivation for enhancing growth and carotenoids production. The authors reported that *n*-hexadecane was proved to be the most beneficial while the addition of 9% (v/v) *n*-hexadecane to the liquid medium at the time of inoculation was found to be optimal, increasing the carotenoids yield by 58% (14.5 mg/L) as compared to the control (9.2 mg/L) with an increased in oxygen transfer rate (OTR) by 90%.

2.5.7 Chemical Enhancer

2.5.7.1 Tricarboxylic Acid (TCA) Cycle Intermediate

The TCA or Kreb's cycle has unique roles and functions in metabolic reactions. The TCA cycle, including the intermediates, i.e., α-ketoglutarate, citrate, malate, fumarate, oxaloacetate and succinate, has been proposed to made available the precursors for the biosynthesis of carotenoids in carotenogenic microorganisms (Nasri Nasrabadi and Razavi, 2010). The major roles of TCA cycle include (i) providing electrons (NADH) for the electron transport chain and biosynthesis, (ii) supplying carbon skeleton or precursors for the biosynthesis of amino acid, nucleic acid, lipids, and polysaccharides, and (iii) generating energy. Moreover, TCA cycle intermediates were known to increase the acetyl-CoA pool, which is known as the origin of isoprene units and, as a result, promoting carotenogenesis (Alcantara and Sanchez, 1999; Flores-Cotera *et al.*, 2001). Therefore, TCA intermediates have been employed as enhancer for carotenoid production in several microorganisms (An, 2001; Bjork and Neujahr, 1969; Flores-Cotera *et al.*, 2001; Nasri Nasrabadi and Razavi, 2010). For example, Nasri Nasrabadi and Razavi (2010) reported that when growth medium was supplemented with α-ketoglutarate, oxaloacetate, and succinate at concentrations of 9.69, 8.68, and 8.51 mM, respectively, the highest canthaxanthin of 13.17 mg/L was achieved by *Dietzia natronolimnaea* HS-1. In addition, Flores-Cotera *et al.* (2001) found that supplementation of 28 mM citrate could also enhance carotenoid production in *X. dendrorhous*. The authors further suggested that citrate could also be the source of carbon for fatty acids as well as carotenoids biosynthesis.

2.5.7.2 Ethanol

Ethanol has been shown to affect morphology, sexual activity, and metabolic activities of various yeasts and fungi (Jones, 1989). Moreover, ethanol has also been reported to increase the acetyl-CoA pool. Ethanol is oxidized to acetaldehyde by either alcohol dehydrogenase or a cytochrome P-450 enzyme system (Jones, 1989). Acetaldehyde is known to be further catabolized by at least three routes:

$$(1)\ \text{Acetaldehyde} + H_2O + O_2 \xrightarrow[\text{aldehyde oxidase}]{} \text{Acetate} + \text{Superoxide}$$

$$(2)\text{Acetaldehyde} + CO_2 \xrightarrow[\text{pyruvate decarboxylase}]{} \text{Pyruvate}$$

$$(3)\ \text{Acetaldehyde} + H_2O + \text{NAD(P)} \xrightarrow[\text{aldehyde dehydrogenase}]{} \text{NAD(P)H} + \text{Acetate} + H^+$$

(Equation 2.2)

The reaction driven by aldehyde dehydrogenase (1) produces NAD(P)H, which when greatly exhibit causing a redox imbalance, then leading to slow activity of the TCA cycle resulting in respiratory inhibition (Gu *et al.*, 1997). In addition, the presence of ethanol (i) increases the formation of reactive oxygen species, (ii) induces specific enzyme involving in oxidative metabolism and acetyl-CoA synthesis such as P-450 systems, oxidases, or glyoxalate cycle enzymes, and (iii) increases a key enzyme, the HMG-CoA reductase activity, in isoprenoid biosynthesis (Gu *et al.*, 1997). The introduction of ethanol to the culture medium has been demonstrated to stimulate microbial carotenogenesis (Gu *et al*, 1997; Yamane *et al.*, 1997a). The supplementation of ethanol (2%, v/v) was reported to stimulate β-carotene and torulene formation in *R. glutinis* (Margalith and Meydav, 1968). Stabnikova *et al.* (1979) described the use of ethanol (3.6%) as the sole source of carbon and energy in a culture medium supporting growth and β-carotene formation by *R. glutinis*. Gu *et al.* (1997) reported that carotenoids production was increased from 1680 to 2240 $\mu g/g_{yeast}$ when 0.2% (v/v) ethanol was added to cultures of the yeast *X. dendrorhous* P-5-6 mutant strain.

2.5.7.3 Pyruvate

Pyruvic acid (pyruvate) is an α-keto acid that plays a central role in various biochemical pathways. It is the end product of the metabolism of glucose known as glycolysis (Figure 2.3). One molecule of glucose breaks down into two molecules of pyruvate, which are then used to provide further energy. Subsequently, it is converted into acetyl-CoA, which is the main input for the Kreb's or Tricarboxylic acid cycle. Carinhas *et al.* (2010) found that supplementation of pyruvate into the cultivation medium of Sf-9 cells resulted in enhancement of carbon flux through the TCA cycle yielding higher efficiency of acetyl-CoA generation. Pyruvate is a key intersection in the network of

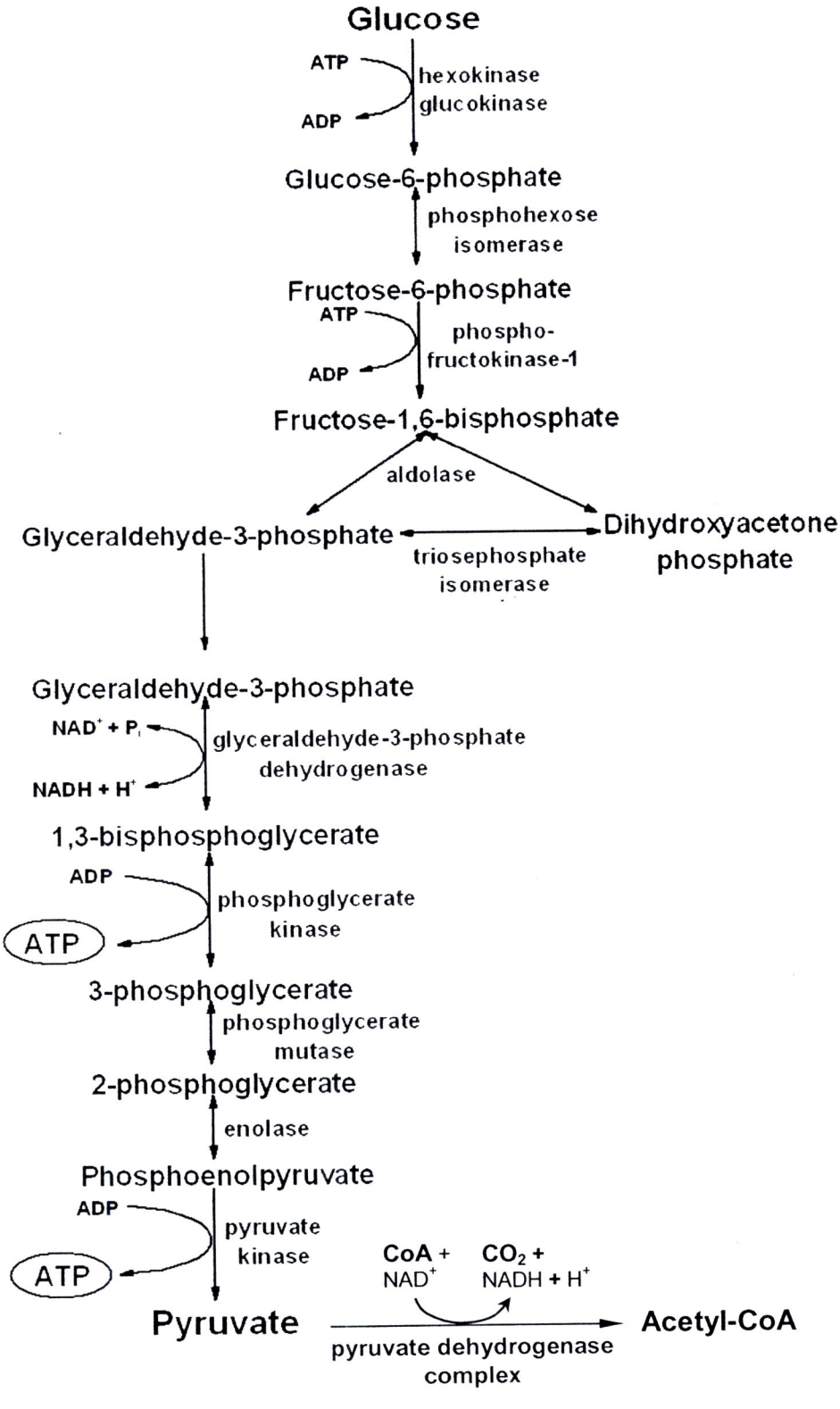


Figure 2.3 Glycolytic pathway and pyruvate decarboxylation (adapt from King, 1996)

metabolic pathways. It can be converted into carbohydrates via gluconeogenesis, to fatty acids or energy through acetyl-CoA, to the amino acid, alanine, and to ethanol. It unites several key metabolic processes; therefore, it may enhance carotenoid production. For example, in *X. dendrorhous* cultivation, Yamane *et al.* (1997a) reported that utilization of pyruvate at 8 g/L as sole carbon source for cultivation of *P. rhodozyma* led to growth and astaxanthin content of 2.8 g/L and 250 $\mu g/g_{yeast}$, respectively, which was, however, lower than that of obtained with glucose at the same concentration which led to biomass and astaxanthin content of 5.7 g/L and 300 $\mu g/g_{yeast}$, respectively.

2.5.7.4 Surfactant

Surfactants are known to increase cell wall permeability, and hence improve substrate uptake and product formation when supplemented into the culture growth medium (Laouar *et al.,* 1996). It was suggested by several studies that surfactants could alter the cell membrane permeability due to their ability to combine with lipid and/or proteins in the membranes (Xu *et al.*, 2007; Choudhari *et al.*, 2008; Kim *et al.*, 1997; Saenge *et al.*, 2011; Laouar *et al.*, 1996). The possible mechanisms of surfactant on biological membranes are proposed to follow a three-stage process of membrane-detergent interaction which are (i) incorporation into the lipid bilayer, (ii) bilayer disruption and (iii) separation of lipid from protein components leading to the formation of transmembrane pores, which would, in turn, facilitate substrate uptake into cell (Laouar *et al.*, 1996). Moreover, surfactant could emulsify the hydrocarbon-based compounds, then breaking down into more manageable molecules, such that the microbes could digest more efficiently; thus, enhancing growth (Bodour *et al.*, 2003). Several studies demonstrated that surfactant favorably influenced cell growth, β-carotene, tricosporic acid, and lycopene production (Kim *et al.*, 1997; Xu *et al.*, 2007; Choudhari *et al.*, 2008). Kim *et al.* (1997) examined effects of nonionic surfactants, i.e., Span 20 and 85, Tween 20 and 85, and Triton X-100, on growth and β-carotene production in *Blakeslea trispora* and found that, with Span 20 supplementation, a high yield of β-carotene (2.16 g/L) of approximately 14 times in comparison with that of the control could be achieved. The authors anticipated that Span 20 could reduce aggregation of *B. trispora*, simultaneously, increase the cell wall permeability, and hence, improve the substrate uptake leading to better β-carotene production. In addition, surfactants such as Tween80, Tween20 and gum arabic at 1% concentration have also

been utilized to increase lipids and carotenoids production in *Rhodotorula glutinis* (Saenge *et al.*, 2011).

2.5.7.5 Metal Ion

Metal ions are common cofactors, which is bound to an enzyme and required for the protein's biological activity. Metal ions are considered helper molecules that assist in biochemical transformations. Several reports found that carotenogenesis of carotenogenic microorganisms was induced in the presence of trace elements. For example, Wang *et al.* (1999) reported that supplementation of heavy metal ions such as lanthanum (La^{3+}), cerium (Ce^{3+}), and neodymium (Nd^{3+}) to the culture medium could enhance almost 40% carotenoids synthesis of the yeast *X. dendrorhous*. Moreover, Flores-Cotera and Sánchez (2001) reported that supplementation of Cu^{2+} at the concentration of lower than 3.2 μM could increase astaxanthin production from 220 to 287 $\mu g/g_{yeast}$; however, with a slight decrease in growth from 11.3 to 10.2 g/L while the presence of Fe^{2+} below 1 μM reduced both the growth and astaxanthin accumulation. There are few reports available on the enhancement of microbial carotenoid production by metal ions and salts in yeasts (Atamanyuk and Razumorskii 1974; Daushvili and Elisashvili 1990). The pink yeast *Rhodotorula rubra* was reported to tolerate metal ions, i.e., copper, cobalt, calcium, and barium (Gammal and Rizk 1989). Similarly, several divalent cations have been demonstrated to act as stimulants for growth of *R. glutinis* (Komemushi *et al.* 1994). Mahattanatavee and Kulprecha (1991) reported that a *Rhodotorula* strain showed marked improvement in carotenoid yield upon supplementation of copper, zinc, and ferrous ions to the growth medium. In a recent study, calcium, zinc, and ferrous salts were shown to have a stimulatory effect on volumetric production (mg/L) as well as cellular accumulation (mg/g) of carotenoids from the yeast *R. glutinis* (Bhosale and Gadre 2001). It was postulated that the positive response was due either to a stimulatory effect of cations on carotenoid-synthesizing enzymes such as Phytoene synthase (Goodwin, 1980), or to the generation of active oxygen radicals in the culture broth.

In yeast and bacteria, Zn^{2+} acts as cofactor of alcohol dehydrogenase enzyme, which plays an important role in break down alcohol molecule to acetaldehyde. Acetaldehyde, then, catabolized into, (1) acetate by aldehyde oxidase and (2) pyruvate by pyruvate decarboxylase, which are substrate capable of triggering carotenoid production.

2.6 Pineapple Juice Concentrate

Pineapples (*Ananas comosus* [L.] Merr.) is the fruit native to Central and South America. It grew in several tropical countries such as Hawaii, India, Malaysia, Philippines, and Thailand. Pineapple varieties are plentiful, but only a few leading types are sold commercially. The large, firm 'Smooth Cayenne' variety, grown in Thailand and Philippines as well as in the Hawaiian Islands, is perhaps the most commonly available.

Pineapple is one of the most produced agricultural product of Thailand (Department of agricultural extension, 1998) of which approximately 2.47 million tons was produced in 2008 (Bureau of Merchandise Trade Administration, 2008). The production of pineapple of Thailand tends to increase every year (in year 2006, 2007, and 2008 which produced 2.60, 2.30, and 2.47 ton million, respectively) (Table 2.3). Approximately 70-80% of pineapple produced was supplied to factories for processing into several products such as canned pineapple, frozen pineapple, pineapple juice concentrate and other products; however, approximately 20-30% are consumed as fresh fruits (Department of Foreign Trade, 2008).

Most pineapples are consumed as either fresh or processed fruit (mainly canned) but only very high-quality fruit (70-80%) is selected for processing and shipment. Whole pineapple fruit can be divided into three parts (Figure 2.4) of which only 52.7% and 19.4% are usable, while 28% are considered waste (Pukrushpan, 1985). Moreover, for second grade pineapple (20-30%) approximately 72% can be used for pineapple juice concentrate production. The pulp from the peel and the pineapple core are the starting materials for the juice production.

Table 2.3 Pineapple production in year 2004-2008

Content/year	2004	2005	2006	2007	2008
1. Harvest area (Rai)	556,275	613,800	629,199	597,467	595,396
2. Productivity (Million ton)	2.10	2.18	2.60	2.30	2.47
3. Productivity per Rai (Kilogram)	3,777	3,557	4,129	3,858	4,159

Source: Bureau of Merchandise Trade Administration (2008)

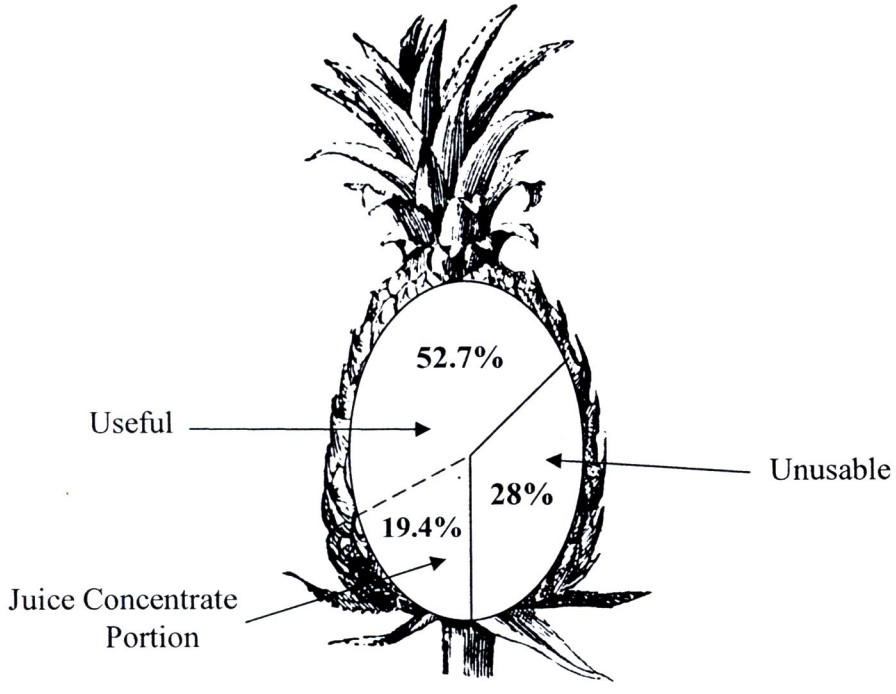


Figure 2.4 Pineapple quantities being used as pineapple juice and residual waste (Adapt from Pukrushpan, 1985)

Pineapple juice concentrate contains several nutrition as shown in Table 2.4 and Table 2.5. Importantly, pineapple juice concentrate consist of high amount of sugar such as sucrose, glucose, and fructose with which microorganism may be utilized as carbon source on growth and maintenance. According to Johnson and Lewis (1979), *X. dendrorhous* could produce invertase enzyme, therefore, capable of utilizing sucrose as sole source. Moreover, citrate and acetic acid are also present in pineapple juice concentrate, which are TCA cycle intermediates responsible for activating astaxanthin production (Flores-Cotera *et al.*, 2001; Meyer and du Preez, 1993). Medium supplemented with copper (Cu) has been shown to enhance astaxanthin content (Flores-Cotera and Sánchez, 2001). Additionally, pineapple juice concentrate provides as well several amino acids (Table 2.5) which actively involves in maintaining microbial growth. Moreover, yeast extract supplemented in traditional medium (YM medium) is costly for up-scale production, thus, pineapple juice concentrate containing high amino acid content may represent a better alternative. Several studies have reported the use of pineapple waste in production of high value products, such as, citric acid (Imandi *et al.*, 2008; Tran *et al.*, 1998), Vinegar (Sossou, 2009), Bioethanol (Tanaka *et al.*, 1999),

including yeast protein (Tuntpatchalern and Vananuvat, 1978). Therefore, it is anticipated that pineapple waste in the form of pineapple juice concentrate may be employed in cultivation of *X. dendrorhous* leading to high astaxanthin content.

Table 2.4 Characteristics of liquid pineapple juice by product

Compositions	Parameters	Abdullah and Mat, 2008	Sasaki, 1991
Sugars (g/L)	Reducing sugar	40.40	39.20
	Sucrose	16.75	40.10
	Glucose	19.72	23.60
	Fructose	20.62	14.00
	Total Sugar	73.76	100.00
Proteins (g/L)	Soluble protein	1.13	0.90
	Kjeldahl nitrogen	0.64	0.20
Organic acids (g/L)	Acidity, as citric acid	2.95	-
	Citric acid	2.18	-
	Malic acid	0.29	-
Cations (mg/L)	Fe	3.30	5.43
	Ca	194.0	3.31
	Mn	3.60	13.97
	Mg	47.70	62.50
	Zn	5.80	-
	Cu	1.40	2.02
	Cd	0.00	0.00
	Na	294.0	8.61
	K	526.0	-
Anions (mg/L)	SO_4^{2-}	25.60	169.7
	PO_4^{3-}	0.00	223.8
	NO_3^{2-}	8.20	-
	Cl^-	256.0	-
	Phosphorus	27.40	-
pH		4.30	4.00

Source: Abdullah and Mat (2008)

Table 2.5 Amino acid profile of pineapple juice concentrate

Amino acid	Amino profile (mg/100 g)
Alanine	52 ±16.7
Arginine	18 ± 5.4
Asparagine	551 ± 183
Aspartic acid	62 ± 16.7
β-Alanine	2.4 ± 0.7
Ethanolamine	15 ± 2.0
GABA	37 ± 9.2
Glutamine	27 ± 18.5
Glutamic acid	28 ± 17.3
Glycine	17 ± 4.2
Histidine	17 ± 7.5
Hydroxyproline	2 ± 0.7
Isoleucine	8 ± 2.1
Leucine	13 ± 3.4
Lysine	18 ± 4.9
Methionine	45 ± 9.1
Phenylalanine	13 ± 2.7
Proline	14 ± 3.7
Serine	92 ± 16.9
Threonine	20 ± 3.93
Tyrosine	20 ± 7.6
Valine	14 ± 3.6
Total amino acids	**1079 ± 284**

Source: Elkins *et al.*, 1997

2.7 Screening Experimental Design

A dramatic increase in the number of possible components (factors) in experiment leads to the need of experimental designs capable of screening a large set of potentially important treatment components quickly, efficiently, and, at the same time, identifying the important ones (Nair *et al.*, 2008). The purpose of screening experiment is to select or screen out the few important main effects from the many less important parameters. Several methods can be used for the screening of the major factors such as Placket-Burman, full factorial design, and fractional factorial design. Firstly, Placket-Burman,

which investigates the dependence of some measured quantities on a number of independent variables (factors) in such a way as to minimize the variance of the estimates of these dependencies using a limited number of experiments (Wikipedia, 2009b). These designs are very useful for detecting large main effects, however, assuming all interactions are negligible when compared with the few important main effects. Secondly, full factorial design is a design in which every setting of every factor appears with every setting of every other factor. This design is very useful for preliminary studies or in the initial steps of an optimization. If there are k factors, each at 2 levels, a full factorial design has 2^k runs. This screening method requires a large number of runs, then not very efficient when many factors are involved (Box *et al.*, 1978). Thirdly, fractional factorial design uses only a fraction of the runs specified by the full factorial design, with which requires the less number of experimental runs, however, provides comparable results with that of full factorial design.

2.7.1 Fractional Factorial Design

Fractional factorial design is an experimental design that consists of a subset run required by full factorial design. It helps reducing the experimental runs called redundancy, of which some higher order interactions tend to become negligible and can properly be disregarded. In addition, when a moderately large number of variables are introduced into a design, it often happens that some have no distinguishable effects. The system is likely to be driven primarily by main effects and low-order interactions in accordance with the so-called "sparsity of effects".

A major use of fractional factorial design is in screening experiment in which many factors are considered and the objective is to identify factors with large effect. The factors identified as important are then investigated more carefully in subsequent experiments (Montgomery, 2005).

Fractional factorial design containing 2^{k-p} runs where k is the number of factors and p is the number of generators with which assignments as effects or interactions are confounded. For example, 2^{k-p} fractional factorial design with 3 variables (2^{3-1}). A full 2^2 design was written for the 2 variables A, and B and interaction C was written, and these were used to define the levels of variable C, thus $C = AB$. The AB is called the generator of C. Sometimes, generator is referred to as a word, which is designated by the letter I, that is $I = ABC$ called the defining relation. Defining relation is the key to

the confounding pattern. For example, A and BC are alias, B and AC are alias, and C and AB are alias. For instance, factor A is confounded with the 2-way interaction BC indicating that an estimate effect for A includes any effect due to BC, and confounding of B with AC indicates that an estimate effect for B includes any effect due to AC. The alias structure may be easily determined by using the defining relation $I=ABC$.

Fractional factorial design helps decreasing the number of experimental runs of full factorial design depending on type of the design, which can be performed particularly with large numbers of variables (Table 2.6). This design may be used as building blocks so that the degree of complexity of the finally constructed design matches the sophistication of the problem.

The most important features of fractional factorial designs is the resolution, namely, III, IV, and V, providing that resolution means ability to separate main effects and low-order interactions from one another. Resolutions below III are not useful because in this design some of the main effects are aliased with each other. However, higher resolution above V are wasteful in that they can estimate very high-order interactions which rarely occur in practice. Characteristic of the resolution is described in Table 2.7.

Iacazio (2005) has reported that medium improvement for quercetinase production by *Penicillium olsonii* could successfully be achieved by a fractional factorial design. Gardeur *et al.* (2007) adopted fractional factorial design to investigate effect of multiple factors on both growth and flesh quality of new aquatic animal perch (*Perca fluviatilis*). The authors found that quality of the aquatic production could be improved efficiently. Chen *et al.* (2009) studied biodegradation of wastewater by microorganisms through fractional factorial design. Result indicated that the fractional factorial design is a useful screening tool for optimizing the microbial community to enhance treatment efficiency.

Table 2.6 Number of experimental runs of fractional factorial design with 9 and 10 factors at different resolutions

Factors	Resolution	Generator	Formula	Number of runs
9	Full	-	2^9	512
	III	5	2^{9-5}_{III}	16
	IV	4	2^{9-4}_{IV}	32
		3	2^{9-3}_{IV}	64
	VI	2	2^{9-2}_{VI}	128
10	Full	-	2^{10}	1024
	III	6	2^{10-6}_{III}	16
	IV	5	2^{10-5}_{IV}	32
		4	2^{10-4}_{IV}	64
	V	3	2^{10-3}_{V}	128

Table 2.7 Characteristics of design resolutions

Resolution	Ability
II	Not useful: main effects are confounded with other main effects
III	No main effects are aliased with any other main effects, but main effects are aliased with two factor interactions. In addition, some two-factor interactions are aliased with each other.
IV	No main effects are aliased with any other main effects or two factor interactions. However, some main effects are aliased with three factor interactions and the two factor interactions are aliased with each other.
V	No main effects or two factor interactions are aliased with any other main effects or two factor interactions. However, some main effects are aliased with four factor interactions and the two factor interactions are aliased with three factor interactions.
VI	The estimated main effects are unconfounded by four-factor (or less) interactions providing further that the two factor interaction effects are also not confounded by three factors (or less) interactions. In addition, the estimated three factor interaction effects may be confounded with other three-factor interaction.

2.8 Optimization Experimental Design

An experimental design technique becomes increasingly widespread in several field of science. Several experimental design models are available which help reducing the number of unnecessary experiments, thus, more economical experimentation. The first step is for detecting appropriate variables affecting desired response while the second step is for optimizing a process with which second-order models should be used. Second-order model is typically employed with more than two factor levels to allow fitting of a full quadratic polynomial.

A second-order polynomial equation was used to express the response as a function of independent variables,

$$R = \beta_0 + \beta_1 X + \beta_2 Y + \beta_{12} XY + \beta_{11} X^2 + \beta_{22} Y^2$$

(Equation 2.3)

where X and Y represents the variables used in the design and β_0, β_1,..., β_{12} represents coefficient estimates with β_0 taking a role of a scaling constant.

Optimization could be used to identify appropriate level of variables identified by screening design that leads to the highest response of interest. This step is carrying out with two or three variables. Several methods can be employed for optimizing of the selected factors. For example, central composite design (CCD) which is a better alternative to the full factorial three-level design because its performance is comparable at a lower cost (Ferreira *et al.,* 2004). Moreover, Box-Behnken design allows the number of design points to increase at the same rate as the number of polynomial coefficients. However, an alternative and very useful experimental design for second-order model is the uniform shell design called Doehlert designs. Doehlert designs are easily applied to optimize variables (Massart *et al.,* 2003) and present advantages over CCD and Box-Behnken designs because fewer experiments are necessary, which are more efficient (Table 2.8) and can move through the experimental domain.

Table 2.8 Comparison of efficiency of central composite design (CCD), Box-Behnken design (BBD) and Doehlert design (DD)

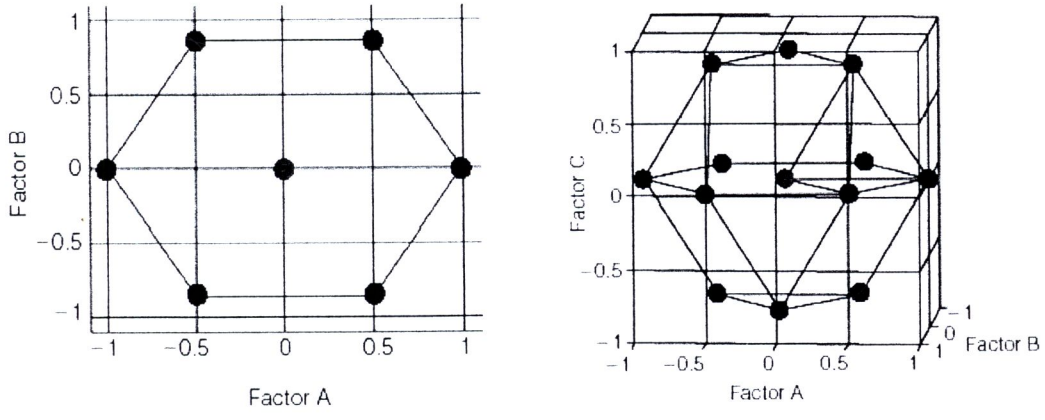
Variables (k)	Number of coefficients (*p*)	Number of experiments (*f*)			Efficiency (*p/f*)		
		CCD	DD	BBD	CCD	DD	BBD
2	6	9	7	-	0.67	0.86	-
3	10	15	13	13	0.67	0.77	0.77
4	15	25	21	25	0.60	0.71	0.60
5	21	43	31	41	0.49	0.68	0.61
6	28	77	43	61	0.36	0.65	0.46
7	36	143	57	85	0.25	0.63	0.42
8	45	273	73	113	0.16	0.62	0.40

2.8.1 Doehlert Design

Doehlert design, an alternative and very useful experimental design for second-order models, is one type of the uniform shell design proposed by Doehlert in 1970 (Doehlert, 1970). Doehlert designs are easily applied to optimize variables and offer advantages in relation to central composite and Box-Behnken designs. The Doehlert design describes a spherical experimental domain and it stresses uniformity in space filling. It does not significantly diverge from the required quality for effective use, although this matrix is neither orthogonal nor rotatable (Massart *et al.*, 2003). For two variables, the Doehlert design consists of one central point and six points forming a regular hexagon, and therefore situated on a circle (Figure 2.5A). In three dimensions, it can be viewed in different ways, depending on the geometric structure selected (Figure 2.6) (García-Campaña *et al.*, 1997).

In Doehlert designs, the number of levels is not the same for all variables. For example, for a two-variable Doehlert design, the most important variable is studied at five levels while the other is studied at only three levels. This property allows a free choice of the factors to be assigned to a large or a small number of levels. Different criteria can be used to assign the factors. Generally, it is preferable to choose the variable with the stronger effect as the factor with five levels in order to obtain most information of the system.

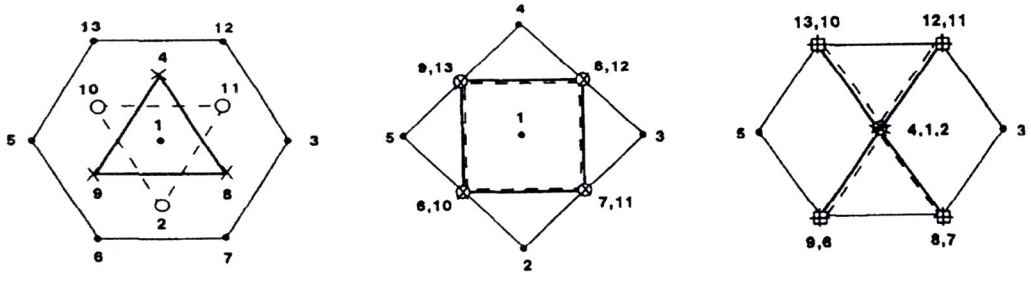
In Doehlert design, the number of experiments required (N) is given by $N = k^2 + k + C_0$, where k is the number of variables and C_0 is the number of center points. Replicates, at least 3-5, at the central level of the variables are supplemented in order to validate the model by means of an estimate of experimental variance (Ferreira *et al.*, 2004).



(A) (B)

Figure 2.5 Doehlert design for two (A) and three (B) variables

Table 2.8 shows the comparison of efficiency among the second-order designs. As can be clearly observed that Doehlert matrices and Box-Behnken designs are more efficient than Central composite designs given that the efficiency of one experimental design is defined as the number of coefficients of the model estimated divided by the number of experiments need be conducted.



(a) (b) (c)

Figure 2.6 Some plane projections of the three-variables Doehlert design leaning: (a) on a square face; (b) on a triangular face; (c) on a vertex. (García-Campaña *et al.*, 1997)

The Lagrange's criteria are applied to characterize the response function obtained through optimization using quadratic model. They are mathematical procedures to determine the nature of the stationary point of a function.

For two variable Doehlert design, the responses could be represented by the function of the form:

$$R(X,Y) = \beta_0 + \beta_1 X + \beta_2 Y + \beta_{12} XY + \beta_{11} X^2 + \beta_{22} Y^2$$

(Equation 2.4)

where R is the experimental response to be optimized, β_0 is the constant term, β_1 and β_2 are coefficients of the linear terms, β_{12} is the coefficient of interaction between the two factors, while β_{11} and β_{22} are coefficients of the quadratic terms.

Lagrange's criteria are based on the calculation of the Hessian determinant (H) of R, given by

$$\Delta_1 = \frac{\partial^2 R}{\partial X^2}$$

$$H = \Delta_2 = \begin{vmatrix} \frac{\partial^2 R}{\partial X^2} & \frac{\partial^2 R}{\partial X \partial Y} \\ \frac{\partial^2 R}{\partial Y \partial X} & \frac{\partial^2 R}{\partial Y^2} \end{vmatrix}$$

The critical point, $(X,Y) = (X_c, Y_c)$ shows three situations as follows:

(1) Relative maximum if : $\Delta_2 > 0$ but $\Delta_1 < 0$

(2) Relative minimum if: $\Delta_2 > 0$ and $\Delta_1 > 0$

(3) A saddle point if: $\Delta_2 < 0$

For three experimental variables, it is possible to estimate the regression coefficients of the design by the function:

$$R(X,Y,Z) = \beta_0 + \beta_1 X + \beta_2 Y + \beta_3 Z + \beta_{12} XY + \beta_{13} XZ + \beta_{23} YZ + \beta_{11} X^2 + \beta_{22} Y^2 + \beta_{33} Z^2$$

(Equation 2.5)

where R is the experimental response; X, Y, and Z are the variables to be optimized; β_0 is the constant term; β_1-β_3 are coefficients of the linear terms; β_{12},..., β_{33} represent coefficients of the quadratic terms.

If the quadratic function only shows one critical point (Xc, Yc, Zc) four situations are possible:

1. There is not any information: $\Delta_2 = 0$.
2. Relative maximum: $\Delta_1 < 0$; $\Delta_2 > 0$; $\Delta_3 < 0$.
3. Relative minimum: $\Delta_1 > 0$; $\Delta_2 > 0$; $\Delta_3 > 0$.
4. Saddle point: none of the above situations applies.

where Δ_3 is the Hessian determinant of the function H(X,Y,Z), Δ_2 and Δ_1 are calculated using the following equations:

$$\Delta_1 = \frac{\partial^2 R}{\partial X^2}$$

$$\Delta_2 = \begin{vmatrix} \frac{\partial^2 R}{\partial X^2} & \frac{\partial^2 R}{\partial X \partial Y} \\ \frac{\partial^2 R}{\partial Y \partial X} & \frac{\partial^2 R}{\partial Y^2} \end{vmatrix}$$

$$\Delta_3 = \begin{vmatrix} \frac{\partial^2 R}{\partial X^2} & \frac{\partial^2 R}{\partial X \partial Y} & \frac{\partial^2 R}{\partial X \partial Z} \\ \frac{\partial^2 R}{\partial Y \partial X} & \frac{\partial^2 R}{\partial Y^2} & \frac{\partial^2 R}{\partial Y \partial Z} \\ \frac{\partial^2 R}{\partial Z \partial X} & \frac{\partial^2 R}{\partial Z \partial Y} & \frac{\partial^2 R}{\partial Z^2} \end{vmatrix}$$

Doehlert experimental design is useful in several application including chemistry and general science. Li *et al.* (2007) studied xylanase production by *Alternaria mali* ND-16 using statistical experimental designs. The major components were optimized using the Doehlert experimental design. The authors found that the optimum conditions for xylanase production were: 11.34 g/L NH_4Cl , 1.26 g/L urea, and 0.98 g/L $MgSO_4$. Under optimal conditions, the xylanase activity from *A. mali* ND-16 reached 30.35 U/mL. Verification of the optimization showed that xylanase production of 31.26 U/mL was achieved through Doehlert design. The authors concluded that Doehlert design is useful technique on optimization capable of improving xylanase production from *Alternaria mali* ND-16. Moreover, Zhou *et al.* (2007) optimized a method for determination of nonextractable deoxynivalenol (DON) in barley grain using trifluoroacetic acid (TFA) and a gas chromatography electron capture detector (GC-ECD). The factors included were TFA concentration, time, and temperature. By using the Doehlert design, optimal conditions were identified as 1.25 N TFA for 54 min at 133 °C, which resulted in the release of an additional 58% DON. Moreover, an increase of 9-88% could be attained during validation.

Examples of Doehlert design for two and three factors are provided in Table 2.9 and Table 2.10, respectively.

Table 2.9 Doehlert design for two factors

Experiment	Factors	
	A	B
1	1	0
2	-1	0
3	0.5	0.866
4	-0.5	0.866
5	0.5	-0.866
6	-0.5	-0.866
7	0	0

Table 2.10 Doehlert design for three factors

Experiment	Factors		
	A	B	C
1	1	0	0
2	0.5	0.866	0
3	0.5	0.289	0.816
4	-1	0	0
5	-0.5	-0.866	0
6	-0.5	-0.289	-0.816
7	0.5	-0.866	0
8	0.5	-0.289	-0.816
9	0	0.577	-0.816
10	-0.5	0.866	0
11	-0.5	0.289	0.816
12	0	-0.577	0.816
13	0	0	0

2.9 Path of Steepest Ascent

If the parameters significantly influencing response of the process of interest have been identified, a response surface design is appropriate. However, if the curvature obtained in the factorial design was insignificant, it indicated that the optimal condition was out of the chosen range. Therefore, another statistical method called steepest ascent may be applied to rapidly approach the optimal condition. First order model from fractional factorial design are generally employed to construct the path of steepest ascent. The path begins at the centre of previous factorial design and stretches well outside the design space. A sequence of equally spaced locations along the path is then selected which form a set of experiments.

The path of steepest ascent has been successfully applied to achieve appropriate range for optimization. Ni *et al.* (2008) investigated the process optimization for astaxanthin extraction using acid. The authors found that screening design yielded an insignificant curvature indicating that optimal range was not included in the design. As a result, a path of steepest ascent was adopted leading to 5.6 mol/L and 18 mL/g of acetic acid and

ethanol, respectively, identified as optimum condition providing that the highest extraction yield of astaxanthin of 1310.8 $\mu g/g_{yeast}$ was achieved. Subsequent optimization of astaxanthin extraction was carried out using CCD. The highest extraction yield of astaxanthin of 1303.7 $\mu g/g_{yeast}$ was resulted at the center point of CCD design obtained from the path of steepest ascent conducted previously, 5.6 mol/L and 18 mL/g of acetic acid and ethanol, respectively.