CHAPTER II
LITERATURE REVIEWS

2.1 Ethanol or ethyl alcohol

Ethanol or ethyl alcohol is a colourless flammable liquid, low in toxicity and agreeable odor. The molecular formula of ethanol is C_2H_5OH. Ethanol belongs to the alcohol family, a group of organic chemical compounds that contain a hydroxyl group, OH, bonded to a carbon atom (Shakhashiri, 2009). The physical and chemical properties are shown in Table 2.1.

Table 2.1 The physical and chemical properties of ethanol

Property	Value
Molecular weight (g/mol)	46
Boiling point	78.5°C
Freezing point	-114.1°C
Btu/lb.	11,500-12,800
Btu/Gal.	90-100,000
Lb/Gal.	6.85
Heat of vaporization (Btu/lb)	410
Specific gravity (at 20°C, water = 1.0)	0.7905
Octane	99

(Johanesen, 1991; Balat, 2011)

2.2 Ethanol production

Ethanol can be produced commercially by chemical synthesis or biochemical synthesis. Chemical synthesis is produced by hydration of ethylene (C_2H_4). For the biosynthesis, ethanol has been produced by anaerobic fermentation of sugar by appropriate microorganisms. As with many microorganisms, *Saccharomyces*

cerevisiae metabolizes glucose by the Embden–Meyerhof-Parnas (EMP) pathway through which one molecule of glucose is metabolized and two molecules of pyruvate are produced (Madigan et al., 2000 cited in Bai et al., 2008). Beside this, the Entner–Doudoroff (ED) pathway is an additional means of glucose consumption in many bacteria, such as *Zymomonas mobilis*, is an anaerobic, Gram-negative bacterium, which produces ethanol from glucose via the ED pathway in conjunction with the enzymes pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH).

Under anaerobic conditions, the pyruvate is further reduced to ethanol with the release of CO_2 in the EMP pathway (Figure 2.1). Theoretically, the yield is 0.511 for ethanol and 0.489 for CO_2 on a mass basis of glucose metabolized. The ATPs produced in the glycolysis are used to drive the biosynthesis of yeast cells, which involves a variety of energy-requiring bioreactions. Therefore, ethanol production is tightly coupled with yeast cell growth. There are many by-products generated during ethanol fermentation. Glycerol, which is the main by-product, is produced at a level of about 1.0% (w/v) from ethanol fermentations. Other by-products such as organic acids and higher alcohols are produced at much lower levels. The production of these by-products as well as the growth of yeast cells inevitably direct some glycolytic intermediates to the corresponding metabolic pathways, decreasing the ethanol yield to some extent. In the industry, the ethanol yield that is calculated based on the total sugar feeding into the fermentation system without deduction of the residual sugar can be as high as 90–93% of its theoretical value of ethanol to glucose (Ingledew, 1999).

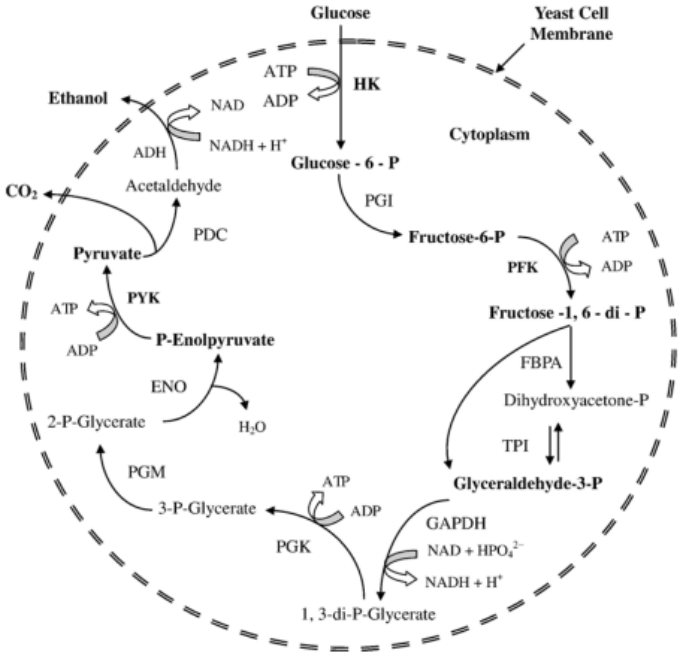

Figure 2.1 EMP pathway: Metabolic pathway of ethanol fermentation in *S. cerevisiae*. Abbreviations: HK: hexokinase, PGI: phosphoglucoisomerase, PFK: phosphofructokinase, FBPA: fructose bisphosphatealdolase, TPI: triose phosphate isomerase, GAPDH: glyceraldehydes-3-phos phate dehydrogenase, PGK: phosphoglycerate kinase, PGM: phosphoglyceromutase, ENO: enolase, PYK: pyruvate kinase, PDC: pyruvate decarboxylase, ADH: alcohol dehydrogenase (Madigan et al., 2000)

The ED pathway (Figure 2.2) forms glyceraldehyde-3-phosphate and pyruvate by the cleavage of 2-keto-3-deoxy-6-phosphogluconate by 2-keto-3-deoxy-gluconate aldolase, yielding only one molecule ATP per glucose molecule. It was reported that the ethanol yield of *Z. mobilis* could be as high as 97% of the theoretical yield of ethanol to glucose (Sprenger, 1996), while only 90–93% can be achieved for *S. cerevisiae*. Also, as a consequence of the low ATP yield, *Z. mobilis* maintains a higher glucose metabolic flux, and correspondingly, guarantees its higher ethanol productivity, normally 3–5 folds higher than that of *S. cerevisiae* (Sprenger, 1996).

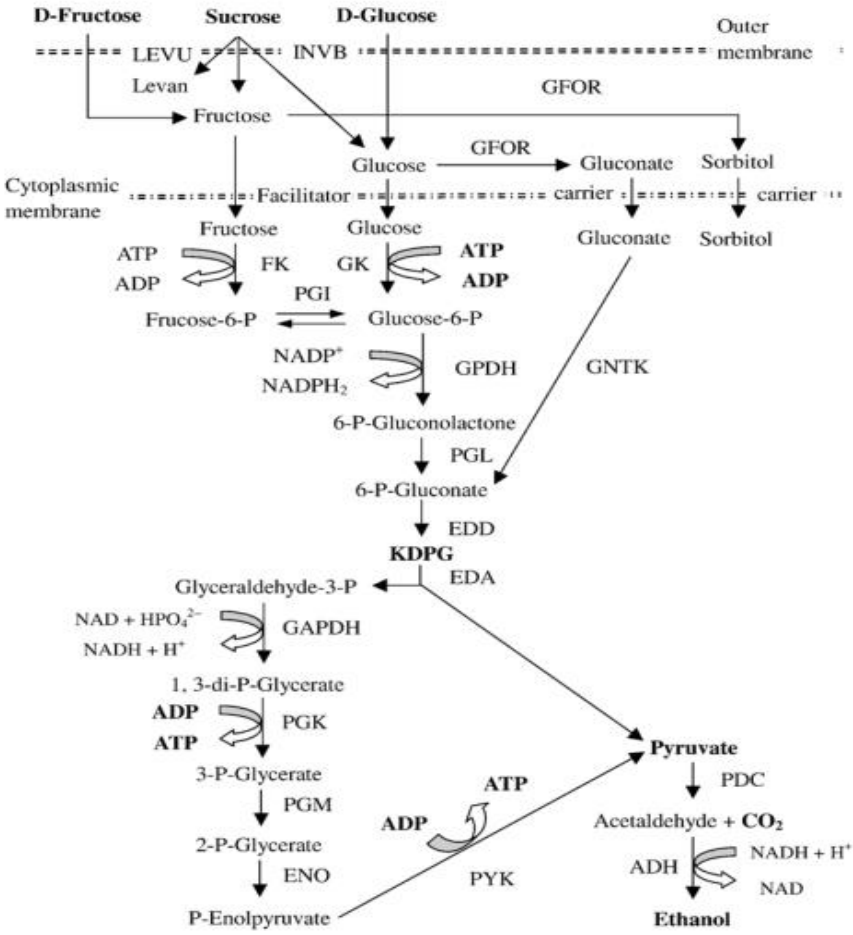

Figure 2.2 ED pathway: Carbohydrate metabolic pathways in *Z. mobilis*. Abbreviations: LEVU: levansucrase, INVB: invertase, GFOR: glucose-fructose oxidoreductase, FK: fructokinase, GK: glucokinase, GPDH: glucose-6-phosphate dehydrogenase, PGL: phosphogluconolactonase, EDD: 6-phosphogluconate dehydratase, KDPG: 2-keto-3-deoxy-6-phosphogluconate, EDA: 2-keto-3-deoxy-gluconate aldolase, GNTK: gluconate kinase. See Figure 2.1 for PGI, GAPDH, PGK, PGM, ENO, PYK, PDC and ADH (Sprenger, 1996)

2.3 Raw materials for bioethanol production

In general, bioethanol can be produced from every sort of carbohydrate materials. These can be divided into three main groups: sugar, starhces, and cellulose materials. Conversion of starch as well as cellulose materials into glucose is usually achieved by thermal process aided by enzymes. Juices from sugar crops contained simple fermentable sugars which can be used directly for ethanol fermentation (Patrascu et al., 2009).

2.3.1 Sugars based

Sugars (from sugarcane, sugar beets, molasses, and fruits) can be directly converted into ethanol without pretreatment. The overall process of fermentation in order to convert glucose to ethanol and carbon dioxide is as follows:

(Sucrose) (Glucose + Fructose) (Ethanol)

$$C_{12}H_{22}O_{11} + H_2O \dashrightarrow 2C_6H_{12}O_6 + \text{Yeast} \dashrightarrow 4C_2H_5OH + 4CO_2$$

2.3.2 Starches based

Most agricultural biomass containing starch can be used as a potential substrate for the ethanol fermentation by microbial processes. Starches (from corn, cassava, potatoes, and root crops) must first be hydrolyzed to fermentable sugars by the action of enzymes or acid, and then the resulting sugars are converted to ethanol and carbon dioxide.

(Starch) (Glucose) (Ethanol)

$$(C_6H_{10}O_5)_N + H_2O \dashrightarrow nC_6H_{12}O_6 + \text{Yeast} \dashrightarrow 2C_2H_5OH + 2nCO_2$$

2.3.3 Cellulose materials based

Cellulose (such as wood, agricultural residues, waste sulfite liquor from pulp, and paper mills) must likewise be converted into sugars, generally by the common methodology which includes an initial pretreatment step to enhance accessibility of cell wall to polysaccharides by hydrolyzing enzymes, followed by enzymatic hydrolysis of polysaccharides into component sugars. Once simple sugars are formed, enzymes from microorganisms can readily ferment them to ethanol.

Fermentative production of ethanol from renewable biomass is an attractive source of energy because plant biomass is the only sustainable source of organic fuels, chemicals, and materials available to humanity.

2.4 Jerusalem artichoke (*Helianthus tuberosus* L.)

Jerusalem artichoke (*Helianthus tuberosus* L.), also called the sunroot or sunchoke, is one of the most interesting materials among those agricultural products. It is a flowering plant native to North America, which has many desirable growing traits such as cold and drought tolerance, wind and sand resistance, saline tolerance, strong fecundity, and high pest and disease resistant. The plant is an annual herb with stems height of 1-3 m height, with vegetation similar to sunflower but perennating by stem tubers. The upper stems are multi branched and slender. Leaves are opposite, ovate and often coarsely toothed, prominently veined with broad winged stalks. The flower heads when produced are much smaller than sunflower, being only 6-8 cm in diameter with yellow disc and ray florets, and are carried individually on branch stems. The whitish/yellow tubers, formed late in the season are numerous, up to 12×6 cm long, very irregular and knobby in unselected forms but almost smooth in others, and with crisp flesh (Khampa et al., 2008). This plant contains nearly 20% of carbohydrates, 70–90% of which is inulin. Inulin consists of linear chains of β (2→1) linked D-fructose units. Each chain is terminated by a D-glucose residue linked to fructose by an α (1→2) bond (Ge and Zhang, 2005), which has potential for ethanol production when fermented by suitable microorganisms. The yield potential for ethanol production is 65 kg per ton of Jerusalem artichoke tuber. A conservative crop yield estimate is 5.4 ton/ha (Li and Chan-Halbrendt, 2009).

2.4.1 Ethanol production from Jerusalem artichoke

Nakamura et al. (1996) reported the simultaneous saccharification and fermentation (SSF) of Jerusalem artichoke tubers under batch fermentation at 30°C using *Aspergillus niger* 817 and *S. cerevisiae* 1200. The results from SSF showed that the maximum ethanol concentrations obtained were 10.4% 15.0% and 20.1% (v/v) from the ground tubers after 15 h, the juice concentrate after 72 h, and the flour after 120 h incubation, respectively.

Szambelan and Chrapkowska (2003) reported the ethanol production by *Z. mobilis*, *S. cerevisiae* and *Kluyveromyces* from Jerusalem artichoke tubers. The authors found that fermentation of Jerusalem artichoke tubers by *Z. mobilis* gave 86.11% and 90.02% theoretical yield of ethanol after acid and enzymatic hydrolysis of inulin, respectively. Fermentation of mash by distillery yeast *S. cerevisiae* or *Kluyveromyces* gave lower ethanol yield (80-84.15% of theoretical yield) than that of Jerusalem artichoke tubers.

Szambelan et al. (2004) reported the ethanol production from Jerusalem artichoke tubers by mixed culture of *Z. mobilis* or *S. cerevisiae* with *K. fragilis.* The ethanol yield from mixed cultures fermentation, expressed as g ethanol per 1 g utilized sugars, was 0.39-0.44 g/g for *S. cerevisiae* and 0.41-0.48 g/g for *Z. mobilis*, while in a single fermentation it was varied from 0.34-0.43 g/g for yeasts and 0.37-0.46 g/g for the bacterium (differences significant statistically at $\alpha = 0.05$).

Onsoy et al. (2007) reported the ethanol production from acid and enzymatic hydrolysate of inulin in Jerusalem artichoke juices in batch fermentation by *Z. mobilis* TISTR548. The results showed that acid hydrolysis of inulin at 80^{o}C for 40 min using concentrated sulfuric acid gave the maximum reducing sugars content as well as ethanol yield (0.42 g/g utilized sugars) and ethanol productivity (0.65 g/l.h) with 83.19% of the theoretical ethanol yield. Under the optimal conditions, i.e., initial pH of 5.0 and 10% inoculum size, the highest ethanol yield (0.47 g/g utilized sugars) and ethanol productivity (1.33 g/l.h) with 92.75% of the theoretical ethanol yield were achieved.

Thuesombat et al. (2007) reported the batch ethanol fermentation of the acid hydrolyzed Jerusalem artichoke juices by *S. cerevisiae*. The maximum ethanol concentration, ethanol yield, and ethanol productivity of 88.1 g/l, 0.45 g/g, and 1.84 g/l/h, respectively, were achieved under the optimal conditions: 250 g/l initial sugar concentration, pH 5.0-5.5 and 10^8 cells/ml initial yeast cell. The conversion efficiency of the fermentation of plant juices was approximately 88% of the theoretical ethanol yield.

Zhang et al. (2010) reported the bioethanol production from hydrolysates of inulin and the tuber meal of Jerusalem artichoke by *Saccharomyces*

sp. W0. The result showed that at the end of the fermentation, over 14.6% (v/v) of ethanol was produced, the ethanol yield was over 0.384 g of ethanol/g of inulin and over 98.8% of total sugar was utilized. When the *Saccharomyces* sp. W0 was grown in the mixture of 4.0% hydrolysate of soybean meal and 20.0% of the hydrolysate of inulin for 120 h, over 14.9% of ethanol was yielded, the ethanol yield was over 0.393 g of ethanol/g of inulin and 98.9% of total sugar was used by the yeast strain. When *Saccharomyces* sp. W0 carrying the same inulinase gene was grown in the medium containing 50% (w/v) of the tuber meal of Jerusalem artichoke for 144 h, over 12.1% (v/v) was yielded, the ethanol yield was 0.319 g of ethanol/g of sugar and 3.7% (w/v) of total sugar and 0.5% (w/v) of reducing sugar were left in the fermented media.

Lim et al. (2010) reported the direct ethanol fermentation from Jerusalem artichoke flour (180 g/l) without any pretreatments by *S. cerevisiae* KCCM50549 in a 5L jar fermentor and showed the maximum ethanol concentration at 36.2 g/l of ethanol within 36 h after fermentation. The conversion efficiency of inulin-type sugars to ethanol was 70%.

Razmovski et al. (2011) investigated the ethanol fermentation of Jerusalem artichoke hydrolysates by *S. cerevisiae* strain DTN. The hydrolysates obtained by acid hydrolysis at 126ºC, hydromodule 1:1, pH 2 and hold time 60 min, resulted in the highest ethanol yield of 7.6% w/w, which corresponds to volumetric productivity of ethanol 1.52 g /l.h and 94.12% (w/w) of theoretical yield of ethanol.

Li et al. (2013) studied the ethanol production from inulin and unsterilized meal of Jerusalem artichoke tubers by *Sacharomyces* sp. W0 expressing the endo-inulinase gene from *Arthrobacter* sp.. The recombinant yeast D5 could produce 13.6% and 10.1% of ethanol from 30% inulin and unsterilized meal of Jerusalem artichoke tubers, respectively.

S. Kim and C.H. Kim (2013) reported the consolidated bioprocessing (CBP) ethanol production of Jerusalem artichoke (*Helianthus tuberosus* L.) by *K. marxianus* CBS 1555. The results showed that the ethanol concentration of 45.3 g/l was obtained after 30 h of fermentation when using 10% (w/v) dried Jerusalem artichoke stalk and 8% (w/v) dried tuber powder as the principal sugar source and a carbon and nutrient source, respectively.

2.5 Thermotolerant yeasts and their ethanol production

Yeasts are ascomycetous or basidiomycetous fungi that reproduce vegetatively by budding or fission, and that form sexual states which are not enclosed in a fruiting body. These eukaryotic organisms have much larger cells than bacteria, which facilitates their separation from the fermentation broth. Yeasts are resistant to virus infection and bacterial contamination, and they are more resistant to ethanol than bacteria (Jeffries and Jin, 2000).

Microorgnisms have generally been divided into three groups: psychophiles, mesophiles and thermophiles depending on their range of temperature for growth. The range of temperature consists of minimum (T_{min}), optimum (T_{opt}) and maximum (T_{max}) temperatures. McCracken and Gong (1982) defined thermotolerant yeasts as those with a T_{min}, T_{opt}, and T_{max} of 20-26°C, 26-35°C, and 37-45°C, respectively, while mesophilic yeasts had a T_{min} of 5-10°C, T_{opt} of 24-30°C, and T_{max} of 35-40°C.

Ethanol production by thermotolerant yeasts has been extensively studied, because thermotolerant yeasts are capable of growth and fermentation during the summer months in non-tropical countries as well as under tropical climates**.** In many warm countries the temperatures frequently reach 35°C and in the typical ethanol fermentation processes carried out at ambient temperatures with no cooling system an increase of up to 11°C can be experienced due to exothermic metabolic reactions (Burrows, 1970). Utilization of thermotolerant microorganisms enables fermentation to be performed at high temperatures, which have several advantages such as reduce cost for cooling system, reduce contamination of mesophilic microorganisms and increase the speed of catalytic reactions related to fermentation.

Many researchers have been reported on screening of thermotolerant yeasts and their potential on ethanol production. For example, Kiran et al. (2000) reported the isolation of thermotolerant yeast *S. cerevisiae* from soil samples collected from a thermal power plant in India. They reported that the maximum ethanol yields obtained from 150 g/l glucose by the isolated yeast VS3 were 75 and 60 g/l at 30 and 40°C, respectively.

Limtong et al. (2007) reported the isolation of the thermotolerant yeast by an enrichment culture technique in a sugar cane juice medium supplemented with 4% (w/v) ethanol at 35°C. One isolate designated as *K. marxianus* DMKU 3-1042 could produce ethanol at both 40 and 45°C. The maximum ethanol concentration of 6.78% (w/v), a productivity of 1.13 g/l/h and a yield of 60.4% of the theoretical yield were obtained at 40°C when sugarcane juice was used as a substrate.

Edgardo et al. (2008) reported the selection of the *S. cerevisiae* strains capable of fermenting sugars obtained from lignocellulosic material at temperatures above 35°C. The yeasts were cultured in liquid medium, and incubated at 35, 40 and 42°C. The results showed that *S. cerevisiae* IR2-9a produced 28 g ethanol/l (62% of the theoretical ethanol yield) and 22 g/l ethanol (73% of the theoretical ethanol yield) from bleached kraft pulp and the organosolv-pretreated *Pinus radiata* chips, respectively. While the ethanol concentration of untreated raw material was 3.5 g ethanol/l (12% of the theoretical ethanol yield).

Dhaliwal et al. (2011) reported the isolation of the thermotolerant yeast from sugarcane juice through enrichment technique. This strain was identified as *Pichia kudriavzevii* (*Issatchenkia orientalis*) through molecular characterization. Fermentation in a laboratory fermenter with galactose adapted *P. kudriavzevii* cells at 40°C resulted in an ethanol concentration and productivity of 71.9 g/l and 4.0 g/l.h, respectively from sugarcane juice composed of about 14% (w/v) sucrose, 2% (w/v) glucose and 1% (w/v) fructose.

Benjaphokee et al. (2012) reported the strain improvement of *S. cerevisiae* and evaluate the performance of such a multiple-tolerant yeast strain of generated by a spore-to-cell hybridization technique without recombinant DNA technology. A heterothallic strain showing a high-temperature (41°C) tolerant (Htg^+) phenotype, a derivative from a strain isolated from nature, was crossed with a homothallic strain displaying high-ethanol productivity (Hep^+), a stock culture at the Thailand Institute of Scientific and Technological Research. The resultant hybrid TJ14 displayed ability to rapidly utilize glucose, and produced ethanol (46.6 g/l) from 10% glucose fermentation medium at high temperature (41°C). Not only ethanol productivity at 41°C but also acid tolerance (Acd^+) was improved in TJ14 as compared with its parental strains, enabling TJ14 to grow in liquid medium even at pH 3. TJ14

maintained high ethanol productivity (46.0 g/l) from 10% glucose when fermentation was done under multiple-stress conditions (41°C and pH 3.5). Furthermore, when TJ14 was subjected to a repeated-batch fermentation scheme, the growth and ethanol production of TJ14 were maintained at excellent levels over ten cycles of fermentation.

de Souza et al. (2012) reported the isolation of *S. cerevisiae* LBM-1, a strain capable of growing at 42°C. In addition, *S. cerevisiae* LBM-1 and *K. marxianus* UFV-3 were able to ferment sugar cane bagasse in SSF processes at 37 and 42°C. Higher ethanol yields were observed when fermentation was initiated after presaccharification at 50°C than at 37 or 42°C. Furthermore, the volumetric productivity of fermentation increased with presaccharification time, from 0.43 g/l.h at 0 h to 1.79 g/l.h after 72 h of presaccharification. The results suggest that the use of thermotolerant yeasts and a presaccharification stage are keys to increasing yields in this process.

Hashem et al. (2013) reported the isolation of the two new thermotolerant yeast strains of *Kluyveromyces* sp. from plum fruit and cantaloupe. The *Kluyveromyces* sp. ZMS1 GU133329 and *Kluyveromyces* sp. ZMS3 GU133331 produced 9.55% (w/v) and 11.72% (w/v) of ethanol at 35ºC, respectively. The maximum ethanol production by these strains was achieved under the appropriate sugar concentration of 20-25% and the pH range 5.0-5.5. In fed-batch culture, the maximum ethanol production was 11.71% (w/v) and 11.62% (w/v) by *Kluyveromyces* sp. ZMS3 GU133331 and *Kluyveromyces* sp. ZMS1 GU133329, respectively.

2.6 Factors influencing ethanol fermentation

There are many factors that influence upon the ethanol yields and fermentation rate in the ethanol fermentation process. A summary of the factors are given in the Table 2.2.

Table 2.2 Factors in the cellar environment that could influence alcoholic fermentation

Cellar Management: Alcoholic Fermentation	
Sections	**Subsections**
Yeast strain	
Yeast preparation	
Yeast nutrition	Nitrogen, Phosphate, Oxygen and other survival factors, Vitamins, Minerals
Inhibitory substances	Ethanol, Acetic acid, Medium chain fatty acids, Toxins and killer toxins, Sulphites, Agricultural residues
Physical factors	Excessive must clarification, pH, Temperature extremes
Microbial incompatibility	
Metabolic basis of stuck and sluggish fermentations	

(Malherbe et al., 2007)

2.6.1 Nitrogen source

Nitrogenous compounds are used by yeast to produce structural and functional proteins that result in increased yeast biomass, fermentation rate, and the production of enzymes that facilitate many biochemical changes occurring during yeast fermentation (Spayd and Andersen-Bagge, 1996 cited in Malherbe et al., 2007; Beltran et al., 2005). Ammonium nitrogen in the form of NH_4NO_3, NH_4Cl and $(NH_4)_2SO_4$ are commonly used as a nitrogen source for yeast growth (Berry et al., 1987). The requirement of nitrogen for protein synthesis may be met by ammonium ion, although amino acids are a preferred source. A wide range of substances (amino acid, peptides, urea, purine, pyrimidines and amines) are able to serve as the nitrogen sources and it can be seen that nitrogen addition during the fermentation showed the maximum of ethanol production (Benerji1 et al., 2010; Yue et al., 2010). Amino

acids are taken up in a sequential manner during fermentation (Jones and Pierce, 1964). This is thought to reflect the properties and specificities of permeases located in the cell membrane. The mixtures of amino acid, such as casein hydrolysate generally allow a higher maximum specific growth rate and cell yield than single amino acid (Cooper, 1982). In brewing, adequate rates of attenuation and complete utilization of fermentable sugars require 150 mg of free amino nitrogen (FAN) per liter in a 12°P (where °P corresponds to grams of dissolved solids expressed as sucrose per 100 g of wort) or 200 mg of FAN per liter in a 16°P. If the FAN content is less than this value, the wort is likely to be deficient in nitrogen and incompleted or protracted (stuck or sluggish) fermentations occur (O'Conner-Cox and Ingledew, 1989). Chang et al. (2011) indicated that SSF configuration with addition of FAN-containing substances results in the highest ethanol yield and the shortest fermentation time. Yeast may use amino acids not only as nitrogen sources but also as redox agents to balance the oxidation-reduction potential under conditions of restricted oxygen (Albers et al., 1996; Mauricio et al., 2001).

2.6.2 Phosphorous

Inorganic phosphates from the nutrient media are a common source of phosphorous in yeast. Phosphorous is assimilated only in the form of the orthophosphate ion ($H_2PO_4^-$). It is a component of sugar phosphate, nucleic acid, nucleoside di- or triphosphate and phospholipids. The content of phosphorus in yeast cells was about 3-5% of dry weight and the major part of this was in the form of orthophosphate (Aiking and Tempest, 1976; Theobald et al., 1996). Inorganic phosphate salts supplemented in the fermentation medium resulted in increase the ethanol production and the formation of adenosine triphosphate (ATP) (Rubio-Arroyo et al., 2011).

2.6.3 Sulphur

The sulphur requirement of yeast can be met by the uptake of sulphates, which can be assimilated through reduction into sulphur amino acids. The most commonly used sulphur source in the media is in the form of K_2SO_4 or more frequently, $(NH_4)_2SO_4$ (Berry et al., 1987).

2.6.4 Magnesium

Magnesium is an important divalent cation in metabolic processes and physiological functions, including cell growth, cell division and enzyme activity in yeast (Walker, 1994). It is a cofactor or effectors for many enzymes in yeast metabolism. Magnesium ions decrease protons and especially, anion permeability of the plasmalemma by interacting with membrane phospholipids, stabilizing the membrane bilayer. The protective effect of magnesium in response to toxic levels of ethanol in *S. carlsbergensis* (Petrov and Okorokov, 1990) has suggested that it plays a crucial role in the cellular protection and recovery from the stress. The main sources of magnesium are from $MgCl_2$ and $MgSO_4$ (Berry et al., 1987). Increasing magnesium levels, extra or intracellularly, may afford some level of protection to stressed cells by maintaining the structural integrity of membranes and supplying cells with an excess of this essential ion for some repair mechanisms or cellular function, such as enzyme activation (Birch and Walker, 2000).

2.6.5 Oxygen

Oxygen is essential for the biosynthesis of sterols and unsaturated fatty acids which are both essential to membrane structure and function (Casey and Ingledew, 1986 cited in Malherbe et al., 2007) and cell viability. Aeration conditions also have an impact on the formation of the by-products such as glycerol, non-fermentable carbon source for many yeast species, which causes difficulty in industrial ethanol recovery. The control of glycerol production using various oxygen levels or controlled oxygenation has been extensively investigated. Alfenore et al. (2004) evaluated the ethanol fermentation under VHG fermentation in two conditions, aeration and micro-aeration using *S. cerevisiae* CBS8066. The results showed that fully aeration led to an increase in the final biomass and ethanol concentration. Moreover, aeration also reduced the concentration of glycerol compared with micro-aeration. Furthermore, Lin et al. (2011) reported the effect of aeration timing and aeration interval on ethanol production during very-high-gravity ethanol fermentation. The result indicated that the aeration rate of 0.82 l/min, and aerating yeast in its log phase (8-18 h) were the most effective aeration schemes among others.

2.6.6 Ethanol concentration

Ethanol exerts a strong inhibition on yeast cell growth and ethanol production, which limits ethanol concentration in the broth to no more than 13% (v/v) for most ethanol production plants at the present time (Bai et al., 2004). The biological effect of high ethanol concentration on cellular growth rates and fermentation efficiency is largely a result of changes in the properties of cellular membranes, in particular an increase in membrane permeability and changes in membrane fluidity to ions and small metabolites (Ingram and Buttke, 1984;) and inhibiting nutrient transport (Van Uden, 1985). Moreover, when ethanol concentration reached up to 84 g/l, it strongly inhibited the assimilation of biotin (Winter et al., 1989).

2.6.7 Substrate concentration

Normally, sugar concentrations above 20% (w/v) are not used under industrial conditions because increasing concentration of sugar and ethanol delays the growth of the yeast, which eventually leads to stuck fermentation (Kalmokoff and Ingledew, 1985; Mota et al., 1987). In addition, D'Amore (1992) reported that as the initial wort gravity was increased, the rate and extent of fermentation decreased and the amount of ethanol produce was lower than theoretically expected. Thus, changes in temperature, oxygen level and cell pitching have been successfully employed to increase the rate of fermentation under high sugar concentrations (Jones and Ingledew, 1994). Ozmihci and Kargi (2007) found that the rate of ethanol production was increased when cheese-whey powder concentration was increased up to 156 g/l (75 g/l of sugar). However, at the high sugar concentrations, biomass concentrations decreased due to cell disruption by high osmotic pressure yielding low biomass and ethanol yields. In addition, high substrate concentrations also cause prolongation of complete sugar utilization and lower final ethanol concentration (Ozmihci and Kargi, 2007). It may be due to substrate inhibition at high sugar concentrations. Ingledew (1999) reported that during ethanol fermentations, sugar concentration above 25% (w/v) affected the osmotic pressure which was a main factor leading to the reduction of yeast viability and ethanol yield.

2.6.8 Fermentation temperature

The fermentation temperature is one of the main factors for ethanol fermentation in terms of both ethanol concentration and rate of ethanol fermentation. In industry, fuel ethanol fermentation was conducted at the fermentation temperature of 30 to 35°C (Jones and Ingledew, 1994). At higher temperatures, the cell membrane fluidity increases and ethanol can enter the cell more readily, adversely affecting metabolism and cell viability. On the other hand, ethanol fermentation at the lower temperature such as 20°C caused the longer fermentation time to complete the conversion of sugar to ethanol (Jones and Ingledew, 1994). Ethanol fermentation at high temperature is a key requirement for effective ethanol production in tropical countries where average day-time temperatures are usually high throughout the year. The advantages associated with the production of ethanol at temperatures higher than those used in conventional systems include reduced risk of contamination and cooling cost, increased productivity and ease with the product may be recovered, particularly at the later stages in batch and fed batch reactor systems (Nolan et al., 1994; Kiran et al., 2000; Limtong et al., 2007).

2.6.9 pH

Hydrogen ion concentration has a significant influence on ethanol fermentation due as much to its importance in controlling bacterial contamination as its effect on growth, fermentation rates and by-product formation. In ethanol fermentation, the pH of medium was in the range of 4.0-5.5, which is a possible way to control bacterial contamination during the fermentation (Narendranath and Power, 2005). The pH of medium is an important factor for ethanol yield. Limtong et al. (2007) reported that the suitable pH for ethanol production from sugarcane juice using thermotolerant yeast, *K. marxianus* DMKU 3-1042 was 5.0. The ethanol concentration produced under this condition reached 8.7% (w/v), with the productivity of 1.45 g/l.h and ethanol yield 77.5% of theoretical yield.