จลนศาสตร์การเจริญของเชื้อ *Saccharomyces cerevisiae* K1-V1116 และการสร้าง Killer toxin ในการผลิตไวน์

เศกสิทธิ์ ชำนาญศิลป์

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GROWTH KINETICS OF *Saccharomyces cerevisiae* K1-V1116 AND KILLER TOXIN PRODUCTION IN WINE MAKING

Sakesit Chumnarnsilpa

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เชื้อ *Saccharomyces cerevisiae* K1-V1116 เป็นเชื้อยีสต์ทางการค้าที่ใช้ในการผลิตไวน์มีความ สามารถในการสร้าง killer toxin ชนิด K2 และได้ถูกนำมาใช้ในการศึกษาจลนศาสตร์การเจริญในน้ำองุ่นพันธ์ Ruby Carbernet โดยเปรียบเทียบกับเชื้อยีสต์สายพันธ์ K1-V1116 HC ซึ่งเป็นสายพันธ์เดียวกันที่ถูกกำจัดความสามารถในการเป็น killer yeast ออกไปด้วยเทคนิค heat curing จากผลการทดลองพบว่า K1-V1116 HC สามารถเจริญและให้ผลผลิตแอลกอฮอล์ที่ดีกว่าที่ระดับความเชื่อมั่น 99% ด้วยค่า การเจริญจำเพาะ (μ_{max}) = 0.15 h^{-1} และ $Y_{Eth/Sugar}$ = 0.47 กรัม/กรัม ในขณะที่ K1-V1116 ให้ค่า μ_{max} = 0.13 h^{-1} และ $Y_{Eth/Sugar}$ = 0.46 กรัม/กรัม

ในการศึกษาความสามารถในการฆ่าของยีตส์ K1-V1116 พบว่า มีค่าสูงสุดเท่ากับ 230 เซลล์ต่อมิลลิลิตร ในวันที่ 3 ของการหมัก จากนั้นความสามารถในการฆ่าจะลดลงอย่างรวดเร็ว

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SAKESIT CHUMNARNSILPA: GROWTH KINETICS OF *Saccharomyces cerevisiae* K1-V1116 and KILLER TOXIN PRODUCTION IN WINE MAKING THESIS ADVISOR; PROF. NANTAKORN BOONKERD, Ph.D.
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KILLER YEAST/HEAT CURING/MAXIMUM SPECIFIC GROWTH RATE/ SPECIFIC GLUCOSE CONSUMPTION RATE/SPECIFIC ETHANOL PRODUCTION RATE/ KILLER ACTIVITY

The commercial K2 killer yeast, *Saccharomyces cerevisiae* K1-V1116 and its heat curing strain, HC were used to study growth kinetics and killer activity in Ruby carbernet grape must fermentation at 25 °C. The killer and non-killer yeast with similar genetic background showed different growth kinetics. The non-killer yeast showed over all better kinetics (maximum specific growth rate, specific rate of ethanol production, specific glucose consumption rate, and yield of ethanol production from reducing sugar) than killer strain. The maximum specific growth rate (μ_{max}) and yield of ethanol from sugar ($Y_{Eth/sugar}$) of killer and non killer are significantly different at 99% confidence. The HC has μ_{max}= 0.146 h^{-1}, $Y_{Eth/sugar}$ = 0.466 gg^{-1}, and K1-V1116 has μ_{max}= 0.135 h^{-1} and $Y_{Eth/sugar}$ = 0.458 gg^{-1}. The K1-V1116 exhibited maximum killer activity (230 cell/ml) on day three and then declined rapidly.

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Chumnarnsilpa

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LIST OF ABBREVIATION

K1-V1116	=	*Saccharomyces cerevisiae* K1-V1116
HC	=	*Saccharomyces cerevisiae* K1-V1116 heat curing strain
μmax	=	Maximum specific growth rate
T_d	=	Doubling time
r_{Eth}	=	Rate of ethanol production
q_{Eth}	=	Specific rate of ethanol production
r_{Gly}	=	Rate of glycerol production
q_{Gly}	=	Specific rate of glycerol production
r_{Glc}	=	Rate of glucose consumption
q_{Glc}	=	Specific rate of glucose consumption
r_{Frc}	=	Rate of fructose consumption
q_{Frc}	=	Specific rate of fructose consumption
EC-1118	=	*Saccharomyces bayanus* EC-1118
67J	=	*Saccharomyces cerevisiae* 67J
7013	=	*Saccharomyces cerevisiae* 7013
7303	=	*Saccharomyces cerevisiae* 7303
Baker	=	*Saccharomyces cerevisiae* (baker yeast)
RS	=	*Saccharomyces cerevisiae* (Pasteur Champagne commercial strain from Red star Co.Ltd. U.S.A.)
$C_{biomass}$	=	Biomass concentration
$C_{ethanol}$	=	Ethanol concentration
VLP	=	Virus like particle
YEPG	=	Yeast extract peptone glucose
L-A	=	L-dsRNA virus
M1	=	M1 dsRNA virus
M2	=	M2 dsRNA virus

PME	=	Pectinmethyl esterase
PG	=	Polygalacturonase
BCP	=	Bromocresol purple
CFA	=	Colony forming ability
HPLC	=	High preformance liquid chromatography
WADY	=	Wine active dried yeast
ORF	=	Open reading frame
PW	=	Peptone water

Chapter1
Introduction

Saccharomyces cerevisiae is the most popular yeast used in alcoholic fermentation step of wine production. The role of yeast in wine production is to converse sugars in grape juice into ethanol. Nowadays, many commercial strains of yeast exit. Approximately 60% of *S. cerevisiae* used in France are killer yeast (Barre, 1980; Barre and Biron, 1982). More than 85% of commercial wine strains in USSR have killing property (Naumov, 1973). Killer yeasts offer advantages over conventional wine yeasts when employed for fermentation. Firstly, they could kill certain wild yeasts which, cause problems such as delay of fermentation, struck fermentation. They also produce off-flavors to wine. Secondly, they could be selected for immunity against any killing action of wild yeasts. It has been reported that there are very high incidences of wild killer yeast observed in Mediterranean (65-90%) and Beaujolais (83%) vineyards (Cuinier and Gros, 1983). This wild killer yeast has greater chance of dominating the fermentation if non-killers which are used for the fermentation. Finally, they could be selected to produce killer toxins that would protect the wine from infection by spoilage yeasts (Graham, 1992). Currently, the killer yeasts belonging to *S. cerevisiae* have been classified into three main groups (K1, K2 and K28) on the basis of their molecular characteristics of the secreted toxins (Walter, 1997). They are constituted by strains producing toxins encoded by dsRNA, but other killer yeasts producing toxins, namely KHR and KHS which are encoded on chromosomal DNA, have been defined (Goto *et al.*, 1990; Goto *et al.*, 1991).

In a survey of natural enological microflora in Geisenheim, Germany in 1985s, four killer strains of *S. cerevisiae* were isolated from natural wine fermentations. All the killer showed the characteristic of K2 killer yeast (Shimizu *et al.,* 1985).

The K2 toxin is encoded by cytoplasmically inherited satellite dsRNA (M2), encapsidated in virus-like particles (VLPs). It dependent on another group of helper yeast viruses (L-A) for their replication and encapsidation (Walter, 1997). The K2 toxin is 16-kDa glycoprotein with an optimum activity at pH 4.2 (Pfeiffer and Radler, 1982). The mechanism of

K2 toxin is similar to K1 toxin. The primary event in the action of K1 toxin is an energy independent binding of toxin to the cell wall receptor (Al-Aidoors and Bussey, 1978; Bussey *et al*, 1979). This is followed by an energy-dependent insertion of the toxin into its action site, the cytoplasmic membrane (Skipper and Bussey, 1977). This action site in the cytoplasmic membrane had been identified as the potassium channel by Aamir *et al.*, in 1999.

All of the commercial killer yeast for winemaking are the K2 type because of the broad spectrum in pH and its stability and high activity (Graham, 1992). Generally, the killer toxin of *S. cerevisiae* become unstable at high ethanol concentrations, high temperatures (Shimizu unpublished data) and very low pH value (Shimizu *et al.*, 1986). The K1, K2 and K28 killer activities of *S. cerevisiae* have been compared at different pH values. The activity of K1 toxin is not observed at pH value less than 3.5 whereas the K2 and K28 toxins show significant activity at pH 2.9. The K2 activity is superior to the K28 activity under acidic conditions. Heard and Fleet (1987) also observed the activity of K2 toxin during experimental wine fermentation at pH 3.0 (Graham, 1992). With respect to stability, K2 killer activity is most stable at pH values less than 4.0 (Shimizu *et al.*, 1986). The K2 killer toxin produced by *S. cerevisiae*, "Prise de Mousse" strain during the fermentation of Koshu white wine was active for more than 30 days during storage at pH 3.1 and 15 °C (Shimizu unpublished data). These facts suggest that K2 killer strains of *S. cerevisiae* should be selected when aiming to exploit the killer phenomenon during commercial wine fermentation.

There are many different techniques to determine killer activity. Each techniques depends on conditions and aim of experiments as well as type of toxins for example BCP (bromocresol purple) fluorescent test, plating (colony forming ability, CFA) test and well test/gel diffusion (Kurzveilova and Sigler, 1995).

In wine fermentation well test is the most well known method that is currently used. This technique is easy with low cost but time consuming. However, it cannot really represent the killer activity in wine must. This technique always uses sensitive strains that are very sensitive to toxin to detect the killer activity in gel. In addition, the killer toxin yeast markedly more stable in gel than in broth. It produces good zone of inhibition on gel while showing little or no activity in broth (Woods, 1968). The environment of killer activity in gel and wine must are very different

therefore the killer activity that observed by clear zone in gel can not show the actual killer activity in wine must.

A. Killer yeast

Bevan and Makover (1963) first reported the killer character in yeasts. The killing action is due to toxin which are produced and secreted by killer strains. Killer toxin are either protein (Palfree and Bussey, 1979) or glycoproteins (Bussey and Skipper, 1975; Pfeiffer and Radler, 1982; Sugisaki *et al.*,1984; Yokomori, *et al.*, 1988) which are lethal to sensitive yeasts. The most thoroughly investigated yeast killer system is that of *Saccharomyces cerevisiae*, which has been described in detail in many reviews. Currently, the killer yeast belonging to this species have been classified into three main groups (K1, K2 and K28) on the basis of the molecular characteristics of the secreted toxins, their killing profiles, the lack of cross immunity, and the encoding genetic deteminants (Magliani, 1997). The K1, K2 and K28 toxins are encoded by different cytoplasmically inherited satellite dsRNA (M1, M2, and M28), encapsidated in virus-like particles (VLPs) and dependent on another group of helper yeast virus (L-A) for their replication and encapsidation. The M dsRNAs are responsible for either killer activity or self-immunity, a phenotype that is characteristic of yeast killer toxin producing strains. The toxin are able to kill non-killer yeasts as well as yeast of the different killer class, while the producing yeast remain immune to their own toxin and to that produce by strains of the same killer group. Killer system, L-A virus is associated with the presence of a satellite M- RNA, packed in a capsid encoded by the helper virus. L-A virus autonomous replicating viruses that do not require M RNA for replication.

L-A mycoviruses are the dsRNA viruses, which have been classified in genus *Totivirus* of families, *Totiviridae* (Buck and Ghabrial, 1991). L-A viruses are 39-nm-diameter isometric particles with no envelope, consisting an undivided dsRNA genome whose complete nucleotide sequence (4,579 bp) has been detemined (Diamond, 1989; Esteban, 1986; and Icho, 1989).

Satellite M dsRNAs are family of RNA molecules present in VLPs persisting in the cytoplasm of *S. cerevisiae* killer strains. They are dependent on L-A helper viruses for their replication and encapsidation (Hannig, and Leibowitz, 1985; Schmitt, and Tipper, 1992; Tipper,

and Bostian, 1984). The presence of a satellite M dsRNA in cells coinfected with an L-A virus is responsible for the killer immune phenotype observed in the killer strains. All three killer toxins of the best known killer strains (K1, K2 and K28) are encoded by different dsRNAs (M1, M2, and M28), differing in size (1.8, 1.5, and 1.9 kb, respectively) and showing similar organization, even without any significant sequence homology (Dignard, et al., 1991; Ghabial, 1994; Schmitt, and Tipper, 1990, 1992,1995; Wickner, 1992,1996).

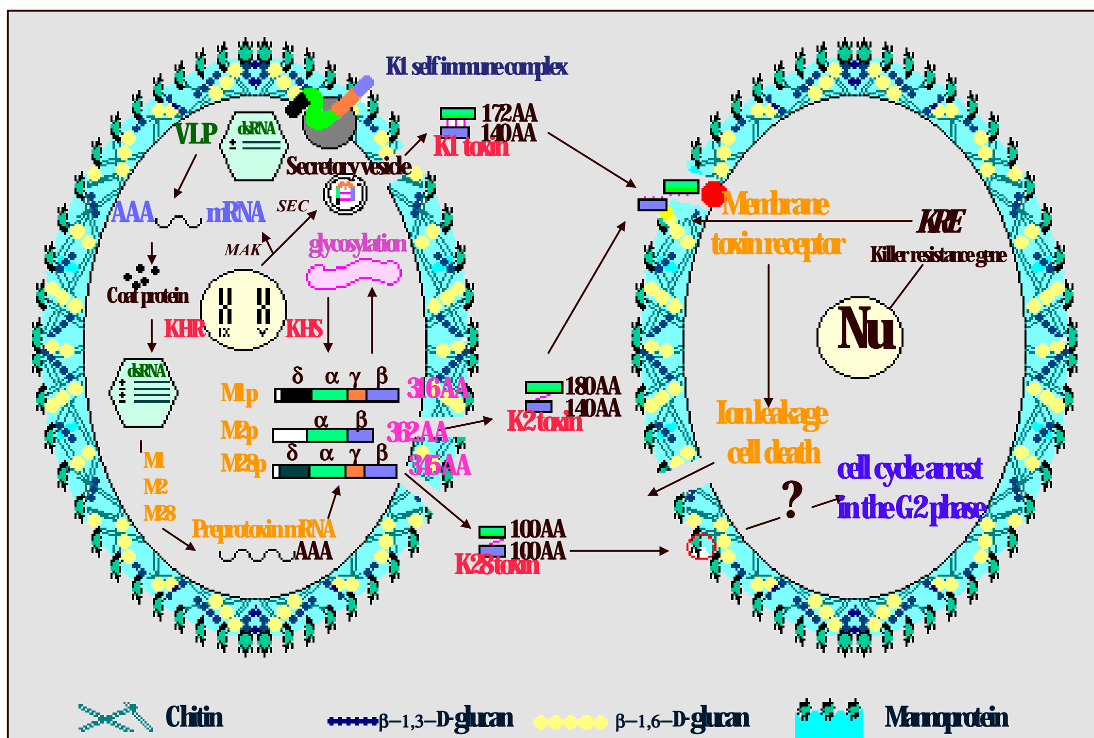

Figure 1 Mechanism of killer toxin encoding by dsRNA virus like particle (Magliani, *et al.* 1997)

K1, K2 and K28 *S. cerevisiae* killer toxin are protein molecules secreted by killer strains carrying a specific satellite dsRNA; killer strain are not susceptible to their own toxin but remain susceptible to other killer toxins. Even though the toxins have different amino acid composition and mode of molecular action, they show some general characteristic in their mechanisms of synthesis, processing, and secretion. Each toxin is encoded by single ORF and synthesized as a single polypeptide preprotoxin comprising larger hydrophobic amino acid temini than normally

found in secreted proteins and potential kex2/kex1 cleavage and N-linked glycosylation sites; the preprotoxin have similar overall structure. The preprotoxins, once synthesized, undergo posttranslation modifications via the endoplasmic reticulum, Golgi apparatus, and secretory vesicles, resulting in the secretion of the mature, active toxin (Magliani, *et al.* 1997).

K2 toxin show a similar overall organization to K1, has been characterized as a 362 amino acid precursor of 38.7 kDa (M2p) containing three potential sites for Asn-linked glycosylation at amino acid residues 177, 214 and 261. It also contain a potential kex1p and kex2p cleavage sites (Dignard, et al. 1991; Meskauskas, and Civitavicius, 1992). During the maturation process, the signal peptide is removed by peptidase cleavage after Ala^{43} and the remaining molecule apparently is cleaved by the kex2p after Arg^{222}, yielding the two subunits (α and β) those constitute the mature secreted toxin. Unlike K1 toxin, a γ domain does not seem to be present in the preprotoxin. The final α and β subunits are larger than those of K1 (172 and 140 amino acid, respectively), and α is N-glycosylated at two positions (positions 177 and 214). Kex1p is also required for the complete processing of α.

Mode of action of K1 and K2 toxins and self-immunity, all the secreted mature toxin can exert killer activity on susceptible cells by difference mechanism that require a specific initial binding to a cell wall receptor, the precise structure of which remain largely unknown. K2 toxin has virtually identical activity to that of K1 toxin, despite a different structure. The first step of binding yeast strongly pH dependent with an optimum at pH 4.6 and is a low-affinity, high-velocity adsorption (1 minute) of the killer toxin to the cell wall receptor, which are presented at an average of $1.1x10^7$ molecules per cell (Bussey, *et al.*, 1979). The second step is a high affinity, low velocity, energy-dependent interaction of the toxin with a probable plasma membrane receptor that leads to the actual lethal effect (Zhu and Bussey, 1989). The constitute of the glucan fraction of the cell wall, mainly β-1,6-D-glucan, have been identified as primary receptors for the toxin and their assembly seems to require a number of yeast *KRE* (killer resistance) gene (Al-Aidroos and Bussey, 1978; Boone, *et al.*, 1990; Brown, *et al.*, 1993; Hill, *et al.*, 1993; Hutchins and Bussey, 1983). After binding to the yeast cell wall, toxin is transferred to the cytoplasmic membrane and acts by forming voltage-independent cation transmembrane channels, which cause ion leakage and subsequence cell death (de la Pena, *et al.*, 1981; Martinac, *et al.*, 1990). Two

strongly hydrophobic regions near the C terminus of the α subunit have an α-helical structure separated by a short, highly hydrophilic segment that may add as membrane-spanning domain responsible for channel formation (Sturley, *et al.*, 1986).

The phenotypes of strains, with regard to their killing ability (K) and resistance to killing (R) are denoted K^+R^+ (normal killer), K^-R^+ (neutral phenotype), or K^-R^- (sensitive non-killer). Treatment of killer strain with cycloheximide (Gerald and Cora, 1972), acridine orange, or growth at elevated temperature converts it into a sensitive non-killer. The cycoheximide affects some protein synthesized on cytoplasmic ribosome and necessary for the replication of the killer determinant (Reed, 1974). Acridine orange, and intercalating dye was treated for curing K1 and K2 killer yeast by the lost of M1 and M2 dsRNAs, respectively (Jose, *et al.*, 1989). Heat curing can eliminate M2 ds-RNA by without interfering with any others genetic backgrounds (Wickner, 1974). The heat curing at 37 ° C for 48 h can was proved that could eliminate M2 dsRNA (Jenny, *et al.* 1991).

B. Must

A number of steps are used to prepare must for winemaking. Crushing and destemming is employed to cause berry breakage and juice release from the grapes. Ordinarily hundred percent of the berry will be broken. It is the beginning of the juices, skin, pulp, and seed contact that will influence the extent of extraction from these grape components. A secondary aspect of the crushing and destemming process is the elimination of the stems from the juice and skin and isolation and collection of them for disposal. The treatment of must prior to fermentation will often include one or more of the following actions: nutrient additions, sulfur dioxide additions, acidity adjustment, juice aeration, thermal treatment of juice, addition of inert solid, and enzyme additions (Boulton, *et al.*, 1995). Some of these treatments are often essential for basic winemaking while others are more appropriately term “stylistic” treatment where the value is more a matter of wine style or individual opinion. The extent to which some of these treatments is necessary or desirable can vary quite widely depending on the cultivars involved and the wine style that is sought. There are often quite different approaches between countries and even regions. Within the stylistic treatment, there is usually a spectrum of opinion ranging from

minimizing the effect to maximizing it, with many level of acceptant in between. Nutrient addition are responsible for adding the substance (S) important to growth of yeast and bacteria in a deficient must including nitrogen sources (both ammonium salts and free amino acid) and the vitamins (biotin, thiamin, panthothenic acid, and inositol). The used of sulfur dioxide to restrict the extent of must browning and to inhibit or kill most of the neutral microflora in the juice. The acidity adjustment will generally be based on the target value for titratable acidity and pH rather than by sensory evaluation, due to the overwhelming influence of sugar levels in the must. The addition of tartaric acid to must can be applied to increase the titratable acidity and reduce the pH. Tartaric acid is the acid of choice since it will not be used by organism at wine pH while both malic and citric acids are substrates for a number of lactic acid bacteria. The aim of must aeration is to oxidize many of the phenolic components, which would normally be the substrates for chemical oxidation (and browning) in the subsequent wine. The brown pigments formed by this action will generally be absorbed to solids and be removed by precipitation during fermentation, living only the light golden, straw-colored pigments in the wine (Cheynier *et al.*, 1990). Thermal treatment of must is employed for two reasons. Firstly, it aims to kill fungi and/or to denature laccase, a potent oxidative enzyme commonly found in grapes infected by mold. Secondly, it is to promote color extraction. This process is often employed with grapes that are poor in pigmentation, caused by either very warm or very cool climatic condition. Addition of inert solids such as bentonite to must to adjust suspended solid content by adsorption of solutes to their surface. This process is effective in small-scale fermentation, which is rarely observed at the commercial scale.

Red wine is made from black grapes. Anthocyanin pigment, tannin and flavonoid phenols in red must make red wine different from white wine, which also effect on yeast. They associated with the skins and seeds as well as other less well defined flavourants (Ramey *et al.*, 1986). Skin contact is one of the techniques for color and flavour extraction. The extraction and retention of the anthocyanin pigments during fermentation is still not completely understood. Enzyme additions have been proposed for application in juices and wine. Pectic enzyme, protease, cellulase, glucosidase, glucanase, and urease are hydrolyzing enzymes necessarily for increase must yield. It helps extract color and flavour from grape fruits and enhance natural

clarification of wine. Juices and wine pose some unusual environmental condition for enzyme activity due to pH, ionic strength and their sulfurdioxide, ethanol and phenol contents (Boulton, *et al.*, 1995).

The addition of a pectic enzyme to must is generally done to enhance the fraction of juice release during draining, reducing the fraction that is release during pressing and facilitate sterilization by membrane filtration. These have also been used in the enhancement of natural clarification of wine. (Hickenbotham and Williams 1940, Besone and Cruess 1941). Pectic enzymes are used to assist the hydrolysis of pectin, a cell wall constituent in most fleshy fruits. In *Vinifera* grapes, the pectin content range between 0.6 and 2.6 g/L (Amerine and Joslyn 1951) depends on the cultivar. Grape pectin includes polymeric galacturonic acid in which approximately two-third of the carboxyl groups has esterified into methoxy rather than the free amino acid forms (Robertson 1987).

Commercial pectic enzymes are generally a mixture of at least two particular enzymes, pectin methly esterase (PME) (EC 3.1.1.11) and poly galacturonase (PG) (EC 3.2.1.15). The PME hydrylyzes the methoxy ester part of the polymer, allowing the PG to break the inter linking bonds (Boulton, *et al.*, 1995). Small amounts of methanol are resulted from this reaction. However most of it is removed with the cabondioxide during fermentation. Both *exo-* and *endo-* forms of PG are commonly used in wine production. The *exo*-forms cleaves galacturonic acid from the terminal of the polymer, while the *endo*-form cleaves random within the polymer. At juice pH and normal temperature these enzymes perform well below their maximum activities, typically at 40-60% in the pH range 3.0 to 3.5 at 25 °C. They can be used in juices either with or without sulfur dioxide since they are not inhibited by it at levels below 400mg/L.

Many commercial preparations also contain other hydrolytic activities such as pectate lyase (EC 4.2.2.2,6,9) and cellulases in an attempt to enhance cell wall breakage. Others can have appreciable levels of β-glucosidase activity and this has been used with limited success for the release of volatile terpenes and anthocyanin from their glucoside forms in juice and wines.

Addition of pectic enzymes usually result in improved clarification by natural settling, but there are other polysaccharides which may be caused of settling characteristics and it would not be addressed by these enzymes.

Although the used of pectic enzymes helps to prevent the development of pectin hazes in wines, the addition is usually made to enhance free-run yield in draining and pressing operations. The hydrolysis of pectin in the cell wall leads to early juice release, and in some case, total yields (free-run plus press fraction) increased (Ough and Berg 1974, Ough and Crowell 1979). They generally contribute to more rapid and extensive natural settling of juice and in some cases improved filterability of the juice.

C. Fermentation

The transformation of must into wine is essentially a microbial process. As such, it is important for enologist to have an understanding of yeast and fermentation biochemistry as the fundamental basis of the winemaking profession. The alcoholic fermentation is the conversion of the principal grape sugars, glucose and fructose, into ethanol and carbon dioxide. This process conducted by yeasts of the genus *Saccharomyces*, generally by *S. cerevisiae* and *S. bayanus* (Boulton 1995). *S. cerevisiae* is the specie par excellence for wine fermentation. The cell is usually spheroid, ovoid, ellipsoid or elongated with a cell size often 3-7 x 4-12 μm. This species may produce up to 18-20% ethanol by volume (Reed and Nagodawithana,1991).

In the 1950s, the practice of using selected wine yeast for inoculation was very common in the United States, Australia, New Zealand, and South Africa. It was also practice in Europe, although the practice was not always admitted. In the 1960s, active dry wine yeast (WADY) was introduced in the United States and its use spread quickly to Australia, New Zealand, and South Africa. Since late 1970s, WADY has been used in Europe. Its used in Germany, Italy and France is now common, and it is being introduced in other wine producing countries of Europe and South Africa (Reed and Nagodawithana 1988).

In the early 1960s, the U.S. wine industry became interested in a commercial source of bulk wine yeasts. Several strains were successfully produce at that time and used in the production of table wines (Thoukis, Reed, and Bouthilet 1963).

Each commercial strain posed their good characteristic such as high sulfur dioxide and alcohol tolerant, good favour, aroma, and body of wine. Killer activity is offered as one of the

good property of wine yeast to protect spontaneous fermentation from wild yeast which cause problems such as delay of fermentation and production of off-flavours (Fleet, 1984).

The *Saccharomyces cerevisiae* killer yeast are infected by M and L viruses. These may bring about worse growth kinetic than uninfected healthy yeast with same genetic background.

Some metabolic parameters, such as specific growth rate (μ_{max}), specific product formation rate, specific substrate consumption rate and yield of product from substrate can be used to compare and represent growth kinetics of different yeast strains (Doran 1995).

D. Killer activity determination

There are many different techniques to determine killer activity. Each techniques depends on conditions and aim of experiments as well as type of toxin for example BCP (bromocresol purple) fluorescent test, plating (colony forming ability, CFA) test and well test/gel diffusion.

Kurzveilova and Sigler developed BCP fluorescent test in 1993. They offered it as a rapid assay for the yeast killer toxin K1 activity in *Saccharomyces cerevisiae*. Under suitable conditions, the test is a universally applicable for determining the proportion of cell with a damaged plasma membrane in a population. BCP is acid-base indicator that carries on positive charge at solution with pH 4.6 which is the pH optimum for the killer toxin. The dye does not stain intact cells during a 7 h incubation. This technique takes 1-2 hr for analysis.

The plating or CFA test is a standard method for estimating killer toxin activity (Bussey and Sherman, 1973; Bussey, *et al.*, 1979). This technique need partial purify and concentrate toxin, and mix with various concentrations of toxin to the susceptible cell. The reaction mixture is incubated for 2-3 h, prior to plate onto solid medium for colony forming. The killer activity is calculated from number of colonies appeared pre- and post-reaction.

The well test is another technique often used for determination of killer toxin activity (Wood and Bevan, 1968; Pena, *et al.*, 1980; Bussey, 1972). Under standardized conditions it seems to be a reliable quantitative assay of killer toxin concentration: at higher toxin concentrations it yields a linear relationship between the diameter of the inhibition zone and the logarithm of toxin concentration. The activity is usually express in arbitrary units different from

LU. These arbitrary units are defined as zones of a certain diameter (Pena, *et al.*, 1980; Bussey, 1972).

In wine fermentation well test is the most well known method that is currently being use. This technique is easy with low cost but time consuming. However, it cannot really represent the killer activity in wine must. This technique always uses sensitive strains that are very sensitive to toxin to detect the killer activity in gel. In addition, the killer toxin yeast markedly more stable in gel than in broth, which produce good zone of inhibition on gel, where it may show little or no activity in broth (Woods, 1968). The environment of killer activity in gel and wine must are very different therefore the killer activity that observed from clear zone in the gel can not show the real killer activity in wine must.

1.1 The problem

As mention above, the killer yeast with killer activity is due to virus infection of host yeast cell. Therefore, the killer yeast should not grow as well as the non-killer yeast. No report has been done to compare the growth kinetics of killer and non-killer yeast with the same genetic background in wine production.

1.2 Objective

To achieve the hypothesis the following specific objectives were investigated

1.2.1 To compare the growth kinetic of killer and non-killer yeast.

1.2.2 To determine the killer activity of killer yeasts in wine must during winemaking.

1.3 Assumption

Killer and non-killer yeast with the similar genetic background should give different growth kinetics because the killer yeast is infected by virus. The virus utilizes energy and nutrient of killer yeast, which should cause different in growth kinetic between killer and non-killer yeast.

Killer toxin is an unstable glycoprotein with very specific optimum condition for activity. The activity of the killer toxin should be either very low or undetectable in must.

1.4 Basic agreement

Saccharomyces cerevisiae K1-V1116, K2 type killer yeast (Graham, 1992) is the killer yeast used in this experiment. It is a commercial strain that is widely used in alcoholic fermentation step of winemaking. Heat curing technique was used for the elimination of virus to produce non-killer strain. The preparation of *S. cerevisiae* K1-V1116 heat curing (HC) strain was discussed in details in chapter 2. *S. cerevisiae* K1-V1116 and *S. cerevisiae* K1-V1116 HC strain represent the killer and non-killer yeast, respectively with same genetic background, were used for determination and comparison of growth kinetics in wine making. The must preparation from Ruby carbernet grape grown in Nakhon Rachasima, Thailand in 2000, were used in these experiments are shown in chapter 2. *S. cerevisiae* RS was used as susceptible strain for killer activity analysis. Fermentations were operated at 25°C in bioreactor. Fermentation broth was sampling at certain period of times for chemical analysis by HPLC. Amount glucose and fructose were monitored to determine the sugar consumption rate. Ethanol and glycerol levels were measured for their production rate. Must was sterilized by membrane filtration technique prior to for fermentation and CFA technique to determine the killer activity. The fermentation of *S. cerevisiae* K1-V1116 and *S. cerevisiae* K1-V1116 HC was carry out at the same period of time, 3 times. Details discussion of the alcoholic fermentation and killer activity analysis were present in Chapter2.

1.5 Scope

The experiments were set up to investigate the growth kinetics and killing activity of *S. cerevisiae* K1-V1116 and *S. cerevisiae* K1-V1116 HC in Ruby carbernet must. The scope of this work was includes the characteristic of growth and killer activity in wine must.

1.6 Benefits

The growth kinetic and killer activity of both killer and non-killer yeast will provide value information when decision making for choosing killer or non-killer yeast in winemaking, especially in black grape must.

Chapter 2
Materials and methods

Materials

Yeast strains

1. *Saccharomyces cerevisiae* K1-V1116 is commercial killer strain from Lalvin Co.Ltd. Canada.
2. *Saccharomyces bayanus* EC-1118 commercial killer strain from Lalvin Co.Ltd. Canada.
3. *Saccharomyces cerevisiae* 67J commercial strain from Fermirouge Holland.
4. *Saccharomyces cerevisiae* 7013 commercial strain from Fermirouge Holland.
5. *Saccharomyces cerevisiae* 7303 commercial strain from Fermirouge Holland.
6. *Saccharomyces cerevisiae* (baker yeast) commercial strain from Fermirouge Holland.
7. *Saccharomyces cerevisiae* (Pasteur Champagne) commercial strain from Red star Co.Ltd. U.S.A.
8. *Saccharomyces cerevisiae* RS (Montrachet) commercial strain from Red star Co.Ltd. U.S.A.

Apparatuses

High-performance liquid chromatography (HPLC)

HPLC (Thermo separation product Inc.) equipped with a refractometer RI-1530 (Jasco, Japan) and prepacked column RT 300-7.8 polyspher OAKC. The column temperature was kept constant at 60°C. The mobile phase was 0.005 N H_2SO_4 with a flow rate of 0.3 ml/min. Before analysis the samples were centrifuged at 4,500 rpm for 10 min at 4°C, and filtered through a Whatman membrane filter (ϕ 0.45 μm).

Glucose and L-Lactate analyzer

Glucose and L-Lactate analyzer model YSI 2300 STAT PLUS YSI Incorporated.

Centrifuge

Labofuge 400R Heraeus Instrument Co. Ltd.

Spectrophotometer

Spectronic 20 Genesys Spectronic instrument Co. Ltd.

Bioreactor

Bioreactor model BIOSTAT B B.Braun Biotech International Company.

Incubator

FOC 225E Refrigerated Incubator VELP scientifica Co. Ltd.

Methods

A. Yeast strain preparation

1. Purification of killer yeast *Saccharomyces cerevisiae* K1-V1116

The 0.5 g of commercial K1-V1116 powder was dissolved into 4.5 ml Peptone W (PW), and incubated at room temperature for10-15 min. Suspension of K1-V1116 was streaked onto 2 plates of Yeast extract Peptone Glucose Agar (YEPGA), and incubated at 25 ºC for 48 hr. Five single colonies were picked from each plate of YEPGA, and kept in YEPG slant. Twenty YEPG slants of K1-V1116 were stored at 25 ºC until used.

2 Killing activity test of K1-V1116 and Screening of susceptible strains.

The eight Commercial strains of unknown killer activity were cultured in 50 ml YEPG broth (pH 4.6) at 25 ºC for 24 hr. Seedling plates of unknown killing activity strain (UN) were prepared on YEPGA plates by pour plate technique (duplicate). The UN strains and K1-V1116 were streaked onto the seedling plates, and incubated at 25ºC for 48 hr. The killer activity was observed by clear zone around the colonies.

3. Heat curing and isolation of susceptible strain (K1-V1116 HC) from killer yeast K1-V1116

Heat curing: K1-V1116 was inoculated into 50 ml of YEPG broth (pH 4.6) in 250 ml flask, and incubated at 42 ºC for 48 hr. then transform to 30 ºC for 24 hr.Isolation of Susceptible strain (*S. ceresiviae* K1-V1116 HC). Culture broth from heat curing step was used to isolate single colonies by streaked on YEPGA plates, and incubated at 25 ºC for 24 hr. The single colonies from 2 plates of YEPGA name HC1-HC12 were transferred onto YEPG slant (duplicate) and stored at 4 ºC until used.

4. Screening of susceptible strains from *Saccharomyces cerevisiae* K1-V1116 HC1-HC20

Twelve heat cured clones (HC1, HC2, HC3, HC4, HC5, HC6, HC7, HC8, HC9, HC10, HC11 and HC12) of *S. ceresiviae* K1-V1116 HC were inoculated into 50 ml YEPG broth (pH 4.6) at 25 °C for 24 hr. The cultures of the unknown killing activity clones (UN) were used to prepare seedling plates by spread or pour plates on YEPG agar medium (duplicate). The killer strains, *S. bayanus* EC1118 and *S. cerevisiae* K1-V1116 were streaked onto the seedling plates, then incubated 25 °C for 48 hr. Observed the clear zone around killer colonies on the susceptible clones.

B. Must preparation for fermentation kinetic and killer activity study

Ruby Carbernet grape was destemmed and crushed. Must was warmed to 45 ^{0}C prior to betreated by 0.01 % pectinase enzyme (Pectinex Ultra SP-L) from NoVo Nordisk Ferment Ltd., Switzerland for 3 hr. Celite 0.3% w/v and 100 ppm SO_2 were applied to must and they were then filtered by whatman number 1 filter papers. Must was divided into 3 parts and was stroed at -20 ^{0}C. Must was sterilized by membrane filter 0.45 µ.

C. Fermentation kinetic

Each set of Ruby Carbernet must 1800 ml was sterilized by membrane filter 0.45 µm and, filled in each reactor. Fermentation was operated at 25 ^{0}C in reactor with 100 rpm agitation. Both *S. cerevisiae* K1-V1116 and *S. cerevisiae* K1-V1116 HC were conducted and sampled for analysis in the same periods. OD_{600} and biomass monitored growths of both strains. Biomass was calculated from OD_{600} and conversion factor (Appendix F). Chemical analysis, glucose was analyzed by Glucose-Lactate analyzer while ethanol, methanol, glycerol and fructose were analyzed by HPLC.

D. Killer activity determination

The 2 sets of fermented musts of *S. cerevisiae* K1-V1116 and *S. cerevisiae* K1-V1116 HC were centrifuged to separate cell. The aliquot of the 2 fermented must was sterilized by membrane filtration (0.45 µm pore size membrane) and divided into 5 test tubes with volume of

2, 4, 6, 8 and 10 ml. Each tube of *S. cerevisiae* K1-V1116 and *S. cerevisiae* K1-V1116 HC fermented must were filled with 50 µl of diluted *S. cerevisiae* RS cell suspension. The 100 µl of must after inoculated *S. cerevisiae* RS cell suspension at time 0 and 2 hr were sampled and counted viable cell by standard plate count with potato dextrose agar medium (triplicate). Number of viable cells of each treatment was count and the killer activities were calculated.

Chapter 3
Results and Discussions

A. Yeast strain preparation

S. cerevisiae K1-V1116 from commercial pack was purified and used for screening of susceptible strains and testing killer activity. It was found that *S. cerevisiae* K1-V1116 and *S. bayanus* EC-1118 were the only 2 strains that showed killer activity. *S. cerevisiae* RS showed non-killer characteristic on the 6 tested strains (Table 1). After *S. cerevisiae* K1-V1116 was heat cured from the killer activity, it was streaked on YEPG agar plate to isolate for single colonies.

Table 1 Codes and Killing activity of 8 yeast strains

Code	Strains	Killer activity
K1 V1116	*Saccharomyces cerevisiae* K1 V1116	Yes
Wine	*Saccharomyces cerevisiae* (wine)	No
67J	*Saccharomyces cerevisiae* 67J	No
7013	*Saccharomyces cerevisiae* 7013	No
7303	*Saccharomyces cerevisiae* 7303	No
EC 1118	*Saccharomyces bayanus* EC 1118	Yes
Baker	*Saccharomyces cerevisiae* (baker)	No
RS	*Saccharomyces cerevisiae* RS	No

The twelve clones of *S. cerevisiae* K1-V1116 HC1-HC12 were picked and stored in YEPG agar slants. The 12 heat cured *S. cerevisiae* K1-V1116 clones (HC1, HC2, HC3, HC4, HC5, HC6, HC7, HC8, HC9, HC10, HC11 and HC12) were tested for killer activity with *S. cerevisiae* RS which was used as susceptible strains. The *S. cerevisiae* K1-V1116 and *S. bayanus* EC1118 were used as reference killer strains. All tested strains could not produce clear zone on RS seedling plates. HC1, HC4, and HC12 were killed by killer strains (*S. cerevisiae* K1-V1116 and *S. bayanus* EC1118). Most of heat curing strains showed neutral character while HC1, HC4

and HC12 showed susceptible character (Table 2). These two characters suggested that the HC1, HC4 and HC12 lost M2 viruses while others heat curing strain have mutation on them. The lost of M2 viruses was the caused of no toxin and immune protein production. These resulted in the HC1, HC4 and HC12 could not kill RS strain and they were killed by killer strain. Other heat curing strains that showed neutral character could not kill RS strain and they were not killed by killer strain suggested that they could not produce toxin to kill RS strain. However the killer strains could not kill them suggested that M2 viruses were still remained in the cell and produced preprotoxin. The preprotoxin production in the cell was modified to immune protein but could not modify to mature toxin and secrete outside the cell (Martinac, 1990).

Table 2 Killer phenotype of *Saccharomyces cerevisiae* K1-V1116 heat curing strains

Heat curing strain	Phenotype	Character
HC1	K^-R^-	Susceptible
HC2	K^-R^+	Neutral
HC3	K^-R^+	Neutral
HC4	K^-R^-	Susceptible
HC5	K^-R^+	Neutral
HC6	K^-R^+	Neutral
HC7	K^-R^+	Neutral
HC8	K^-R^+	Neutral
HC9	K^-R^+	Neutral
HC10	K^-R^+	Neutral
HC11	K^-R^+	Neutral
HC12	K^-R^-	Susceptible

Conclusion

The curing method was used to produce a sensitive strain of *S. cerevisiae* K1-V1116 (HC1). The K1-V1116 and HC1 should have similar genetic background with different in only the M2 virus in K1-V1116 strain. The HC1 was changed to sensitive strain which was observed

by it could be killed by the K1-V1116 and EC-1118 killer strain, and it could not kill the RS susceptible strain. Therefore the K1-V1116 and HC1 were used to study of growth kinetic and killer activity in wine must.

B. Must preparation for fermentation kinetic and killer activity study

After destemming and cru shing, must was treated with 0.1% pectinase at 45 °C for 3 h., Then it was added with 0.3% cellite and filtered by filter paper no 1 and micro filtration (0.45μ m) respect ively. The must was divided into 3 parts and kept at -20 ^{0}C for triplicate experiments. It was found that character of must was not change in each step of preparation (Table 3).

Table 3 Must character in each step of preparation

Treatment	pH	Total soluble solid (^{0}Brix)
Crushing	3.48	21.0
Pectinase	3.48	20.8
Paper filtration	3.48	20.8
Sulfitation	3.48	20.8
Micro-filtration	3.48	20.8

Total soluble solids and pH were in the range of suitable must for fermentation. Must from crushing step give more total soluble solid than other steps. The total soluble solids and pH of each steps are similar except in crushing step. The breakage and lysis of tissue and cell should be due to higher total soluble solid in this step. The character of must before and after freezing at -20 °C were observed no different. The pectinase treatment of the Ruby carbernet grape gave 59.25% must.

Conclusion

The preparation of must by pectinase treatment gave good characteristic of must that is suitable for sterilization by membrane filtration and fermentation under the same condition of general red wine fermentation.

C. Fermentation kinetics

The *S. cerevisiae* K1-V1116 HC obtained from *S. cerevisiae* K1-V1116 that eliminated killer activity by heat curing technique, was used for comparing the growth kinetic with *S. cerevisiae* K1-V1116. Both yeast strains were used in the fermentation of Ruby cabernet grape must. It was found that growth patterns of both strains are similar. The lag and accelerated phase take 3 and 4 hours respectively. The exponential phase was in 10 – 32 hours of fermentation. The stationary phase lasted for between 32 – 72 hours, and followed by rapid cells died after 72 hours. Glucose and fructose were major sugar in must which were utilized by yeast as carbon sources. These sugars were used to characterize for substrates consumption. Ethanol and glycerol is the main product and by product of yeast in winemaking respectively. Each of them was analyzed and was used for comparison of specific product formation rates. *S. cerevisiae* K1-V1116 HC gave better growth kinetic in all parameters as shown in Table 4. *S. cerevisiae* K1-V1116 HC gave better specific growth rate, and production yield than *S. cerevisiae* K1-V1116 with 99% confidence. Glucose and fructose consumption rates of S. cerevisiae K1-V1116 HC were observed to be faster than *S. cerevisiae* K1-V1116.

Transportation of hexose monosaccharide across plasma membrane of *S. cerevisiae* can be carried out by two systems, high affinity (energy dependent or active transport) and low affinity (free mechanism or passive transport) (Walker 1998). These were regulated by availability of extracellular sugars. Glucose and fructose are major carbon sources in must, therefore the translocation across plasma membrane are responsible for ethanol production.

The glucose transport in *S. cerevisiae* can be referred to the high-affinity system which is absent in cell growing in the high level of glucose (2% w/v). Under these conditions low-affinity-system are operable which are constitutive and independent of phosphorylation (Walker 1998). In this experiment glucose concentration was in excess approximately 8-9 % w/v, hence, it was possible that some glucose transport should be only by low-affinity system in both strains. This hypothesis is supported by Fuhrmann and volker 1992 Gamo *et al.* 1995. Specific glucose consumption rates of them both strains might be similar os slightly faster in *S. cerevisiae* K1-V1116 HC because they transport glucose by the same system, or *S. cerevisiae* K1-V1116 HC does not have parasite in its cell, therefore, they consume glucose faster and grow better. This

was confirmed by the results of this work as evidence in higher specific glucose consumption rate.

S. cerevisiae fructose transport is facilitated by diffusion rather than by active transport. There are at least 20 known hexose transporters, they are responsible for other hexose including fructose. By this transport system, specific fructose consumption rate should be lower than glucose consumption rate. The result showed the same trend of glucose and fructose consumption rate in both strains. Their specific fructose consumption rates of both strains were slower than the specific glucose consumption rate because the frequency of fructose transporters is lower than glucose transporters (Walker 1998).

In alcoholic fermentation of winemaking, yeast generated energy by anaerobic pathway only because *S. cerevisiae* is 'Crabtree-positive' yeast. Yeast actively ferments glucose under aerobic condition and high concentration of glucose (Van Urk *et al.* 1989) because it exhibits glucose catabolite repression and proteolytic inactivation of the high-affinity glucose transporter (Does and Bisson 1989). Glucose sensing may itself be regulated by repression and inactivation mechanisms (Thevelein 1991; Lagunsu 1993). In this way, the high-affinity glucose carrier is repressed or switched off during growth in high concentration of glucose (0.18 g/L). Although, there was dissolved oxygen in must approximately 3-4 hours at the beginning in this experiment, all of energy (ATP) was produced from anaerobic pathway. Yeast can generated only 2 ATP from 1 molecule of glucose, which was very low when, compared to the respiration pathway. These should be reason of slower growing and small number of cell cycle ($\cong$ 4). The result showed smaller specific growth rate of in *S. cerevisiae* K1-V1116 (killer) when compared with the *S. cerevisiae* K1-V1116 HC (non-killer) as shown in Table 4.1. These could be postulated that *S. cerevisiae* K1-V1116 and in *S. cerevisiae* K1-V1116 HC have some difference in energy consumption that reflected in their growth rates.

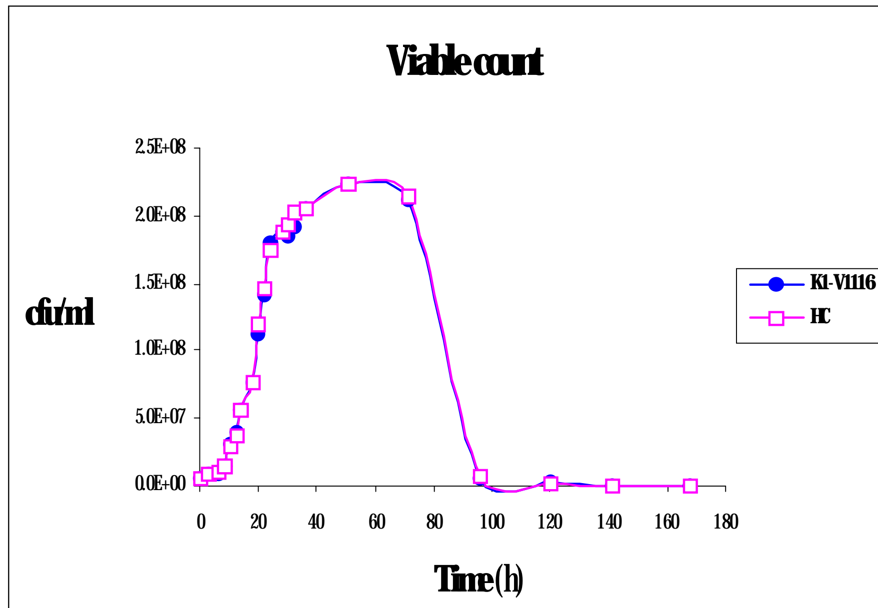

Figure 2 Growth curve (viable cell VS time) of K1-V1116 and HC in first trial

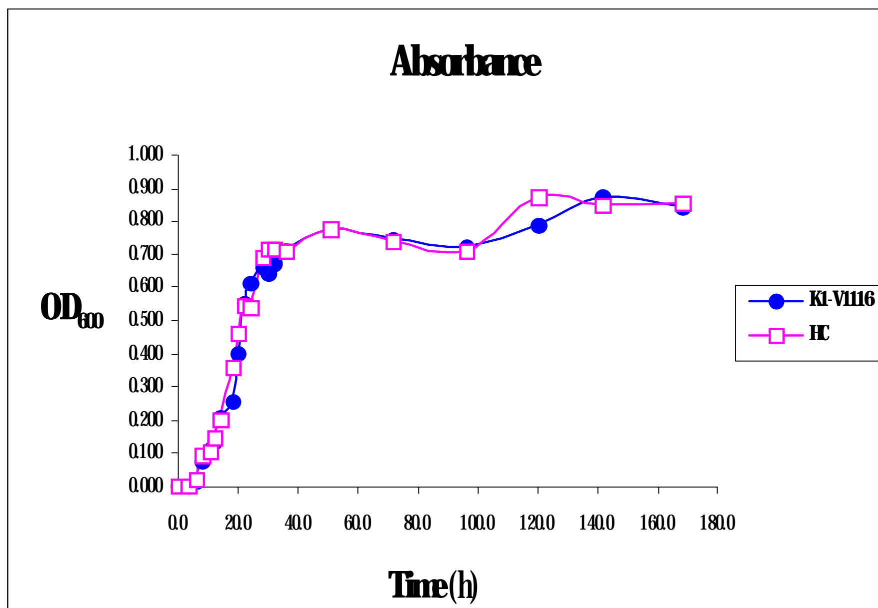

Figure 3 Growth curve (optical density VS time) of K1-V1116 and HC in first trial

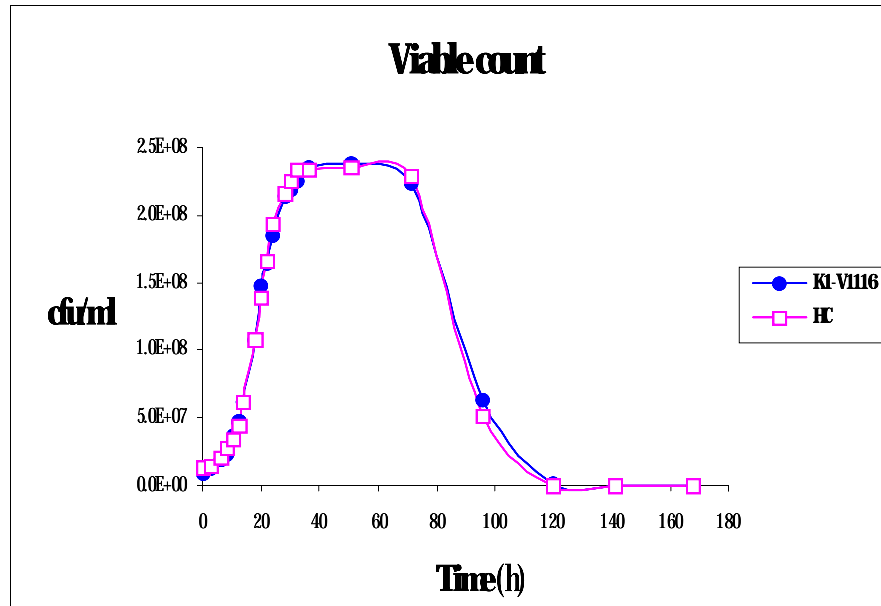

Figure 4 Growth curve (viable cell VS time) of K1-V1116 and HC in second trial

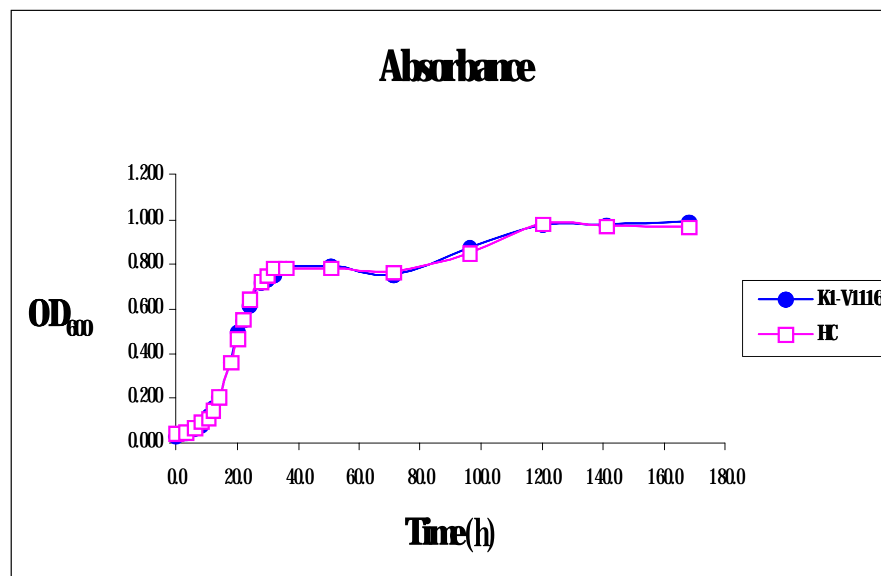

Figure 5 Growth curve (optical density VS time) of K1-V1116 and HC in second trial

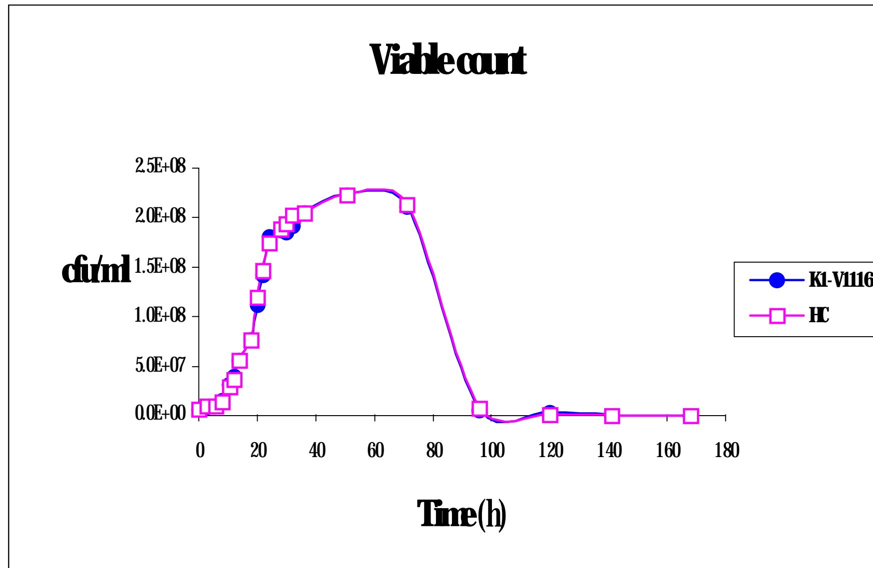

Figure 6 Growth curve (viable cell VS time) of K1-V1116 and HC in third trial

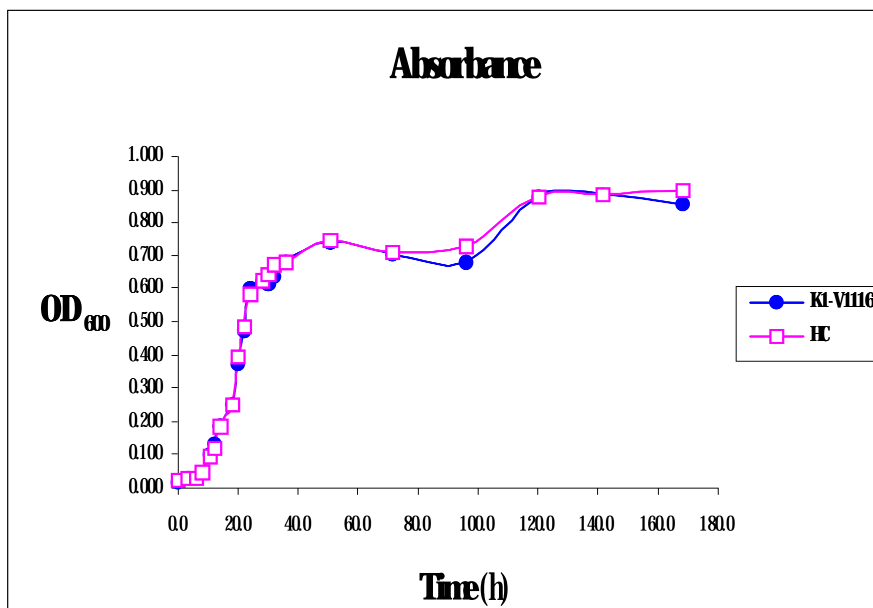

Figure 7 Growth curve (optical density VS time) of K1-V1116 and HC in third trial

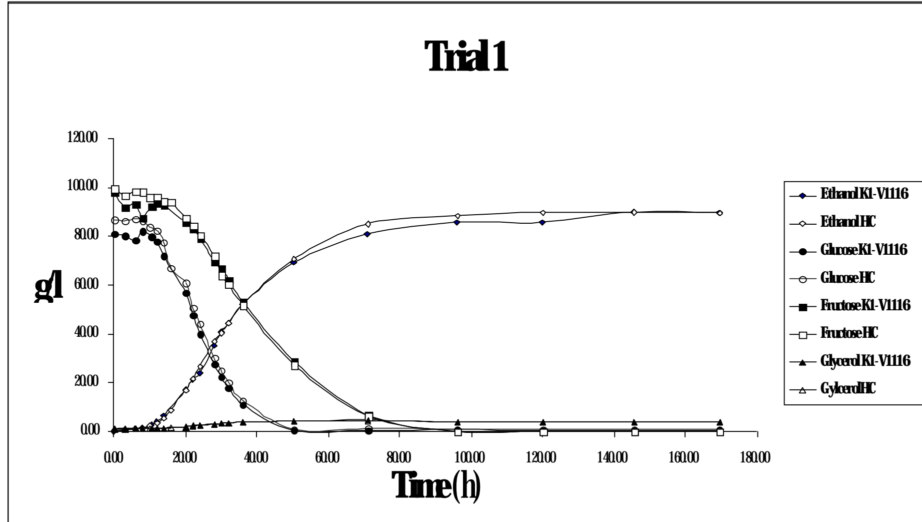

Figure 8 The change in chemical composition of must during fermented in first trail

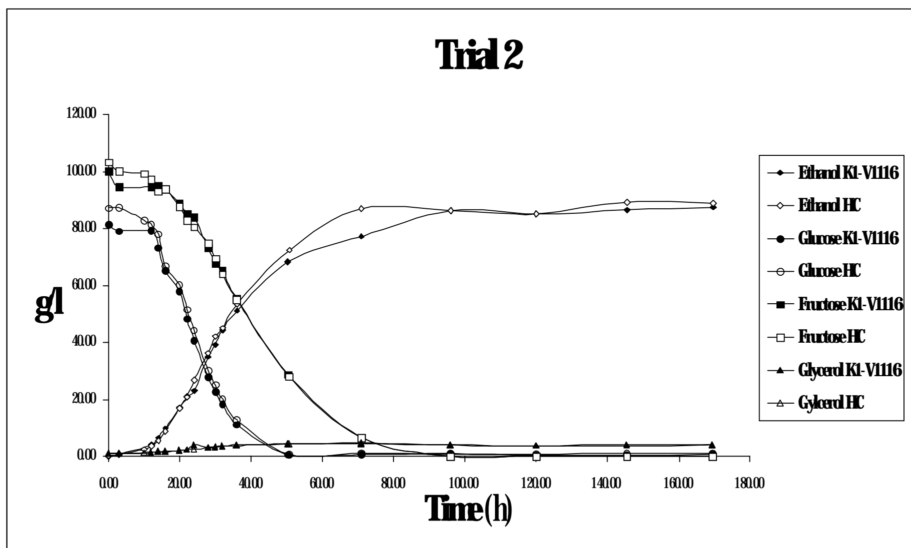

Figure 9 The change in chemical composition of must during fermented in second trai

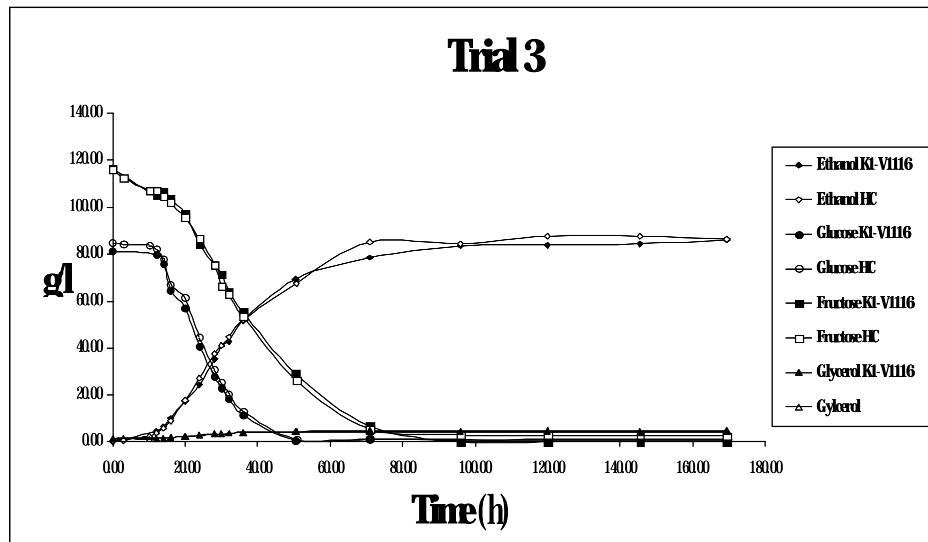

Figure 10 The change in chemical composition of must during fermented in third trail

S. cerevisiae K1-V1116, K2 killer yeast is infected by L and M2 VLP. These viruses consume energy and others material from hosts for their reproduction, especially M2 consume a lot of energy to produce 362 amino acid preprotoxin (Magliani, *et al.*, 1997). This protein is constitutive express in log phase of yeast growth with different processing to perform mature toxin and immune protein to kill susceptible yeast and protect them from their own toxin (Palfree and Bussey, 1979). Mature toxin is released to environment for killing other yeast while immune protein is in cytoplasm to induce immunity (Heslot and Gaillardin, 1992). The production of preportoxin the cell must spend at least 362 ATP/molecule. From this evident, high amount energy were used in producing and processing of preprotoxin. Bussey reported in 1972 that uptake or metabolism of the sugar is necessary for the increase killing ability. Not only in preprotoxin production but energy also used in viral propagation.

Nowadays, the mechanism of immunity is still unclear in all *S. cerevisiae* killer yeast especially K2 killer type but there are two possible mechanisms have been advocated for K1 killer type. Firstly, alter or masking of the KRE3 receptor by the immunity determinant, masking the receptor unavailable to the toxin (Boone, *et al.*, 1986). Secondly, remove of KRE3 receptor from the plasma membrane by the immune determinant (Heslot and Gaillardin, 1992). In 1999

Table 4 Growth kinetic parameter of *S. cerevisiae* K1-V1116 and *S. cerevisiae* K1-V1116 HC

Parameter	K1-V1116		HC	
	Average	Sd	Average	Sd
Maximum specific growth rate (h^{-1})	0.135	0.002	0.146	0.001
Doubling time (h)	5.117	0.066	4.755	0.016
Specific rate of ethanol production ($g_{Eth}g_{biomass}^{-1}h^{-1}$)	0.383	0.006	0.400	0.026
Specific rate of glycerol production ($g_{Gly}g_{biomass}^{-1}h^{-1}$)	0.020	0.000	0.020	0.000
Specific rate of glucose consumption ($g_{Glc}g_{biomass}^{-1}h^{-1}$)	0.563	0.015	0.590	0.046
Specific rate of fructose consumption ($g_{Frc}g_{biomass}^{-1}h^{-1}$)	0.390	0.086	0.410	0.072
Observe yield of ethanol from sugar ($g_{Eth}g^{-1}_{sugar}$)	0.458	0.009	0.466	0.007

Aamir Ahmed and his colleague reported that TOK1 potassium channels are the molecular targets for K1 viral killer toxin and kill target cell by inducing cell loss of potassium ion. Since potassium channels play an important role of life, thus killed cell cannot survive if immunity occurs by removing of receptor from the plasma membrane so the immunity mechanism should be the masking of toxin receptor of killer cell. However, up to the present, there has no publications to prove the molecular target for the immune proteins of the killer cell. This report revealed the relationship of killing and immunity of K2 killer yeast. Firstly, specific growth rate of killer was lower than non-killer strain this was possible that the binding of immune protein to receptor was energy dependent as the binding of toxin. A molecule of toxin uses one ATP for binding. Some $6x10^3$-$2.8x10^4$ molecules of toxin are needed to kill a cell of the sensitive strain. If binding of immune protein to the same receptor site of toxin to protect cell, it would pay approximately $1.7x10^3$ molecules of glucose in anaerobic pathway for binding to a receptor. Not only for binding of immune proteins to receptors but also for production of theire cell consume a lot of energy. These might be the reason for slower growth rate of killer as compared to the non-killer strain of the similar genetic background that was shown in this experiment. Secondly, slower glucose consumption rate but same fructose consumption rate of killer strain might propose two evidences. 1) Secretion of K2 killer toxin bound to other membrane from outside the

cell by the same mechanism of killer activity at different receptors. Those receptors may be hexose transporter especially glucose related transporters. Binding of toxin induces changing in conformation and properties. 2) It is possible that immune protein is non specific binding to others membrane protein from inside the cell. The immune proteins contain α domain that is highly hydrophobic region and move freely, so it is highly incident to insert the membrane and interact with other membrane proteins. If they are hexose transporters the binding may effect their structures and functions.

Jenny and her colleagues (1991) had compared the growth kinetic of killer and its heat curing strain in Rhine Riesling grape must. They observed from growth curve and reported that there were no significant different in the growth rates between them which was contrary to this report. It was possible for the difference that the fermentation was conducted at 18 °C, and they considered only from growth curve. They did not analyze killer toxin in must their finding could not be proved that killer gave the same growth rate as their heat curing strain while killer strain grew with toxin producing gene.

The experiment of Silva in 1996 showed that the killer activity of yeast depended on the incubation temperature. Some killer yeast did not show killer activity at 18 °C and 28 °C. Their experiment also showed that the killer strains had smaller maximum specific growth rate than sensitive strain YEPD-MB medium when incubated at 18 °C with aeration. The result could support this result which was found that non-killer (heat-curing) strains had higher maximum specific growth rate than killer strain.

Conclusion

Heat curing of viruses from commercial killer strain, *Saccharomyces cerevisiae* K1-V1116 gave better growth kinetic in Ruby carbernet grape must fermentation when compared with the similar genetic background original strain. The specific growth rate, ethanol production rate, and yield of ethanol from reducing sugar of heat curing strain was obviously better than original killer strain. The rate of glucose consumption of heat curing strain was faster while there was no different observed in fructose consumption rate.

D. Killer activity determination

Killer toxin production of K1-V1116 was monitored from its killing activity. The killer activity of K1-V1116 in Ruby carbernet showed the same pattern as described by Shimizu (unpublished data). The killer activity was first observed on the first day of fermentation, then reached maximum on day 3, and decreased rapidly after day 4 and 5 of fermentation (Figure9). The killer activity was calculated from the multiplicity (m), obtained from the relationship $S/S_0 = e^{-m}$, where S/S_0 is the survival ratio (S_0 is the original number of viable yeast cell and S is the number of viable cells after killer toxin treatment). The term multiplicity is used in analogy with the practice in bacteriophage studies, to express the ratio between the number of LU and the number of cells

Table 5 Killer activity of K1-V1116 in must

Time	Killer activity (cell/ml)		
(day)	**Ruby carbernet**	**Black pop**	**White malaga**
1	48	5	6
2	49	87	105
3	230	305	350
4	46	112	114
5	37	30	102

in the sample (Kurzwielova and Sigler, 1995). Multiplicity 1 means the corresponding volume of killer toxin (must) can just kill all cells present (refer to the curve in appendix X, where m = ln (S_0/S) is plotted against toxin volume). For determination of the number of lethal units in a toxin preparation, cell killing is performed with the volume of toxin to such an extent that no free toxin remain in the suspension after the killing (i.e. the observed extent of killing is perceptibly below 100%). The toxin preparation can then be assumed to have killed a number of sensitive cells, which is proportional to the number of lethal units it contained. The maximum killer activity on day 3 is 230 cell/ml of must, which was lower than described by Graham in 1992. Generally, the population of contamination yeast (*S. cerevisiae*) in clarified was less than $5x10^2$ cell/ml (Shimizu

unpublished data). From the results suggested that killer activity of K1-V1116 in Ruby Carbernet must might not protect must from normal contamination of wild yeasts. Tyurina and colleagues reported in 1980 that a commercial K2 killer could be used to control the growth of undesirable wild yeast during fermentation and protect the fermented wine against the growth of spoilage yeast, these data apply only to the production of white wines.

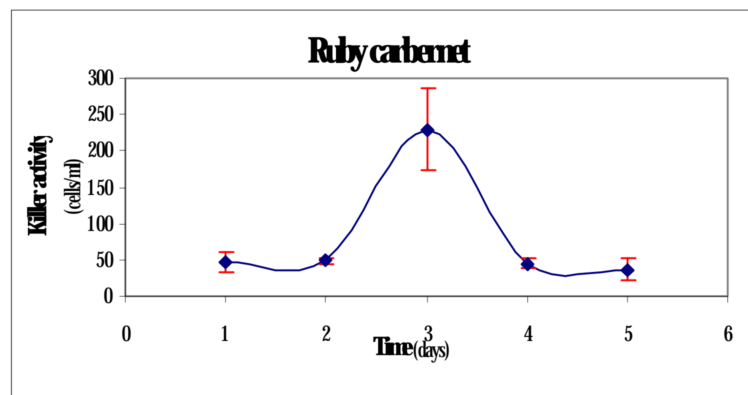

Figure 11 Killer activity of K1-V1116 in Ruby carbernet must

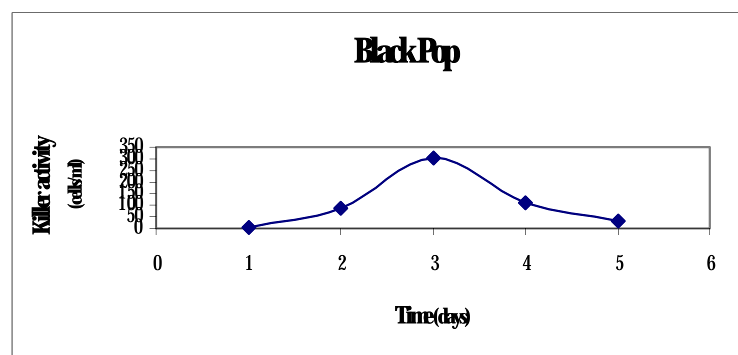

Figure 12 Killer activity of K1-V1116 in Black pop must

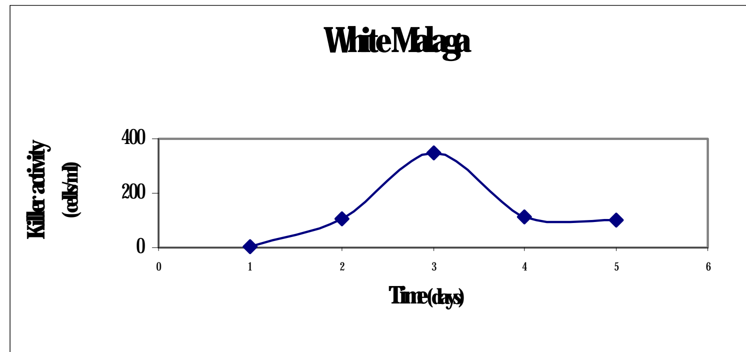

Figure13 Killer activity of K1-V1116 in White malaga must

In red wine fermentation, K2 killer toxin activity disappeared within three days after inoculation of the yeast into the musts and, presumably, this phenomenon might be related to complexing of the toxin protein with tannins. This was proved by these experiments using Black pop (high tannin) and White malaga (low tanin) for fermenting and analyzing killer activity by our method. The results showed higher killer activity was than found White Malaga and Black Pop e(Table5 and Fig 9-11). The killer activity pattern in Black pop and Ruby carbemet must were similar to other red must as reported above. White Malaga must give the higher and longer killer activity as showed in Figure11. This result was similar to the previous reported (Tyurina, *et al*, 1980). The results obtained from this experiment supported the hypothesis that tannin can get rid of K2 killer activity from must that was earlier proposed by Graham in 1992.

Conclusion

Killer activity of K1-V1116, the K2 killer yeast, occurred in Ruby cabemet. The killer activity could directly be determined from must by CFA technique. The killer activity was found in the maximum activity, which was 230 cell/ml, on the third day of fermentation, and decreased rapidly in day four and day five.

Summary

The heat curing method was used to produce a sensitive strain of *S. cerevisiae* K1-V1116 (HC). The K1-V1116 and HC had the same genetic background with different only in the M2 virus in K1-V1116 strain. This HC was changed to sensitive strain which was proved by it could be killed by K1-V1116 and EC-1118 killer strain, and could not be killed by RS susceptible strain. K1-V1116 and HC were then used for the study of growth kinetic and killer activity in wine must.

The preparation of must by pectinase gave good characteristic that suitable for sterilization by membrane filtration and fermentation with the same conditions of general red wine.

Heat curing of viruses from commercial killer strain, *Saccharomyces cerevisiae* K1-V1116 gave better growth kinetics in Ruby Carbernet grape must fermentation comparing to the same genetic background of original strain. The specific growth rate, ethanol production rate, and yield of ethanol from reducing sugar of heat curing strain were better than original killer strain. The rate of glucose consumption of heat curing strain was faster while the fructose consumption rate was not different.

Killer activity of K1-V1116, the K2 killer yeast, occurred in Ruby cabernet. The killer activity could directly be determined from must by CFA technique. The killer activity was found that is the maximum activity, which is equal to 230 cell/ml on third day of fermentation, and decreased rapidly after day four and five, respectively.

Suggestion

Our results showed the effect of M1dsRNA virus in growth of killer yeast. Cloning of the killer toxin gene into yeast genome might be an alternative expression system to avoid the effect of virus.

Killer activity may be effected by tannin or other unknown components in the must. The studying this might give some information to improve the killer activity. The engineering of killer toxin may be another technique for the improvement of the killer activity.

The result in lower sugars transportation rate of killer yeast hint that the effect of killer toxin might be on hexose transporter or other membrane proteins. This suggested that K2 killer toxin an interesting protein to further the study. The study of its mechanism may give valueable information in protein-protein interaction.

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Appendix A
Growth data

Table A.1 Growth data of first trial

Time (h)	K1-V1116			HC		
	Abs	cfu/ml	Biomass(g/l)	Abs	cfu/ml	Biomass(g/l)
0.0	0.005	1.5E+06	0.11	0.005	1.5E+06	0.11
3.0	0.008	2.4E+06	0.17	0.007	2.1E+06	0.15
6.0	0.018	5.4E+06	0.38	0.022	6.6E+06	0.46
8.0	0.078	2.3E+07	1.64	0.094	2.8E+07	1.98
10.5	0.117	3.5E+07	2.47	0.111	3.3E+07	2.34
12.0	0.140	4.2E+07	2.95	0.150	4.5E+07	3.16
14.0	0.211	6.3E+07	4.45	0.207	6.2E+07	4.37
18.0	0.260	7.8E+07	5.48	0.360	1.1E+08	7.59
20.0	0.403	1.2E+08	8.50	0.465	1.4E+08	9.81
22.0	0.555	1.7E+08	11.70	0.551	1.7E+08	11.62
24.0	0.616	1.8E+08	12.99	0.545	1.6E+08	11.49
28.0	0.663	2.0E+08	13.98	0.694	2.1E+08	14.63
30.0	0.647	1.9E+08	13.64	0.715	2.1E+08	15.08
32.0	0.676	2.0E+08	14.25	0.717	2.2E+08	15.12
36.0	0.709	2.1E+08	14.95	0.708	2.1E+08	14.93
50.7	0.779	2.3E+08	16.43	0.779	2.3E+08	16.43
71.0	0.747	2.2E+08	15.75	0.743	2.2E+08	15.67
96.0	0.725	6.4E+07	15.29	0.713	5.3E+07	15.04
120.0	0.789	8.0E+05	16.64	0.871	5.0E+05	18.37
141.0	0.876	5.0E+05	18.47	0.848	2.0E+05	17.88
168.0	0.842	4.6E+05	17.76	0.853	1.9E+05	17.99

Table A.2 Growth data of second trial

Time	K1-V1116			HC		
(h)	Abs	cfu/ml	Biomass(g/l)	Abs	cfu/ml	Biomass(g/l)
0.0	0.031	9.3E+06	0.65	0.042	1.3E+07	0.89
3.0	0.042	1.3E+07	0.89	0.049	1.5E+07	1.03
6.0	0.064	1.9E+07	1.35	0.068	2.0E+07	1.43
8.0	0.078	2.3E+07	1.64	0.094	2.8E+07	1.98
10.5	0.123	3.7E+07	2.59	0.112	3.4E+07	2.36
12.0	0.160	4.8E+07	3.37	0.150	4.5E+07	3.16
14.0	0.205	6.2E+07	4.32	0.207	6.2E+07	4.37
18.0	0.360	1.1E+08	7.59	0.360	1.1E+08	7.59
20.0	0.495	1.5E+08	10.44	0.465	1.4E+08	9.81
22.0	0.547	1.6E+08	11.53	0.551	1.7E+08	11.62
24.0	0.616	1.8E+08	12.99	0.645	1.9E+08	13.60
28.0	0.713	2.1E+08	15.04	0.718	2.2E+08	15.14
30.0	0.732	2.2E+08	15.44	0.749	2.2E+08	15.79
32.0	0.750	2.3E+08	15.82	0.781	2.3E+08	16.47
36.0	0.782	2.3E+08	16.49	0.781	2.3E+08	16.47
50.7	0.792	2.4E+08	16.70	0.782	2.3E+08	16.49
71.0	0.747	2.2E+08	15.75	0.765	2.3E+08	16.13
96.0	0.872	6.4E+07	18.39	0.849	5.3E+07	17.90
120.0	0.974	8.0E+05	20.54	0.980	5.0E+05	20.67
141.0	0.974	5.0E+05	20.54	0.968	2.0E+05	20.41
168.0	0.991	4.6E+05	20.90	0.966	1.9E+05	20.37

Table A.3 Growth data of third trial

Time	K1-V1116			HC		
(h)	Abs	cfu/ml	Biomass(g/l)	Abs	cfu/ml	Biomass(g/l)
0.0	0.021	6.3E+06	0.44	0.022	6.6E+06	0.46
3.0	0.030	9.0E+06	0.63	0.032	9.6E+06	0.67
6.0	0.031	9.3E+06	0.65	0.033	9.9E+06	0.70
8.0	0.049	1.5E+07	1.03	0.048	1.4E+07	1.01
10.5	0.105	3.2E+07	2.21	0.097	2.9E+07	2.05
12.0	0.132	4.0E+07	2.78	0.121	3.6E+07	2.55
14.0	0.185	5.6E+07	3.90	0.185	5.6E+07	3.90
18.0	0.255	7.7E+07	5.38	0.255	7.7E+07	5.38
20.0	0.375	1.1E+08	7.91	0.397	1.2E+08	8.37
22.0	0.474	1.4E+08	10.00	0.487	1.5E+08	10.27
24.0	0.603	1.8E+08	12.72	0.582	1.7E+08	12.27
28.0	0.624	1.9E+08	13.16	0.628	1.9E+08	13.24
30.0	0.617	1.9E+08	13.01	0.646	1.9E+08	13.62
32.0	0.640	1.9E+08	13.50	0.674	2.0E+08	14.21
36.0	0.683	2.0E+08	14.40	0.683	2.0E+08	14.40
50.7	0.743	2.2E+08	15.67	0.745	2.2E+08	15.71
71.0	0.704	2.1E+08	14.85	0.713	2.1E+08	15.04
96.0	0.679	5.5E+06	14.32	0.727	7.5E+06	15.33
120.0	0.878	3.1E+06	18.51	0.878	8.4E+05	18.51
141.0	0.888	4.4E+05	18.73	0.888	1.4E+05	18.73
168.0	0.855	1.8E+05	18.03	0.897	8.7E+04	18.92

Appendix B

Chemical Analysis Data

Table B.1 Chemical compositions of must during fermentation by K1-V1116 in first trial

Time(h)	Ethanol (g/l)	Methanol (g/l)	Glucose (g/l)	Fructose (g/l)	Glycerol (g/l)
0.00	0.00	0.00	80.83	98.19	1.03
3.00	0.64	0.00	80.38	92.15	1.27
6.00	0.92	0.00	78.25	93.23	1.32
8.00	1.34	0.00	82.14	87.68	1.06
10.50	2.69	0.00	79.94	92.19	1.17
12.00	4.14	0.00	78.03	93.63	1.29
14.00	6.44	0.00	72.13	92.83	1.42
20.00	17.23	0.00	56.74	85.81	1.98
22.00	21.45	0.00	47.74	83.20	2.33
24.00	23.95	0.00	39.99	79.40	2.49
28.00	35.11	0.00	27.57	69.81	3.02
30.00	40.79	0.00	22.30	66.91	3.33
32.00	44.35	0.00	17.90	62.16	3.48
36.00	52.00	0.00	10.94	53.37	3.81
50.40	69.25	0.00	0.39	28.92	4.29
71.00	80.88	0.00	0.43	6.69	4.54
96.00	85.71	0.00	0.74	0.00	3.85
120.00	85.67	0.00	0.31	0.00	3.89
145.50	90.04	0.00	0.46	0.00	3.95
169.50	89.62	0.00	0.66	0.00	3.94

Table B.2 Chemical compositions of must during fermentation by HC in first trial

Time (h)	Ethanol (g/l)	Methanol (g/l)	Glucose (g/l)	Fructose (g/l)	Glycerol (g/l)
0.00	0.24	0.24	86.86	99.59	1.10
3.00	0.58	0.00	86.30	96.72	1.05
6.00	0.97	0.00	87.21	98.58	1.17
8.00	1.32	0.00	86.40	98.19	1.18
10.00	2.34	0.00	83.83	96.14	1.21
12.00	3.50	0.00	82.35	96.26	1.33
14.00	5.58	0.00	77.70	94.30	1.40
16.00	8.80	0.00	67.07	93.94	1.61
20.00	17.01	0.00	61.02	87.70	1.99
22.00	21.75	0.00	50.78	84.53	2.43
24.00	26.76	0.00	44.11	80.55	2.59
28.00	36.97	0.00	30.18	72.05	3.17
30.00	40.54	0.00	24.84	64.16	3.29
32.00	44.54	0.00	20.15	60.34	3.52
36.00	51.80	0.00	12.67	51.87	3.82
50.40	70.72	0.00	0.73	27.44	4.26
71.00	85.11	0.00	1.19	6.43	4.43
96.00	88.32	0.00	0.97	0.00	3.93
120.00	89.72	0.00	0.94	0.00	3.95
145.50	89.97	0.00	0.75	0.00	4.00
169.50	89.59	0.00	0.80	0.00	3.98

Table B.3 Chemical compositions of must during fermentation by K1-V1116 in second trial

Time(h)	Ethanol (g/l	Methanol (g/l)	Glucose (g/l)	Fructose (g/l)	Glycerol (g/l)
0.00	0.00	0.00	81.62	100.09	1.08
3.00	0.66	0.00	79.21	94.53	1.09
12.00	4.13	0.00	79.22	94.78	1.33
14.00	6.45	0.00	73.10	95.10	1.47
16.00	9.76	0.00	65.44	93.87	1.65
20.00	17.13	0.00	57.93	88.84	2.05
22.00	20.96	0.00	48.23	85.10	2.32
24.00	22.92	0.00	40.68	83.90	3.99
28.00	34.96	0.00	27.77	73.33	3.07
30.00	39.18	0.00	22.61	67.85	3.26
32.00	44.33	0.00	18.36	65.28	3.59
36.00	51.13	0.00	11.23	55.24	3.81
50.40	68.36	0.00	0.55	28.88	4.32
71.00	77.26	0.00	0.66	6.68	4.36
96.00	86.32	0.00	0.99	0.00	3.87
120.00	85.09	0.00	0.37	0.00	3.85
145.50	86.50	0.00	0.58	0.00	3.84
169.50	87.51	0.00	0.66	0.00	3.88

Table B.4 Chemical compositions of must during fermentation by HC in second trial

Time(h)	Ethanol (g/l)	Methanol (g/l)	Glucose (g/l)	Fructose (g/l)	Glycerol (g/l)
0.00	0.26	0.00	87.26	103.42	1.11
3.00	0.61	0.00	87.39	100.06	1.14
10.00	2.38	0.00	82.99	99.20	1.25
12.00	3.57	0.00	81.42	97.25	1.35
14.00	5.53	0.00	77.97	93.13	1.63
16.00	8.80	0.00	66.75	93.94	1.61
20.00	17.01	0.00	60.05	87.70	1.99
22.00	20.81	0.00	51.44	82.91	2.32
24.00	26.76	0.00	44.12	80.55	2.59
28.00	36.29	0.00	30.00	74.80	3.17
30.00	42.12	0.00	25.12	69.61	3.44
32.00	45.06	0.00	20.03	63.97	3.60
36.00	53.42	0.00	12.70	55.11	3.99
50.70	72.46	0.00	0.77	28.04	4.37
71.00	87.11	0.00	1.23	6.58	4.60
96.00	86.32	0.00	0.92	0.00	3.87
120.00	85.09	0.00	0.81	0.00	3.85
145.50	89.33	0.00	1.05	0.00	4.04
169.50	88.87	0.00	1.10	0.00	4.01

Table B.5 Chemical compositions of must during fermentation by K1-V1116 in third trial

Time(h)	Ethanol (g/l)	Methanol (g/l)	Glucose (g/l)	Fructose (g/l)	Glycerol (g/l)
0.00	0.00	0.00	81.33	116.28	1.16
12.00	4.30	0.00	80.11	105.07	1.52
14.00	6.47	0.00	75.83	106.48	1.47
16.00	9.99	0.00	64.50	103.45	1.71
20.00	17.38	0.00	57.16	97.25	2.15
24.00	24.28	0.00	40.92	84.38	2.56
28.00	35.23	0.00	27.77	75.15	3.08
30.00	40.86	0.00	23.04	71.28	3.40
32.00	42.78	0.00	18.41	64.01	3.50
36.00	51.73	0.00	11.34	55.41	3.86
50.40	69.20	0.00	0.59	29.13	4.32
71.00	78.69	0.00	1.01	6.74	4.45
96.00	83.50	0.00	1.05	0.00	4.43
120.00	83.89	0.73	0.46	0.00	4.53
145.50	84.43	0.68	0.76	0.00	4.40
169.50	86.15	0.64	0.72	0.00	4.48

Table B.6 Chemical compositions of must during fermentation by HC in third trial

Time(h)	Ethanol (g/l)	Methanol (g/l)	Glucose (g/l)	Fructose (g/l)	Glycerol (g/l)
0.00	0.21	0.00	84.84	115.96	1.15
3.00	0.66	0.00	84.11	112.34	1.42
10.00	2.25	0.00	83.82	107.28	1.12
12.00	3.64	0.00	82.17	106.97	1.46
14.00	5.86	0.00	78.03	104.54	1.44
16.00	8.84	0.00	67.01	101.81	1.61
20.00	17.66	0.00	61.16	95.37	2.10
24.00	27.27	0.00	44.61	86.66	2.64
28.00	37.58	0.00	30.78	75.52	3.26
30.00	41.08	0.00	25.07	66.34	3.34
32.00	44.60	0.00	20.24	62.60	3.53
36.00	51.89	0.00	12.78	53.26	3.86
50.70	67.62	0.00	0.75	26.22	4.07
71.00	85.31	0.00	1.21	5.43	4.47
96.00	84.40	0.00	0.92	2.88	4.40
120.00	87.76	0.00	1.02	2.81	4.28
145.50	87.83	0.00	1.02	2.71	4.03
169.50	86.40	0.00	1.10	2.40	4.44

Appendix C

Killer Activity

The calculation of killer activity

Killer activity of must was calculated from the multiplicity (m), obtained from the relationship $S/S_0 = e^{-m}$ when S/S_0 is the survival ratio (S_0 is the original number of viable cells and S is the number of viable cells after the killer toxin treatment). The multiplicity is used to express the ratio between the number of lethal unit (LU) and the number of the cell in the sample. Multiplicity 1 mean that the corresponding volume (V_{m1}) of the must that killed all cells presened. The plotted of $\ln(S/S_0)$ against must volume gave the linear relation when the concentration of toxin is low. The slope from the plot (trend line) was used to calculate the volume (V_{m1}) of the must at multiplicity ($\ln(S/S_0) = 1$). The LU was calculated from total cells divided by volume (V_{m1}).

Table C.6 Killer activity day 5 batch 1

2 ml	1	2	3	Avg	SD	Cell / ml	Total cell
So	120	150	168	146	24		
S	154	162	207	174	29	1460	2920
4 ml	1	2	3	Avg	SD	Cell / ml	Total cell
So	90	95	107	97	9		
S	68	80	95	81	14	973	3893
6 ml	1	2	3	Avg	SD	Cell / ml	Total cell
So	60	72	90	74	15		
S	62	71	72	68	6	740	4440
8 ml	1	2	3	Avg	SD	Cell / ml	Total cell
So	50	55	56	54	3		
S	43	45	49	46	3	537	4293
10 ml	1	2	3	Avg	SD	Cell / ml	Total cell
So	40	43	43	42	2		
S	36	37	39	37	2	420	4200

Volume(ml)	ln (So)	ln(S)	ln(So/S)
2	4.98	5.16	-0.18
4	4.58	4.39	0.18
6	4.30	4.22	0.08
8	3.98	3.82	0.16
10	3.74	3.62	0.12

Killer Activity		
ln(So/S)	m* (ml)	Lethal Unit(cell / ml)
1	66.23	59.63

m= 1/slope of trend line

Killing Activity = Avg total cell / m

* : multiplicity

Average Total Cell
3949

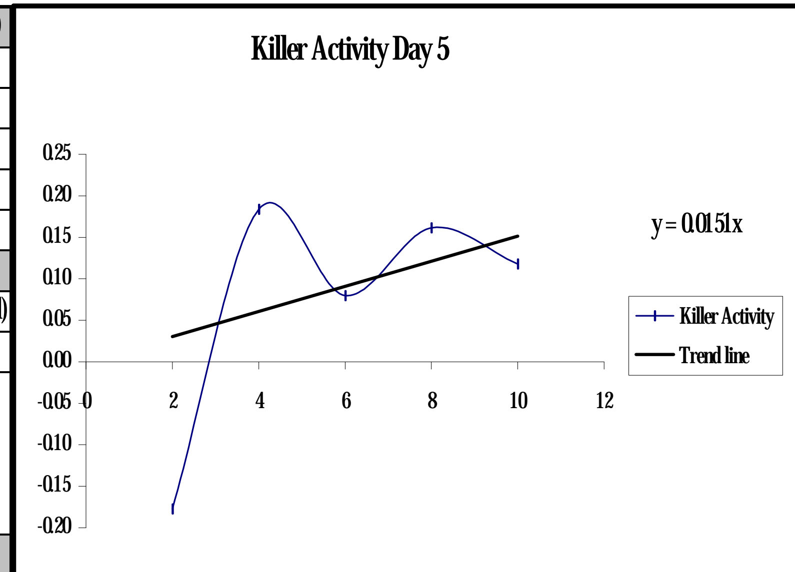


Figure C.6 Killer activity day 5 batch 1

Table C.9 Killer activity day 4 of second trial

2ml	1	2	3	Avg	SD	Cell / ml	Total cell	Volume(ml)	ln (So)	ln(S)	ln(So/S)
So	121	127	125	124	3			2	4.82	4.77	0.05
S	113	121	121	118	5	1243	2487	4	4.25	4.17	0.07
4ml	1	2	3	Avg	SD	Cell / ml	Total cell	6	3.96	3.84	0.13
So	67	69	74	70	4			8	3.84	3.67	0.16
S	64	64	67	65	2	700	2800	10	3.34	3.21	0.14
6ml	1	2	3	Avg	SD	Cell / ml	Total cell	Killer Activity			
So	57	52	49	53	4			ln(So/S)	m* (ml)	Lethal Unit(cell / ml)	
S	46	48	45	46	2	527	3160	1	57.14	52.45	
8ml	1	2	3	Avg	SD	Cell / ml	Total cell	m = 1/slope of trend line			
So	43	47	49	46	3			Killing Activity = Avg total cell / m			
S	35	35	48	39	8	463	3707				
10ml	1	2	3	Avg	SD	Cell / ml	Total cell	* : multiplicity			
So	32	31	22	28	6			Average Total Cell			
S	24	24	26	25	1	283	2833	2997			

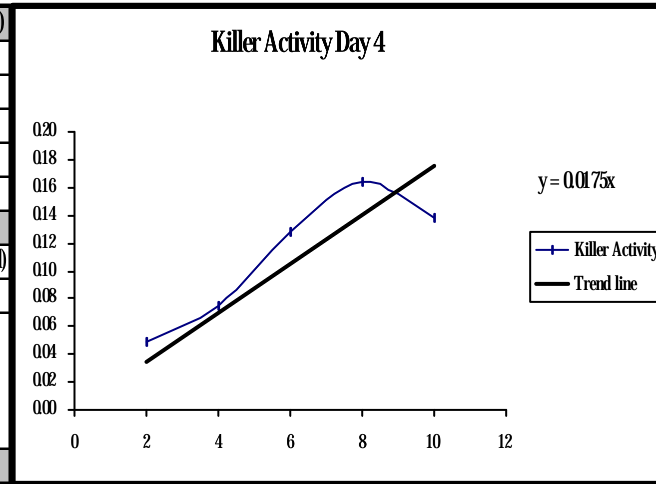


Figure C.9 Killer activity day 4 of second trial

Table C.10 Killer activity day 5 of second trial

2ml	1	2	3	Avg	SD	Cell / ml	Total cell	Volume(ml)	ln (So)	ln(S)	ln(So/S)
So	151	145	158	151	7			2	5.02	5.04	-0.02
S	154	152	157	154	3	1513	3027	4	4.46	4.45	0.00
4ml	1	2	3	Avg	SD	Cell / ml	Total cell	6	4.07	4.03	0.03
So	84	86	89	86	3			8	3.78	3.74	0.04
S	88	80	90	86	5	863	3453	10	3.52	3.42	0.09
6ml	1	2	3	Avg	SD	Cell / ml	Total cell	Killer Activity			
So	59	59	57	58	1			ln(So/S)	m* (ml)	Lethal Unit(cell / ml)	
S	53	57	59	56	3	583	3500	1	153.85	21.89	
8ml	1	2	3	Avg	SD	Cell / ml	Total cell	m = 1/slope of trend line			
So	43	43	45	44	1			Killing Activity = Avg total cell / m			
S	40	43	43	42	2	437	3493				
10ml	1	2	3	Avg	SD	Cell / ml	Total cell	* : multiplicity			
So	35	33	33	34	1			Average Total Cell			
S	31	31	30	31	1	337	3367	3368			

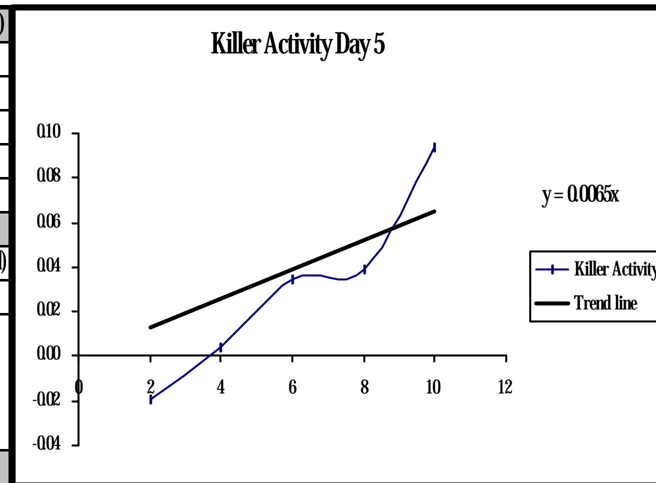


Figure C.10 Killer activity day 5 of second trial

Table C.14 Killer activity day 4 of third trial

2ml	1	2	3	Avg	SD	Cell / ml	Total cell	Volume(ml)	ln (So)	ln(S)	ln(So/S)
So	111	107	105	108	3			2	4.68	4.69	-0.01
S	113	112	101	109	7	1077	2153	4	4.24	4.19	0.04
4ml	1	2	3	Avg	SD	Cell / ml	Total cell	6	3.82	3.68	0.14
So	65	69	74	69	5			8	3.56	3.42	0.14
S	61	61	77	66	9	693	2773	10	3.18	3.09	0.09
6ml	1	2	3	Avg	SD	Cell / ml	Total cell	Killer Activity			
So	48	47	42	46	3			ln(So/S)	m* (ml)	Lethal Unit(cell / ml)	
S	40	44	35	40	5	457	2740	1	72.99	35.33	
8ml	1	2	3	Avg	SD	Cell / ml	Total cell	m = 1/slope of trend line			
So	36	34	36	35	1			Killing Activity = Avg total cell / m			
S	32	29	31	31	2	353	2827				
10ml	1	2	3	Avg	SD	Cell / ml	Total cell	* : multiplicity			
So	22	24	26	24	2			Average Total Cell			
S	24	20	22	22	2	240	2400	2579			

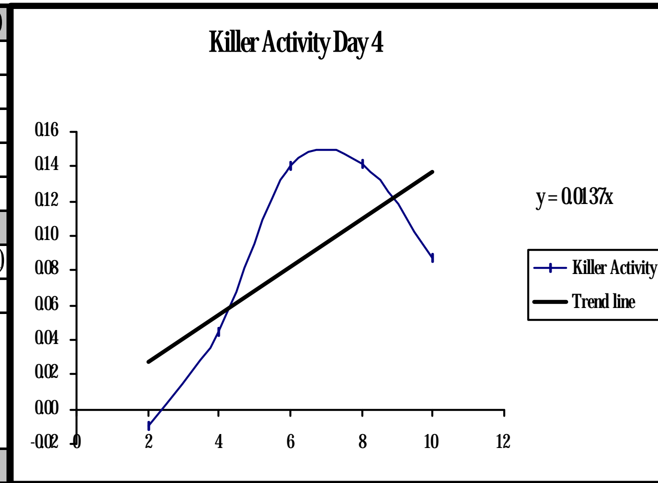


Figure C.14 Killer activity day 4 of third trial

Table C.15 Killer activity day 5 of third trial

2ml	1	2	3	Avg	SD	Cell / ml	Total cell	Volume(ml)	ln (So)	ln(S)	ln(So/S)
So	160	150	168	159	9			2	5.07	5.05	0.02
S	154	152	162	156	5	1593	3187	4	4.54	4.54	0.01
4ml	1	2	3	Avg	SD	Cell / ml	Total cell	6	4.21	4.16	0.05
So	90	95	97	94	4			8	3.86	3.78	0.09
S	95	90	95	93	3	940	3760	10	3.67	3.61	0.06
6ml	1	2	3	Avg	SD	Cell / ml	Total cell	Killer Activity			
So	60	72	70	67	6			ln(So/S)	m* (ml)	Lethal Unit(cell / ml)	
S	61	71	61	64	6	673	4040	1	133.33	28.10	
8ml	1	2	3	Avg	SD	Cell / ml	Total cell	m = 1/slope of trend line			
So	49	45	49	48	2			Killing Activity = Avg total cell / m			
S	40	45	46	44	3	477	3813				
10ml	1	2	3	Avg	SD	Cell / ml	Total cell	* : multiplicity			
So	40	39	39	39	1			Average Total Cell			
S	36	38	37	37	1	393	3933	3747			

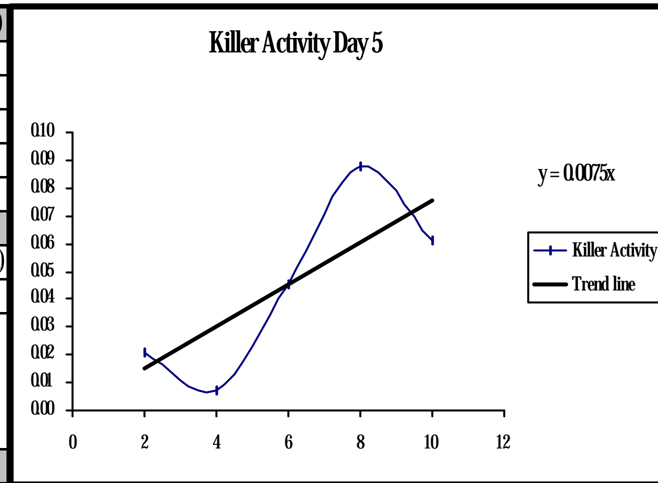
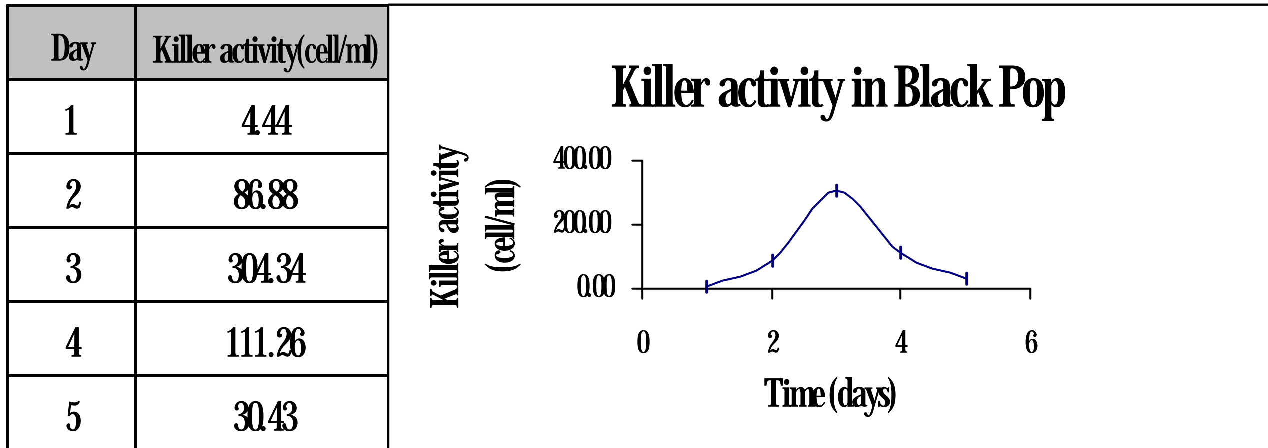
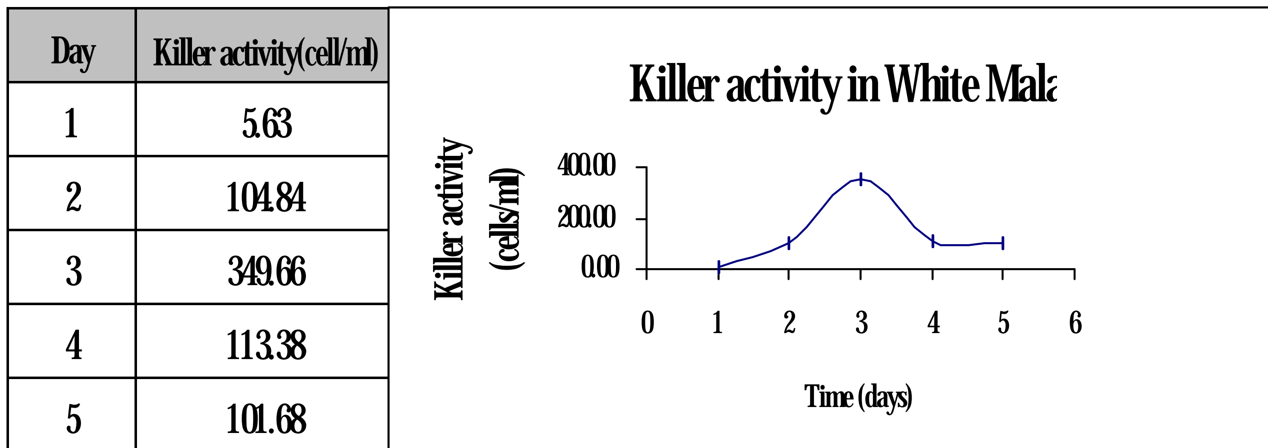


Figure C.15 Killer activity day 5 of third trial



Day	Killer activity(cell/ml)
1	4.44
2	86.88
3	304.34
4	111.26
5	30.43

Figure C.27 Killer activity in Black Pop must



Day	Killer activity(cell/ml)
1	5.63
2	104.84
3	349.66
4	113.38
5	101.68

Figure C.28 Killer activity in White Malaga must

Table C.25 Killer activity day 4 White Malaga

2ml	1	2	3	Avg	SD	Cell / ml	Total cell	Volume(ml)	ln(So)	ln(S)	ln(So/S)
So	123	127	125	125	2			2	4.83	4.81	0.02
S	122	122	125	123	2	1250	2500	4	4.45	4.16	0.29
4ml	1	2	3	Avg	SD	Cell / ml	Total cell	6	3.84	3.73	0.11
So	87	85	86	86	1			8	3.48	3.31	0.17
S	67	62	64	64	3	860	3440	10	3.18	2.59	0.59
6ml	1	2	3	Avg	SD	Cell / ml	Total cell	Killer Activity			
So	47	48	45	47	2			ln(So/S)	m* (ml)	Lethal Unit(cell / ml)	
S	42	41	42	42	1	467	2800	1	24.21	113.39	
8ml	1	2	3	Avg	SD	Cell / ml	Total cell	m = 1/slope of trend line			
So	32	32	33	32	1			Killing Activity = Avg total cell / m			
S	29	28	25	27	2	323	2587				
10ml	1	2	3	Avg	SD	Cell / ml	Total cell	* : multiplicity			
So	24	23	25	24	1			Average Total Cell			
S	13	13	14	13	1	240	2400	2745			

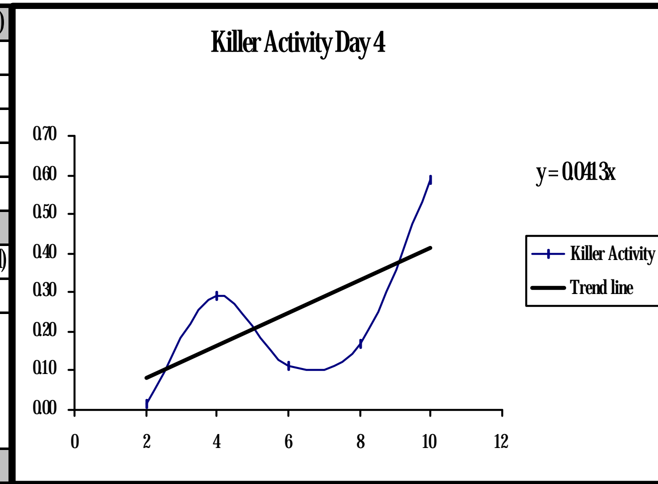


Figure C.25 Killer activity day 4 White Malaga

Table C.26 Killer activity day 5 White Malaga

2ml	1	2	3	Avg	SD	Cell / ml	Total cell	Volume(ml)	ln(So)	ln(S)	ln(So/S)
So	150	155	151	152	3			2	5.02	4.99	0.04
S	145	147	147	146	1	1520	3040	4	4.45	4.38	0.07
4ml	1	2	3	Avg	SD	Cell / ml	Total cell	6	4.21	4.10	0.11
So	84	86	87	86	2			8	4.03	3.71	0.33
S	79	79	81	80	1	857	3427	10	3.71	3.40	0.31
6ml	1	2	3	Avg	SD	Cell / ml	Total cell	Killer Activity			
So	65	68	69	67	2			ln(So/S)	m* (ml)	Lethal Unit(cell / ml)	
S	60	61	60	60	1	673	4040	1	32.57	117.36	
8ml	1	2	3	Avg	SD	Cell / ml	Total cell	m = 1/slope of trend line			
So	56	57	56	56	1			Killing Activity = Avg total cell / m			
S	40	41	41	41	1	563	4507				
10ml	1	2	3	Avg	SD	Cell / ml	Total cell	* : multiplicity			
So	40	41	42	41	1			Average Total Cell			
S	29	30	31	30	1	410	4100	3823			

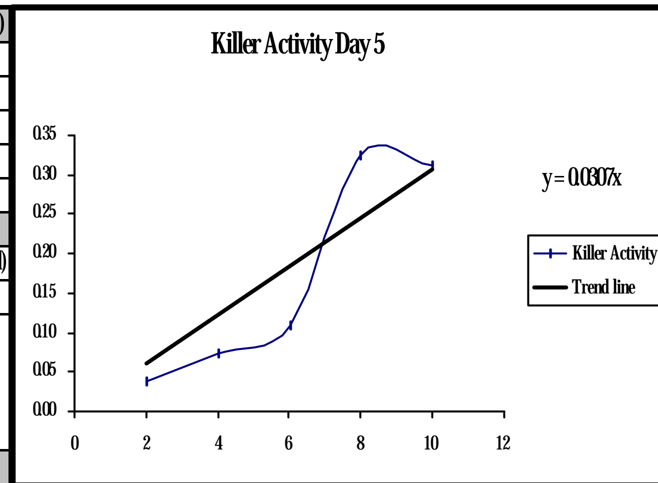


Figure C.26 Killer activity day 5 White Malaga

Appendix D

The calculation of specific growth rate

Table D.1 Maximum Specific Growth Rates Determination (Trial 1)

Time (h)	ΔTime (h)	K1 V1116		HC	
		OD'_{600}	$\ln(x/x_o)$	OD'_{600}	$\ln(x/x_o)$
10.5	0.000	0.117	0.000	0.111	0.000
12.0	1.500	0.140	0.179	0.150	0.301
14.0	3.500	0.211	0.590	0.207	0.623
20.0	9.500	0.403	1.237	0.465	1.433
22.0	11.500	0.555	1.557	0.551	1.602

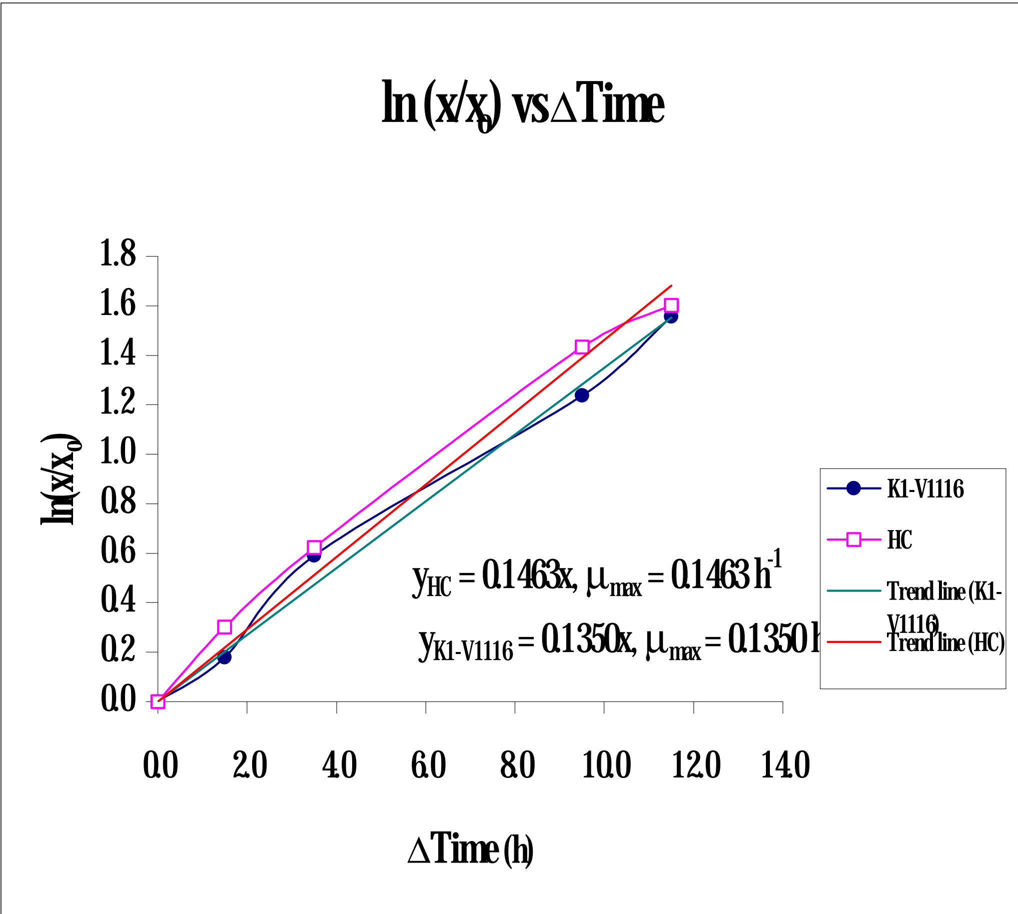


Table D.2 Maximum Specific Growth Rates Determination (Trial 2)

Time (h)	ΔTime (h)	K1 V1116		HC	
		OD'_{600}	$\ln(x/x_0)$	OD'_{600}	$\ln(x/x_0)$
10.5	0.000	0.123	0.000	0.112	0.000
12.0	1.500	0.160	0.263	0.150	0.292
14.0	3.500	0.205	0.511	0.207	0.614
20.0	9.500	0.495	1.392	0.465	1.424
22.0	11.500	0.547	1.492	0.551	1.593

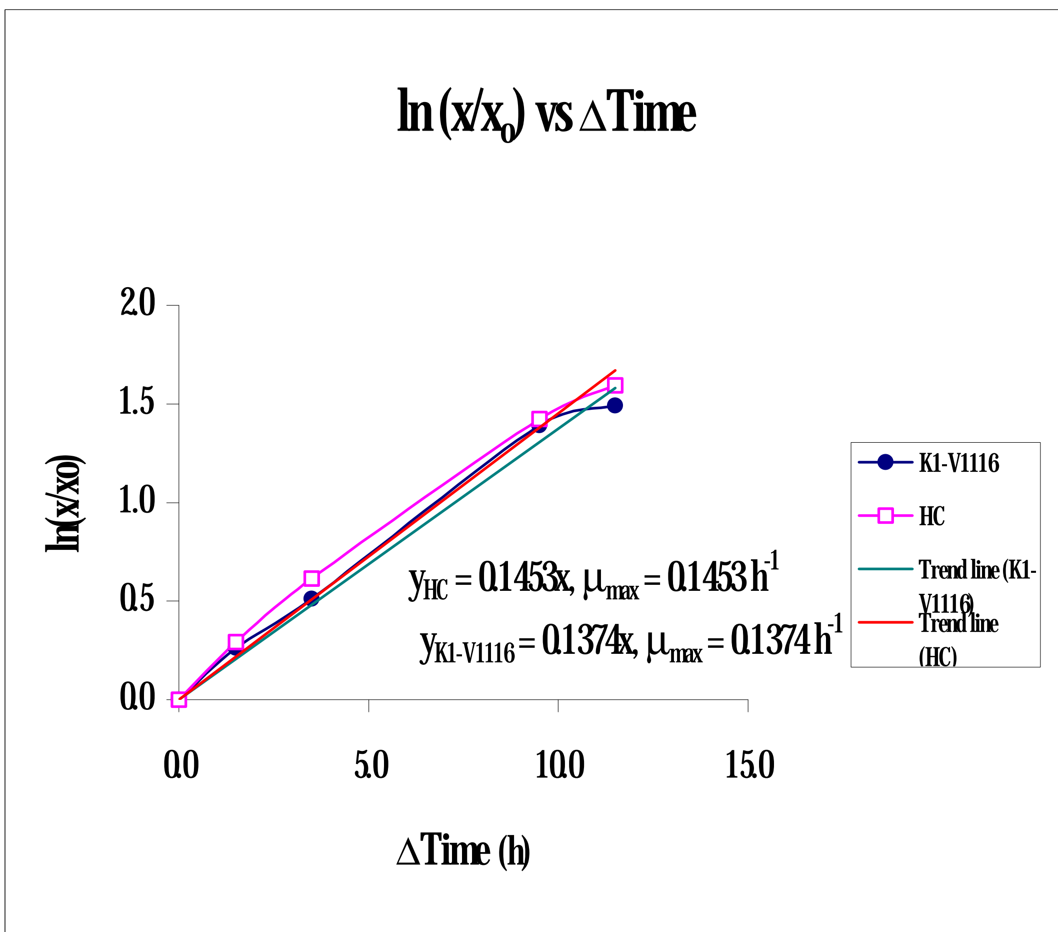


Figure D.2 Plot of $\ln(x/x_0)$ vs ΔTime (Trial 2)

Table D.3 Maximum Specific Growth Rates Determination (Trial 3)

Time (h)	ΔTime (h)	K1 V1116		HC	
		OD'_{600}	$\ln(x/x_0)$	OD'_{600}	$\ln(x/x_0)$
10.5	0.000	0.105	0.000	0.097	0.000
12.0	1.500	0.132	0.229	0.121	0.221
14.0	3.500	0.185	0.566	0.185	0.646
20.0	9.500	0.375	1.273	0.397	1.409
22.0	11.500	0.474	1.507	0.487	1.614

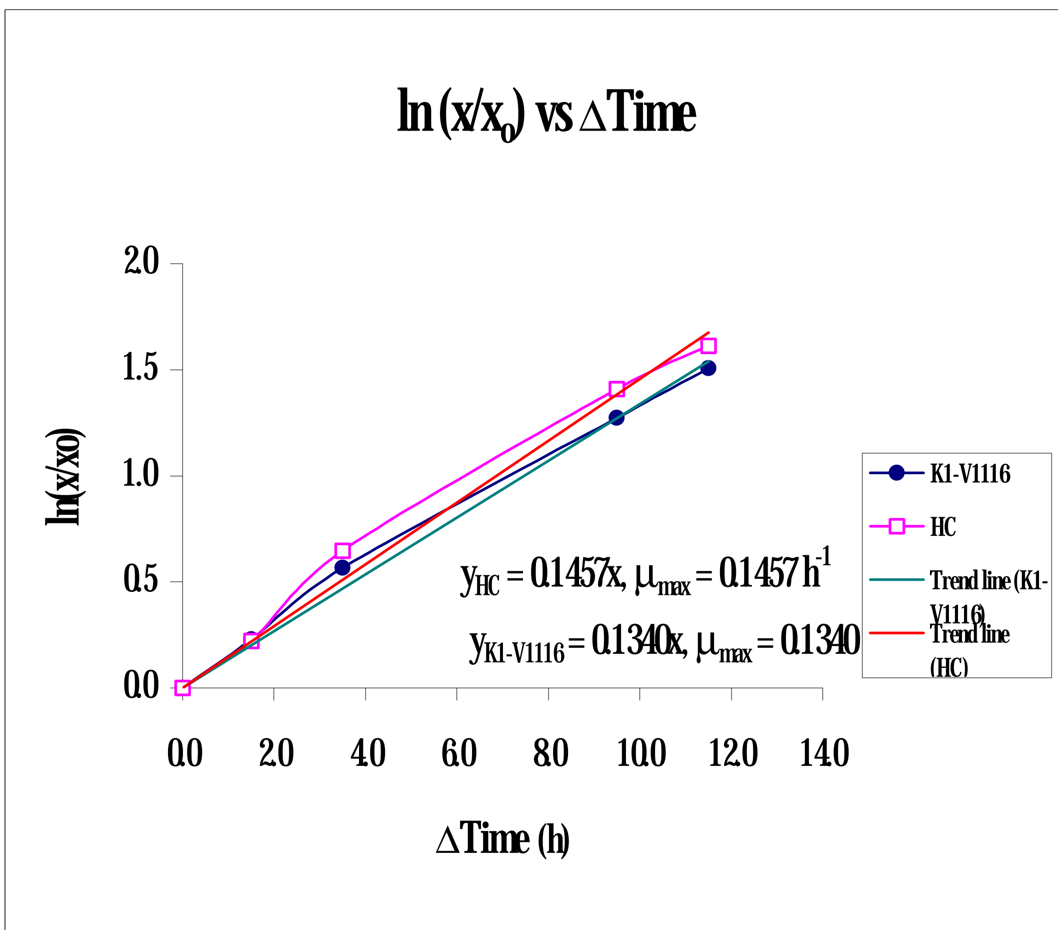
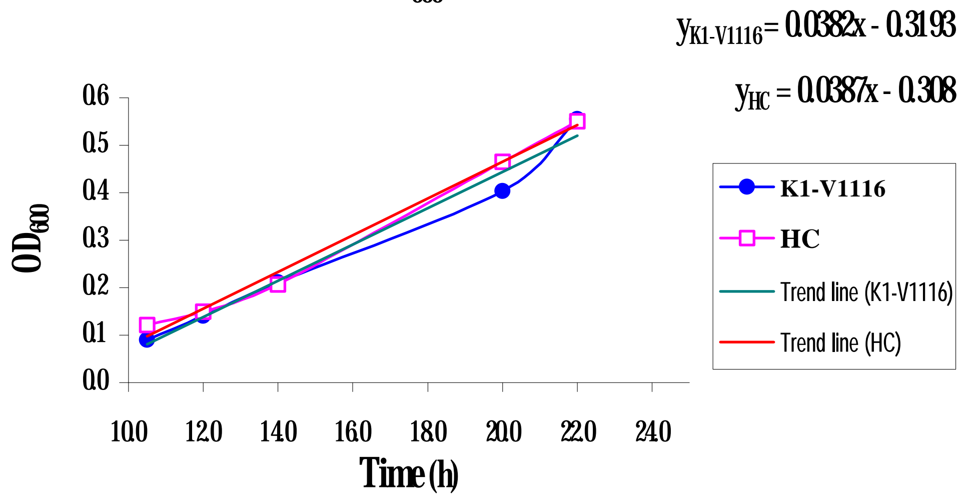
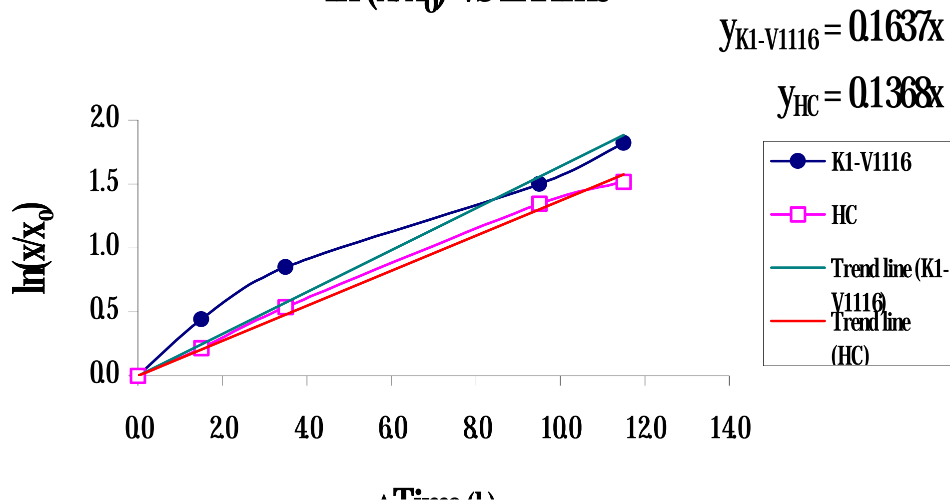


Figure D.3 Plot of $\ln(x/x_0)$ vs ΔTime (Trial 3)

Growth DATA							
Time (h)	ΔTime (h)	K1 V1116			HC		
		OD'_{600}	OD_{600}	$\ln(x/x_0)$	OD'_{600}	OD_{600}	$\ln(x/x_0)$
10.5	0.000	0.090	0.082	0.000	0.121	0.098	0.000
12.0	1.500	0.140	0.139	0.531	0.150	0.156	0.464
14.0	3.500	0.211	0.216	0.969	0.207	0.234	0.866
20.0	9.500	0.403	0.445	1.693	0.465	0.466	1.556
22.0	11.500	0.555	0.521	1.852	0.551	0.543	1.709

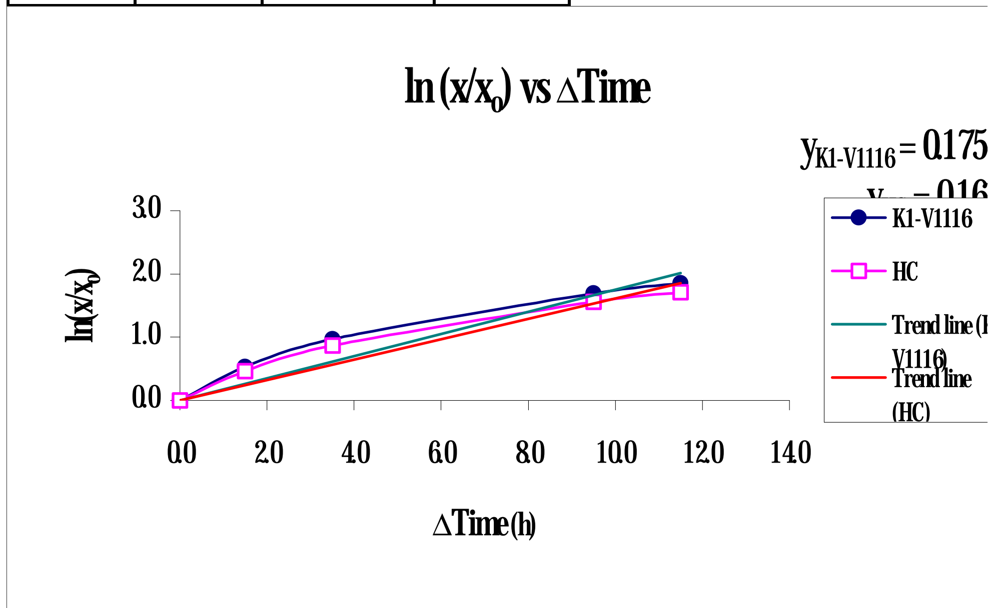
Batch I





ΔTime (h)

K1		HC	
0.090	0.000	0.121	0.000
0.140	0.442	0.150	0.215
0.211	0.852	0.207	0.537
0.403	1.499	0.465	1.346
0.555	1.819	0.551	1.516



1754x
0161x
116

line (K1-
5)
line

Appendix E

The Calculation of relevant Parameters

E.1 The calculation of Maximum Specific Growth Rate (μ_{max})

The maximum specific growth rate exhibit in the exponential phase. If growth is optimal and cells double logarithmically, then

$$\frac{dx}{dt} = \mu_{\max}.X \qquad \textbf{(1)}$$

When integrated, this yields

$$x = x_0 e^{(\mu_{max} t)} \qquad \textbf{(2)}$$

where x_0 = the initial cell mass

x = the cell mass at any time

t = time

or

$$\ln(x/x_0) = \ln x - \ln x_0 = \mu_{max}.t \qquad \textbf{(3)}$$

Figure A.1 to A.6 showed exponential phase of 3 batches existed in 10-32 h of fermentation. The plotted of $\ln(x/x_0)$ against time in this period were plotted expressed trend line by Excel program. These are for elimination of the human error (manual plot) as show in figures D.1-D.3. The maximum specific growth rate existed as slope of the trend lines.

E.2 The calculation of Production Rate (r_p)

Production rate is the differentiation of product concentration per time (in Log phase), which can be expressed as a function of product concentration and time:

$$r_p = \frac{[P_2]-[P_1]}{t_2 - t_1} = \frac{\Delta[P]}{\Delta t} \qquad \textbf{(4)}$$

where r_p = production rate; $g_{product} l^{-1} h^{-1}$

P = product concentration; $g_{product} l^{-1}$

t = time; h

In addition, production rate can be expressed in a function of biomass concentration:

$$r_p = q_p(x_2 - x_1) \qquad (5)$$

where r_p = production rate; $g_{product} l^{-1} h^{-1}$
x = biomass concentration; $g_{biomass} l^{-1}$
q_p = specific rate of product formation; $g_{product}\, g_{biomass}^{-1}\, h^{-1}$

From figure B.1, the change of product concentration existed in hour 20 – 72 of fermentation. The hour 20 and 32 of each batch were used to calculate the production rate.

Sample of calculation (K1-V1116 Batch 1)

$[Eth_1] = 17.23\ g_{Eth} l^{-1}$ $\qquad [Eth_2] = 44.35\ g_{Eth} l^{-1}$
$[Gly_1] = 1.98\ g_{Gly} l^{-1}$ $\qquad [Gly_2] = 3.48\ g_{Gly} l^{-1}$
$x_1 = 8.50\ g_{biomass} l^{-1}$ $\qquad x_2 = 14.25\ g_{biomass} l^{-1}$
$t_1 = 20$ h $\qquad t_2 = 32$ h

Ethanol production rate (r_{Eth})

From equation (4), we get;

$$r_{Eth} = \frac{[Eth_2] - [Eth_1]}{t_2 - t_1} \qquad (6)$$

Substitute $[Eth_1]$, $[Eth_2]$, t_1 and t_2 into equation (6);

$$r_{Eth} = \frac{44.35 - 17.23}{32 - 20} \qquad \frac{g_{Eth} l^{-1}}{h}$$

$$\therefore r_{Eth} = 2.26 \quad g_{Eth} l^{-1} h^{-1}$$

From equation (5);

$$r_{Eth} = q_{Eth}(x_2 - x_1) \qquad \textbf{(7)}$$

$$q_{Eth} = \frac{r_{Eth}}{(x_2 - x_1)}$$

Substitute r_{Eth}, x_1 and x_2 into equation
( )

$$q_{Eth} = \frac{2.26}{(14.25 - 8.50)} \qquad \frac{g_{Eth} l^{-1} h^{-1}}{g_{biomass} l^{-1}}$$

$$\therefore q_{Eth} = 0.39 \quad g_{Eth} g_{biomass}{}^{-1} h^{-1}$$

Glycerol production rate (r_{Gly})

From equation (4), we get;

$$r_{Gly} = \frac{[Gly_2] - [Gly_1]}{t_2 - t_1} \qquad \textbf{(8)}$$

Substitute $[Gly_1]$, $[Gly_2]$, t_1 and t_2 into equation (8);

$$r_{Gly} = \frac{3.48 - 1.98}{32 - 20} \qquad \frac{g_{Gly} l^{-1}}{h}$$

$$\therefore r_{Gly} = 0.12 \quad g_{Gly} l^{-1} h^{-1}$$

From equation (5);

$$r_{Gly} = q_{Gly}(x_2 - x_1) \qquad (9)$$

$$q_{Gly} = \frac{r_{Gly}}{(x_2 - x_1)}$$

Substitute r_{Gly}, x_1 and x_2 into equation

$$q_{Gly} = \frac{0.12}{(14.25 - 8.50)} \qquad \frac{g_{Gly} l^{-1} h^{-1}}{g_{biomass} l^{-1}}$$

$$\therefore q_{Gly} = 0.02 \qquad g_{Gly} g_{biomass}{}^{-1} h^{-1}$$

E.3 The calculation of Consumption Rate (r_s)

Consumption rate is the differentiation of substrate concentration per time (in Log phase), which can be expressed as function of substrate concentration and time:

$$r_s = \frac{[S_1] - [S_2]}{t_2 - t_1} = \frac{\Delta[S]}{\Delta t} \qquad (10)$$

where r_s = consumption rate; $g_{substrate} l^{-1} h^{-1}$
S = substrate concentration; $g_{substrate} l^{-1}$
t = time; h

In addition, consumption rate can be expressed in a function of biomass concentration:

$$r_s = q_s(x_2 - x_1) \qquad (11)$$

where r_s = consumption rate; $g_{substrate} l^{-1} h^{-1}$
x = biomass concentration; $g_{biomass} l^{-1}$
q_s = specific rate of substrate consumption; h^{-1}

From figure B.1, the change of substrate concentration existed in hour 20 – 72 of fermentation. The hour 20 and 32 of each batch were used to calculate the consumption rate.

Sample of calculation (K1-V1116 Batch 1)

$[Glc_1] = 56.74\ g_{Glc} l^{-1}$	$[Glc_2] = 17.90\ g_{Glc} l^{-1}$
$[Frc_1] = 85.81\ g_{Frc} l^{-1}$	$[Frc_2] = 62.16\ g_{Frc} l^{-1}$
$x_1 = 8.50\ g_{biomass} l^{-1}$	$x_2 = 14.25\ g_{biomass} l^{-1}$
$t_1 = 20\ h$	$t_2 = 32\ h$

Glucose consumption rate (r_{Glc})

From equation (10), we get;

$$r_{Glc} = \frac{[Glc_1] - [Glc_2]}{t_2 - t_1} \qquad (12)$$

Substitute $[Glc_1]$, $[Glc_2]$, t_1 and t_2 into equation (12);

$$r_{Glc} = \frac{56.74 - 17.90}{32 - 20} \qquad \frac{g_{Glc} l^{-1}}{h}$$

$$\therefore r_{Glc} = 3.24 \qquad g_{Glc} l^{-1} h^{-1}$$

From equation (11);

$$r_{Glc} = q_{Glc}(x_2 - x_1) \qquad (13)$$

$$q_{Glc} = \frac{r_{Glc}}{(x_2 - x_1)}$$

Substitute r_{Glc}, x_1 and x_2 into equation

$$q_{Glc} = \frac{3.24}{(14.25 - 8.50)} \qquad \frac{g_{Glc} l^{-1} h^{-1}}{g_{biomass} l^{-1}}$$

$$q_{Glc} = 0.56 \qquad g_{Glc} g_{biomass}{}^{-1} h^{-1}$$

Fructose consumption Rate (r_{Frc})

From equation (10), we get;

$$r_{Frc} = \frac{[Frc_1] - [Frc_2]}{t_2 - t_1} \qquad \textbf{(14)}$$

Substitute [Frc_1], [Frc_2], t_1 and t_2 into equation (14);

$$r_{Frc} = \frac{85.81 - 62.16}{32 - 20} \qquad \frac{g_{Frc} l^{-1}}{h}$$

$$\therefore r_{Frc} = 1.97 \qquad g_{Frc} l^{-1} h^{-1}$$

From equation (11);

$$r_{Frc} = q_{Frc}(x_2 - x_1) \qquad \textbf{(15)}$$

$$q_{Frc} = \frac{r_{Frc}}{(x_2 - x_1)}$$

$$q_{Frc} = \frac{1.97}{(14.25 - 8.50)} \qquad \frac{g_{Frc} l^{-1} h^{-1}}{g_{biomass} l^{-1}}$$

Substitute r_{Frc}, x_1 and x_2 into equation

$$q_{Frc} = 0.34 \qquad g_{Frc} g_{biomass}{}^{-1} h^{-1}$$

Table E.1 Production and consumption rates of Batch 1

Ethanol production Batch 1 K1-V1116				Ethanol production Batch 1 HC			
T_1	T_2	$\Delta T(h)$	$r_{Eth}(g_{Eth} l^{-1} h^{-1})$	T_1	T_2	$\Delta T(h)$	$r_{Eth}(g_{Eth} l^{-1} h^{-1})$
20	32	12.00	2.26	20	32	12.00	2.29
$[Eth]_1$	$[Eth]_2$	$\Delta P(g_{Eth} l^{-1})$	$q_{Eth}(g_{Eth} g_{biomass}^{-1} h^{-1})$	$[Eth]_1$	$[Eth]_2$	$\Delta P(g_{Eth} l^{-1})$	$q_{Eth}(g_{Eth} g_{biomass}^{-1} h^{-1})$
17.23	44.35	27.12	0.39	17.10	44.54	27.44	0.43
$[Biomass]_1$	$[Biomass]_2$	$\Delta x(g_{biomass} l^{-1})$	$\mu(h^{-1})$	$[Biomass]_1$	$[Biomass]_2$	$\Delta x(g_{biomass} l^{-1})$	$\mu(h^{-1})$
8.50	14.25	5.75	0.146	9.81	15.12	5.31	0.135
Glycerol production Batch 1 K1-V1116				**Glycerol production Batch 1 HC**			
T_1	T_2	$\Delta T(h)$	$r_{Gly}(g_{Gly} l^{-1} h^{-1})$	T_1	T_2	$\Delta T(h)$	$r_{Gly}(g_{Gly} l^{-1} h^{-1})$
20	32	12.00	0.12	20	32	12.00	0.13
$[Gly]_1$	$[Gly]_2$	$\Delta P(g_{Gly} l^{-1})$	$q_{Gly}(g_{Gly} g_{biomass}^{-1} h^{-1})$	$[Gly]_1$	$[Gly]_2$	$\Delta P(g_{Gly} l^{-1})$	$q_{Gly}(g_{Gly} g_{biomass}^{-1} h^{-1})$
1.98	3.48	1.50	0.02	1.99	3.52	1.53	0.02
$[Biomass]_1$	$[Biomass]_2$	$\Delta x(g_{biomass} l^{-1})$	$\mu(h^{-1})$	$[Biomass]_1$	$[Biomass]_2$	$\Delta x(g_{biomass} l^{-1})$	$\mu(h^{-1})$
8.50	14.25	5.75	0.146	9.81	15.12	5.31	0.135
Glucose consumption Batch 1 K1-V1116				**Glucose consumption Batch 1 HC**			
T_1	T_2	$\Delta T(h)$	$r_{Glc}(g_{Glc} l^{-1} h^{-1})$	T_1	T_2	$\Delta T(h)$	$r_{Glc}(g_{Glc} l^{-1} h^{-1})$
20	32	12.00	3.24	20	32	12.00	3.41
$[Glc]_1$	$[Glc]_2$	$\Delta S(g_{Glc} l^{-1})$	$q_{Glc}(g_{Glc} g_{biomass}^{-1} h^{-1})$	$[Glc]_1$	$[Glc]_2$	$\Delta S(g_{Glc} l^{-1})$	$q_{Glc}(g_{Glc} g_{biomass}^{-1} h^{-1})$
56.74	17.90	38.84	0.56	61.02	20.15	40.87	0.64
$[Biomass]_1$	$[Biomass]_2$	$\Delta x(g_{biomass} l^{-1})$	$\mu(h^{-1})$	$[Biomass]_1$	$[Biomass]_2$	$\Delta x(g_{biomass} l^{-1})$	$\mu(h^{-1})$
8.50	14.25	5.76	0.146	9.81	15.12	5.31	0.135
Fructose consumption Batch 1 K1-V1116				**Fructose consumption Batch 1 HC**			
T_1	T_2	$\Delta T(h)$	$r_{Frc}(g_{Frc} l^{-1} h^{-1})$	T_1	T_2	$\Delta T(h)$	$r_{Frc}(g_{Frc} l^{-1} h^{-1})$
20	32	12.00	1.97	20	32	12.00	2.28
$[Frc]_1$	$[Frc]_2$	$\Delta S(g_{Frc} l^{-1})$	$q_{Frc}(g_{Frc} g_{biomass}^{-1} h^{-1})$	$[Frc]_1$	$[Frc]_2$	$\Delta S(g_{Frc} l^{-1})$	$q_{Frc}(g_{Frc} g_{biomass}^{-1} h^{-1})$
85.81	62.16	23.65	0.34	87.70	60.34	27.36	0.43
$[Biomass]_1$	$[Biomass]_2$	$\Delta x(g_{biomass} l^{-1})$	$\mu(h^{-1})$	$[Biomass]_1$	$[Biomass]_2$	$\Delta x(g_{biomass} l^{-1})$	$\mu(h^{-1})$
8.50	14.25	5.75	0.146	9.81	15.12	5.31	0.135

Table E.2 Production and consumption rates of Batch 2

Ethanol production Batch 2 K1-V1116				Ethanol production Batch 2 HC			
T_1	T_2	ΔT(h)	$r_{Eth}(g_{Eth} l^{-1} h^{-1})$	T_1	T_2	ΔT(h)	$r_{Eth}(g_{Eth} l^{-1} h^{-1})$
20	32	12.00	2.27	20	32	12.00	2.33
$[Eth]_1$	$[Eth]_2$	$\Delta P(g_{Eth} l^{-1})$	$q_{Eth}(g_{Eth} g_{biomass}^{-1} h^{-1})$	$[Eth]_1$	$[Eth]_2$	$\Delta P(g_{Eth} l^{-1})$	$q_{Eth}(g_{Eth} g_{biomass}^{-1} h^{-1})$
17.13	44.33	27.20	0.38	17.10	45.06	27.96	0.39
$[Biomass]_1$	$[Biomass]_2$	$\Delta x(g_{biomass} l^{-1})$	$\mu(h^{-1})$	$[Biomass]_1$	$[Biomass]_2$	$\Delta x(g_{biomass} l^{-1})$	$\mu(h^{-1})$
9.85	15.82	5.97	0.137	10.44	16.47	6.03	0.145
Glycerol production Batch 2 K1-V1116				**Glycerol production Batch 2 HC**			
T_1	T_2	ΔT(h)	$r_{Gly}(g_{Gly} l^{-1} h^{-1})$	T_1	T_2	ΔT(h)	$r_{Gly}(g_{Gly} l^{-1} h^{-1})$
20	32	12.00	0.13	20	32	12.00	0.13
$[Gly]_1$	$[Gly]_2$	$\Delta P(g_{Gly} l^{-1})$	$q_{Gly}(g_{Gly} g_{biomass}^{-1} h^{-1})$	$[Gly]_1$	$[Gly]_2$	$\Delta P(g_{Gly} l^{-1})$	$q_{Gly}(g_{Gly} g_{biomass}^{-1} h^{-1})$
2.05	3.59	1.54	0.02	1.99	3.60	1.61	0.02
$[Biomass]_1$	$[Biomass]_2$	$\Delta x(g_{biomass} l^{-1})$	$\mu(h^{-1})$	$[Biomass]_1$	$[Biomass]_2$	$\Delta x(g_{biomass} l^{-1})$	$\mu(h^{-1})$
9.85	15.82	5.97	0.137	10.44	16.47	6.03	0.145
Glucose consumption Batch 2 K1-V1116				**Glucose consumption Batch 2 HC**			
T_1	T_2	ΔT(h)	$r_{Glc}(g_{Glc} l^{-1} h^{-1})$	T_1	T_2	ΔT(h)	$r_{Glc}(g_{Glc} l^{-1} h^{-1})$
20	32	12.00	3.30	20	32	12.00	3.34
$[Glc]_1$	$[Glc]_2$	$\Delta S(g_{Glc} l^{-1})$	$q_{Glc}(g_{Glc} g_{biomass}^{-1} h^{-1})$	$[Glc]_1$	$[Glc]_2$	$\Delta S(g_{Glc} l^{-1})$	$q_{Glc}(g_{Glc} g_{biomass}^{-1} h^{-1})$
57.93	18.36	39.57	0.55	60.05	20.03	40.02	0.55
$[Biomass]_1$	$[Biomass]_2$	$\Delta x(g_{biomass} l^{-1})$	$\mu(h^{-1})$	$[Biomass]_1$	$[Biomass]_2$	$\Delta x(g_{biomass} l^{-1})$	$\mu(h^{-1})$
9.85	15.82	5.97	0.137	10.44	16.47	6.03	0.145
Fructose consumption Batch 2 K1-V1116				**Fructose consumption Batch 2 HC**			
T_1	T_2	ΔT(h)	$r_{Frc}(g_{Frc} l^{-1} h^{-1})$	T_1	T_2	ΔT(h)	$r_{Frc}(g_{Frc} l^{-1} h^{-1})$
20	32	12.00	1.96	20	32	12.00	1.98
$[Frc]_1$	$[Frc]_2$	$\Delta S(g_{Frc} l^{-1})$	$q_{Frc}(g_{Frc} g_{biomass}^{-1} h^{-1})$	$[Frc]_1$	$[Frc]_2$	$\Delta S(g_{Frc} l^{-1})$	$q_{Frc}(g_{Frc} g_{biomass}^{-1} h^{-1})$
88.84	65.28	23.56	0.33	87.70	63.97	23.73	0.33
$[Biomass]_1$	$[Biomass]_2$	$\Delta x(g_{biomass} l^{-1})$	$\mu(h^{-1})$	$[Biomass]_1$	$[Biomass]_2$	$\Delta x(g_{biomass} l^{-1})$	$\mu(h^{-1})$
9.85	15.82	5.97	0.137	10.44	16.47	6.03	0.145

Table E.3 Production and consumption rates of Batch 3

Ethanol production Batch 3 K1-V1116				**Ethanol production Batch 3 HC**			
T_1	T_2	$\Delta T(h)$	$r_{Eth}(g_{Eth} l^{-1} h^{-1})$	T_1	T_2	$\Delta T(h)$	$r_{Eth}(g_{Eth} l^{-1} h^{-1})$
20	32	12.00	2.12	20	32	12.00	2.25
$[Eth]_1$	$[Eth]_2$	$\Delta P(g_{Eth} l^{-1})$	$q_{Eth}(g_{Eth} g_{biomass}^{-1} h^{-1})$	$[Eth]_1$	$[Eth]_2$	$\Delta P(g_{Eth} l^{-1})$	$q_{Eth}(g_{Eth} g_{biomass}^{-1} h^{-1})$
17.38	42.78	25.40	0.38	17.66	44.60	26.94	0.38
$[Biomass]_1$	$[Biomass]_2$	$\Delta x(g_{biomass} l^{-1})$	$\mu(h^{-1})$	$[Biomass]_1$	$[Biomass]_2$	$\Delta x(g_{biomass} l^{-1})$	$\mu(h^{-1})$
7.91	13.50	5.59	0.134	8.37	14.21	5.84	0.146
Glycerol production Batch 3 K1-V1116				**Glycerol production Batch 3 HC**			
T_1	T_2	$\Delta T(h)$	$r_{Gly}(g_{Gly} l^{-1} h^{-1})$	T_1	T_2	$\Delta T(h)$	$r_{Gly}(g_{Gly} l^{-1} h^{-1})$
20	32	12.00	0.11	20	32	12.00	0.12
$[Gly]_1$	$[Gly]_2$	$\Delta P(g_{Gly} l^{-1})$	$q_{Gly}(g_{Gly} g_{biomass}^{-1} h^{-1})$	$[Gly]_1$	$[Gly]_2$	$\Delta P(g_{Gly} l^{-1})$	$q_{Gly}(g_{Gly} g_{biomass}^{-1} h^{-1})$
2.15	3.50	1.35	0.02	2.10	3.53	1.43	0.02
$[Biomass]_1$	$[Biomass]_2$	$\Delta x(g_{biomass} l^{-1})$	$\mu(h^{-1})$	$[Biomass]_1$	$[Biomass]_2$	$\Delta x(g_{biomass} l^{-1})$	$\mu(h^{-1})$
7.91	13.50	5.59	0.134	8.37	14.21	5.84	0.146
Glucose consumption Batch 3 K1-V1116				**Glucose consumption Batch 3 HC**			
T_1	T_2	$\Delta T(h)$	$r_{Glc}(g_{Glc} l^{-1} h^{-1})$	T_1	T_2	$\Delta T(h)$	$r_{Glc}(g_{Glc} l^{-1} h^{-1})$
20	32	12.00	3.23	20	32	12.00	3.41
$[Glc]_1$	$[Glc]_2$	$\Delta S(g_{Glc} l^{-1})$	$q_{Glc}(g_{Glc} g_{biomass}^{-1} h^{-1})$	$[Glc]_1$	$[Glc]_2$	$\Delta S(g_{Glc} l^{-1})$	$q_{Glc}(g_{Glc} g_{biomass}^{-1} h^{-1})$
57.16	18.41	38.75	0.58	61.16	20.24	40.92	0.58
$[Biomass]_1$	$[Biomass]_2$	$\Delta x(g_{biomass} l^{-1})$	$\mu(h^{-1})$	$[Biomass]_1$	$[Biomass]_2$	$\Delta x(g_{biomass} l^{-1})$	$\mu(h^{-1})$
7.91	13.50	5.59	0.134	8.37	14.21	5.84	0.135
Fructose consumption Batch 3 K1-V1116				**Fructose consumption Batch 3 HC**			
T_1	T_2	$\Delta T(h)$	$r_{Frc}(g_{Frc} l^{-1} h^{-1})$	T_1	T_2	$\Delta T(h)$	$r_{Frc}(g_{Frc} l^{-1} h^{-1})$
20	32	12.00	2.77	20	32	12.00	2.73
$[Frc]_1$	$[Frc]_2$	$\Delta S(g_{Frc} l^{-1})$	$q_{Frc}(g_{Frc} g_{biomass}^{-1} h^{-1})$	$[Frc]_1$	$[Frc]_2$	$\Delta S(g_{Frc} l^{-1})$	$q_{Frc}(g_{Frc} g_{biomass}^{-1} h^{-1})$
97.25	64.01	33.24	0.50	95.37	62.60	32.77	0.47
$[Biomass]_1$	$[Biomass]_2$	$\Delta x(g_{biomass} l^{-1})$	$\mu(h^{-1})$	$[Biomass]_1$	$[Biomass]_2$	$\Delta x(g_{biomass} l^{-1})$	$\mu(h^{-1})$
7.91	13.50	5.59	0.134	8.37	14.21	5.84	0.146

E.4 The calculation of yields of ethanol from sugar

Final concentration of ethanol of each treatment were used to calculate yeild. Average initial quantity of glucose and fructose were combine, and used for the calculation of each yield.

Sample of calculation

1. Average inintial sugar concentration (data from Table B.1-B.6)

Sugar	1	2	3	4	5	6	Avg
Glucose (gl^{-1})	86.80	86.30	87.20	87.39	81.33	80.11	84.86
Fructose (gl^{-1})	99.59	96.72	103.42	100.06	116.28	105.07	103.52

Total sugar concentration = 84.86+103.52 = 188.38 gl^{-1}

2. Average final concentrations of ethanol (gl^{-1})

Treatment	1	2	3	4	Avg
Batch 1 K1-V1116	85.710	85.670	90.640	89.620	87.910
Batch 1 HC	88.320	89.720	89.970	89.590	89.400
Batch 2 K1-V1116	86.320	85.090	87.510	87.510	86.608
Batch 2 HC	86.320	85.090	88.870	88.870	87.288
Batch 3 K1-V1116	83.500	83.890	86.150	86.150	84.923
Batch 3 HC	84.400	87.760	86.400	86.400	86.240

3. Calculations of yields ($g_{Eth} g_{Sugar}^{-1}$)

Treatment	[Initial sugar](gl^{-1})	[Final ethanol](gl^{-1})		$Yield_{Eth/Sugar}$
Batch 1 K1-V1116	188.380	87.760		0.466
Batch 1 HC	188.380	89.355		0.474
Batch 2 K1-V1116	188.380	86.355		0.458
Batch 2 HC	188.380	87.403		0.464
Batch 3 K1-V1116	188.380	84.493		0.449
Batch 3 HC	188.380	86.598		0.460

4. Average yields

Batch	1	2	3	Avg	Sd
K1-V1116	0.466	0.458	0.449	0.458	0.009
HC	0.474	0.464	0.460	0.466	0.007

Appendix F

Standard Curve

F.1 Standard curve of biomass and OD_{600}

K1-V1116 cells were collected from YEPG broth and dissolved in distilled water. The weight of dry cells (biomass) in 1 ml of cell suspension were determined according to A.O.A.C. The cells in 1 ml of cell suspension were collected and diluted in Ruby Carbernet must as shown in Table F.1 and F.2. Each dilution of cells were measured the absorbance (OD_{600}). Biomass in each dilution of cell suspension were calculated and plotted against OD_{600} (Figure F.1). The slopes of each curve were used as conversion factor for the calculation of biomass in the experiment.

Table F.1 Detemination of biomass in cell suspension

Number	Paper mass (mg)	Paper + Dry mass (mg)	Dry mass (mg)	Sample Volume (mg)	Biomass (mg/ml)
1	475.2	500.4	25.2	0.5	50.4
2	440.7	468.4	27.7	0.5	55.4
3	466.3	494.8	28.5	0.5	57.0
4	465.7	492.5	26.8	0.5	53.6
5	473.4	500.9	27.5	0.5	55.0
6	464.2	492.7	28.5	0.5	57.0
				Average	**54.73**

Table F.2 Detemination of biomass suspension and optical density

Dilution	Biomass (mg/ml)	OD_{600}
0.2500	13.6825	0.645
0.1250	6.84125	0.345
0.0625	3.420625	0.135
0.0313	1.710313	0.014

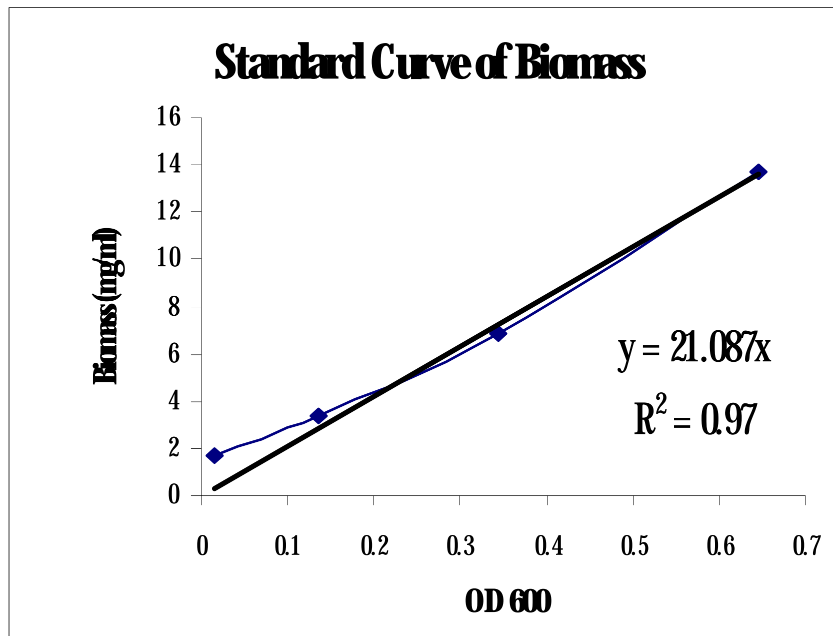


Figure F.1 Plot of Biomass vs OD_{600}

F.2 Standard curve of viable cells and OD_{600}

Viable cells of K1-V1116 and HC were counted by standard plate count on YEPG medium. Viable cell of each strain was plotted against OD_{600}.

Table F.3 The determination of viable cell of K1-V1116 and optical density

Abs600	1	2	3	4	Average
0.031	131	138	127	127	1.308E+06
0.113	227	185	190	193	1.988E+07
0.36	747	767	779	858	7.878E+07
0.547	170	162	177	149	1.645E+08
0.713	217	217	222	228	2.210E+08

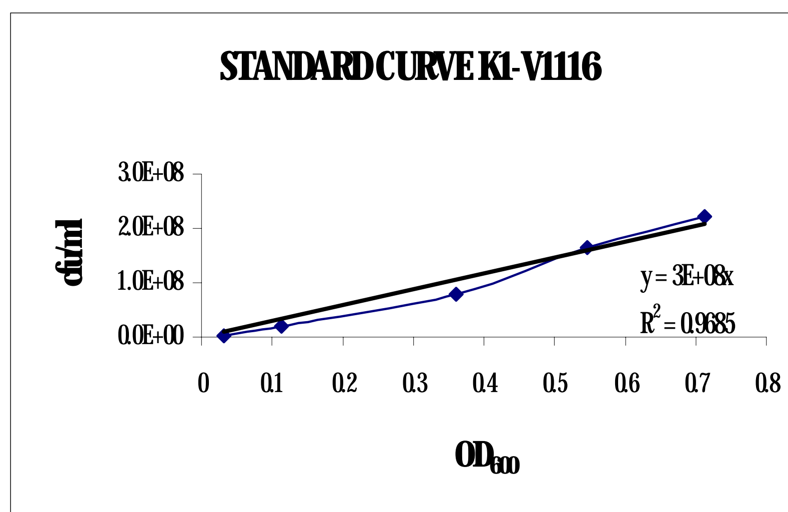


Figure F.2 Plot of K1-V1116 cell vs OD_{600}

Table F.4 The determination of viable cell of HC and optical density

OD_{600}	1	2	3	4	Average
0.042	117	114	110	151	1.230E+06
0.121	183	183	172	207	1.863E+07
0.360	831	708	732	823	7.735E+07
0.551	153	158	158	156	1.563E+08
0.718	193	212	211	225	2.103E+08

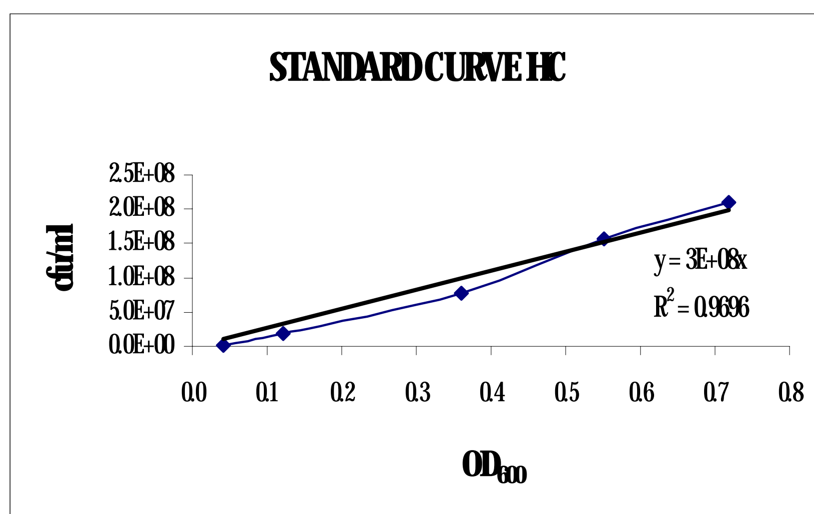


Figure F.3 Plot of HC cell vs OD_{600}

Table F.5 Viable cell of both strains and optical density

OD_{600}	1	2	3	4	Average
0.031	131	138	127	127	1.308E+06
0.042	117	114	110	151	1.230E+06
0.113	227	185	190	193	1.988E+07
0.121	183	183	172	207	1.863E+07
0.547	170	162	177	149	1.645E+08
0.551	153	158	158	156	1.563E+08
0.713	217	217	222	228	2.210E+08
0.718	193	212	211	225	2.103E+08

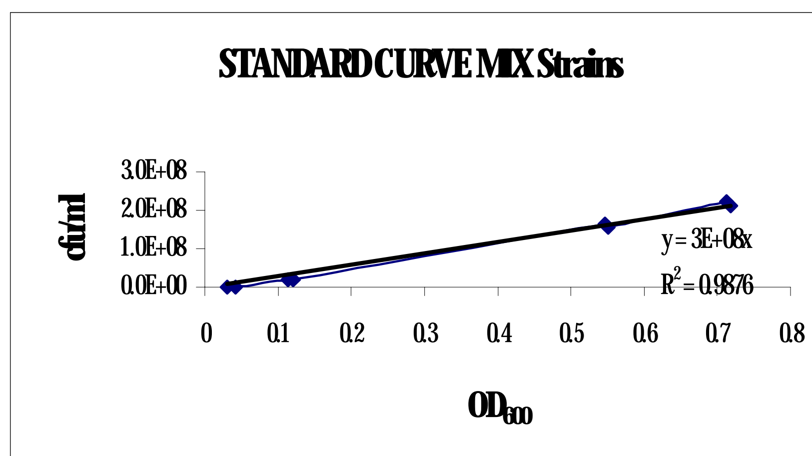


Figure F.4 Plot of cell vs OD_{600}

Appendix G

Analysis of Variances

G.1 Analysis of variances (ANOVA) of the maximum specific growth rate

Formula

CM (Correction for Mean) = $(\sum\sum x_{ij})^2/n = (\sum T_i)^2/n$

SST (Sum of Square Total) = $\sum\sum x^2_{ij}$ - CM

SSTrt (Sum of Square for Treatment) = $\sum T^2_i/n_i$ - CM

SSE (Sum of Square for Error) = SST - SSTrt

MSE (Mean Square for Treatment) = SSE/(n - k)

MSTrt (Mean Square for Treatment) = SSTrt/(k - 1)

Probable Treatment (F- Test) **F** = MSTrt/MSE ~ $F_{k-1;n-k}$

The experiments were designed as complete randomized design (CRD). The results of treatment are shown in table below.

K1-V1116	0.1350	0.1370	0.1340	**0.4060**
HC	0.1460	0.1453	0.1457	**0.4370**

Assumption for analysis

H_o: specific growth rates of both strains are not different.

H_1: specific growth rates of both strains are different.

or

H_o: $\mu_{K1\text{-}V1116} = \mu_{HC}$

H_1: $\mu_{K1\text{-}V1116} \neq \mu_{HC}$

Populations of both treatments have normal distribution and same variation ($\sigma^2_{K1\text{-}V1116} = \sigma^2_{HC}$)

$n = n_{K1\text{-}V1116} + n_{HC} = 3 + 3 = 6$

$T_{K1\text{-}V1116} = 0.4060$, $T_{HC} = 0.4370$

$\sum\sum x_{K1\text{-}V1116, HC} = \sum T = T_{K1\text{-}V1116} + T_{HC} = 0.4060 + 0.4370 = 0.8430$

$CM = (\sum T)^2/n = 0.8430^2/6 = 0.1184$

$\sum\sum x^2_{K1\text{-}V1116, HC} = 0.1350^2 + 0.1370^2 + 0.1340^2 + 0.1460^2 + 0.1453^2 + 0.1457^2 = 0.1186$

$SST = \sum\sum x^2_{K1\text{-}V1116, HC} - CM = 0.1186 - 0.1184 = 0.0002$

$SSTrt = (\sum T_i)^2/n_i - CM = [(0.4060^2/3) + (0.4370^2/3)] - 0.1184 = 0.1186 - 0.1184$

$= 0.0002$

$SSE = SST - SSTrt = 0.0002 - 0.0002 = 0$

$MSTrt = SSTrt/(k-1) = 0.0002/(2-1) = 0.0002/1 = 0.0002$

$MSE = SSE/(n-k) = 0/(6-2) = 0/4 = 0$

$F = MSTrt/MSE = 0.0002/0 = \infty$

H_0 will be refused when $F > F_{1,\,4;\,0.99}$ and from table $F_{1,4;0.99} = 21.20 < \infty$.

Therefore, $\mu_{K1\text{-}V1116}$ and μ_{HC} are significant difference at 0.01 confidence level.

G.2 Analysis of variances (ANOVA) of the specific ethanol production rate

Formula

CM (Correction for Mean) = $(\sum\sum x_{ij})^2/n = (\sum T_i)^2/n$

SST (Sum of Square Total) = $\sum\sum x^2_{ij}$ - CM

SSTrt (Sum of Square for Treatment) = $\sum T^2_i/n_i$ - CM

SSE (Sum of Square for Error) = SST - SSTrt

MSE (Mean Square for Treatment) = SSE/(n - k)

MSTrt (Mean Square for Treatment) = SSTrt/(k - 1)

Probable Treatment (F- Test) **F** = MSTrt/MSE ~ $F_{k-1;n-k}$

The experiments were designed as complete randomized design (CRD). The results of treatment are shown in table below.

K1-V1116	0.390	0.380	0.380	**1.150**
HC	0.430	0.390	0.380	**1.200**

Assumption for analysis

H_o: specific ethanol production rates of both strains are not different.

H_1: specific ethanol production rates of both strains are different.

or

H_o: $q_{Eth, K1-V1116} = q_{Eth, HC}$

H_1: $q_{Eth, K1-V1116} \neq q_{Eth, HC}$

Populations of both treatments have normal distribution and same variation ($\sigma^2_{K1-V1116} = \sigma^2_{HC}$)

$n = n_{K1-V1116} + n_{HC} = 3 + 3 = 6$

$T_{K1-V1116} = 1.150$, $T_{HC} = 1.200$

$\sum\sum x_{K1-V1116, HC} = \sum T = T_{K1-V1116} + T_{HC} = 1.150 + 1.200 = 2.350$

$CM = (\sum T)^2/n = 2.350^2/6 = 0.9204$

$\sum\sum x^2_{K1-V1116, HC} = 0.390^2 + 0.380^2 + 0.380^2 + .0430^2 + 0.390^2 + 0.380^2 = 0.9223$

$SST = \sum\sum x^2_{K1\text{-}V1116, HC} - CM = 0.9223 - 0.9204 = 0.0019$

$SSTrt = (\sum T_i)^2/n_i - CM = [(1.150^2/3) + (1.200^2/3)] - 0.9204 = 0.9208 - 0.9204$

$= 0.0004$

$SSE = SST - SSTrt = 0.0019 - 0.0004 = 0.0015$

$MSTrt = SSTrt/(k\text{-}1) = 0.0004/(2\text{-}1) = 0.0004/1 = 0.0004$

$MSE = SSE/(n\text{-}k) = 0.0015/(6\text{-}2) = 0.0015/4 = 0.000375$

$F = MSTrt/MSE = 0.0004/0.000375 = 1.067$

H_o will be refused when $F > F_{1, 4; 0.99}$ and from table $F_{1,4;0.99} = 21.20 > 1.067$.

Therefore, $q_{Eth, c, K1\text{-}V1116}$ and $q_{Eth, HC}$ are not significant difference at 0.01 confidence level.

G.3 Analysis of variances (ANOVA) of the specific glucose consumption rate

Formula

CM (Correction for Mean) = $(\sum\sum x_{ij})^2/n = (\sum T_i)^2/n$

SST (Sum of Square Total) = $\sum\sum x^2_{ij}$ - CM

SSTrt (Sum of Square for Treatment) = $\sum T^2_i/n_i$ - CM

SSE (Sum of Square for Error) = SST - SSTrt

MSE (Mean Square for Treatment) = SSE/(n - k)

MSTrt (Mean Square for Treatment) = SSTrt/(k - 1)

Probable Treatment (F- Test) **F** = MSTrt/MSE ~ $F_{k-1;n-k}$

The experiments were designed as complete randomized design (CRD). The results of treatment are shown in table below.

K1-V1116	0.560	0.550	0.580	**1.690**
HC	0.640	0.550	0.580	**1.770**

Assumption for analysis

H_o: specific glucose consumption rates of both strains are not different.

H_1: specific glucose consumption rates of both strains are different.

or

H_o: $q_{Glc, K1-V1116} = q_{Glc,HC}$

H_1: $q_{Glc,K1-V1116} \neq q_{Glc,HC}$

Populations of both treatments have normal distribution and same variation ($\sigma^2_{K1-V1116} = \sigma^2_{HC}$)

$n = n_{K1-V1116} + n_{HC} = 3 + 3 = 6$

$T_{K1-V1116} = 1.690$, $T_{HC} = 1.770$

$\sum\sum x_{K1-V1116, HC} = \sum T = T_{K1-V1116} + T_{HC} = 1.690 + 1.770 = 3.460$

$CM = (\sum T)^2/n = 3.460^2/6 = 1.995$

$\sum\sum x^2_{K1-V1116, HC} = 0.560^2 + 0.550^2 + 0..580^2 + 0.640^2 + 0.550^2 + 0.580^2 = 2.001$

$SST = \sum\sum x^2_{K1-V1116, HC} - CM = 2.001 - 1.995 = 0.006$

$SSTrt = (\sum T_i)^2/n_i - CM = [(1.690^2/3) + (1.770^2/3)] - 1.995 = 1.996 - 1.995$

$= 0.001$

$SSE = SST - SSTrt = 0.003 - 0.001 = 0.005$

$MSTrt = SSTrt/(k-1) = 0.001/(2-1) = 0.001/1 = 0.001$

$MSE = SSE/(n-k) = 0.005/(6-2) = 0.005/4 = 0.00125$

$F = MSTrt/MSE = 0.001/0.00125 = 0.8$

H_o will be refused when $F > F_{1, 4; 0.99}$ and from table $F_{1,4;0.99} = 21.20 > 0.8$

Therefore, $q_{Glc, K1-V1116}$ and $q_{Glc, HC}$ are not significant difference at 0.01 confidence level.

G.4 Analysis of variances (ANOVA) of the specific fructose consumption rate

Formula

CM (Correction for Mean) = $(\sum\sum x_{ij})^2/n = (\sum T_i)^2/n$

SST (Sum of Square Total) = $\sum\sum x^2_{ij}$ - CM

SSTrt (Sum of Square for Treatment) = $\sum T^2_i/n_i$ - CM

SSE (Sum of Square for Error) = SST - SSTrt

MSE (Mean Square for Treatment) = SSE/(n - k)

MSTrt (Mean Square for Treatment) = SSTrt/(k - 1)

Probable Treatment (F- Test) **F** = MSTrt/MSE ~ $F_{k-1;n-k}$

The experiments were designed as complete randomized design (CRD). The results of treatment are shown in table below.

K1-V1116	0.340	0.330	0.500	**1.170**
HC	0.430	0.330	0.470	**1.230**

Assumption for analysis

H_o: specific fructose consumption rates of both strains are not different.

H_1: specific fructose consumption rates of both strains are different.

or

H_o: $q_{Frc, K1-V1116} = q_{Frc, HC}$

H_1: $q_{Frc, K1-V1116} \neq q_{Frc, HC}$

Populations of both treatments have normal distribution and same variation ($\sigma^2_{K1-V1116} = \sigma^2_{HC}$)

$n = n_{K1-V1116} + n_{HC} = 3 + 3 = 6$

$T_{K1-V1116} = 1.170$, $T_{HC} = 1.230$

$\sum\sum x_{K1-V1116, HC} = \sum T = T_{K1-V1116} + T_{HC} = 1.170 + 1.230 = 2.400$

$CM = (\sum T)^2/n = 2.40^2/6 = 0.960$

$\sum\sum x^2_{K1-V1116, HC} = 0.340^2 + 0.330^2 + 0.500^2 + 0.430^2 + 0.330^2 + 0.470^2 = 0.9892$

$SST = \sum\sum x^2_{K1-V1116, HC} - CM = 0.9892 - 0.960 = 0.0292$

$SSTrt = (\sum T_i)^2/n_i - CM = [(1.17^2/3) + (1.23^2/3)] - 0.960 = 0.9606 - 0.960$

$= 0.0006$

$SSE = SST - SSTrt = 0.0292 - 0.0006 = 0.0286$

$MSTrt = SSTrt/(k-1) = 0.0006/(2-1) = 0.0006/1 = 0.0006$

$MSE = SSE/(n-k) = 0.0286/(6-2) = 0.0268/4 = 0.00715$

$F = MSTrt/MSE = 0.0006/0.00715 = 0.0839$

H_o will be refused when $F > F_{1, 4; 0.99}$ and from table $F_{1,4;0.99} = 21.20 > 0.0839$

Therefore, $q_{Frc, K1-V1116}$ and $q_{Frc, HC}$ are not significant difference at 0.01 confidence level.

G.5 Analysis of variances (ANOVA) of the yeild of ethanol from sugar

Formula

CM (Correction for Mean) = $(\sum\sum x_{ij})^2/n = (\sum T_i)^2/n$

SST (Sum of Square Total) = $\sum\sum x^2_{ij}$ - CM

SSTrt (Sum of Square for Treatment) = $\sum T^2_i/n_i$ - CM

SSE (Sum of Square for Error) = SST - SSTrt

MSE (Mean Square for Treatment) = SSE/(n - k)

MSTrt (Mean Square for Treatment) = SSTrt/(k - 1)

Probable Treatment (F- Test) **F** = MSTrt/MSE ~ $F_{k-1;n-k}$

The experiments were designed as complete randomized design (CRD). The results of treatment are shown in table below.

K1-V1116	0.466	0.458	0.449	**1.373**
HC	0.474	0.464	0.460	**1.398**

Assumption for analysis

H_o: Yields of ethanol from sugar of both strains are not different.

H_1: Yields of ethanol from sugar of both strains are different.

or

H_o: $Y_{K1-V1116} = Y_{HC}$

H_1: $Y_{K1-V1116} \neq Y_{HC}$

Populations of both treatments have normal distribution and same variation ($\sigma^2_{K1-V1116} = \sigma^2_{HC}$)

$n = n_{K1-V1116} + n_{HC} = 3 + 3 = 6$

$T_{K1-V1116} = 1.373, T_{HC} = 1.398$

$\sum\sum x_{K1-V1116, HC} = \sum T = T_{K1-V1116} + T_{HC} = 1.373 + 1.398 = 2.771$

$CM = (\sum T)^2/n = 2.771^2/6 = 1.279$

$\sum\sum x^2_{K1-V1116, HC} = 0.466^2 + 0.458^2 + 0.449^2 + 0.474^2 + 0.464^2 + 0.460^2 = 1.280$

$SST = \sum\sum x^2_{K1\text{-}V1116, HC} - CM = 1.280 - 1.279 = 0.001$

$SSTrt = (\sum T_i)^2/n_i - CM = [(1.373^2/3) + (1.398^2/3)] - 1.280 = 1.280 - 1.279$

$= 0.001$

$SSE = SST - SSTrt = 0.001 - 0.001 = 0$

$MSTrt = SSTrt/(k\text{-}1) = 0.001/(2\text{-}1) = 0.001/1 = 0.001$

$MSE = SSE/(n\text{-}k) = 0/(6\text{-}2) = 0/4 = 0$

$F = MSTrt/MSE = 0.001/0 = \infty$

H_0 will be refused when $F > F_{1, 4; 0.99}$ and from table $F_{1,4;0.99} = 21.20 < \infty$

Therefore, $Y_{K1\text{-}V1116}$ and Y_{HC} are significant difference at 0.01 confidence level.

BIBLIOGRAPHY

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