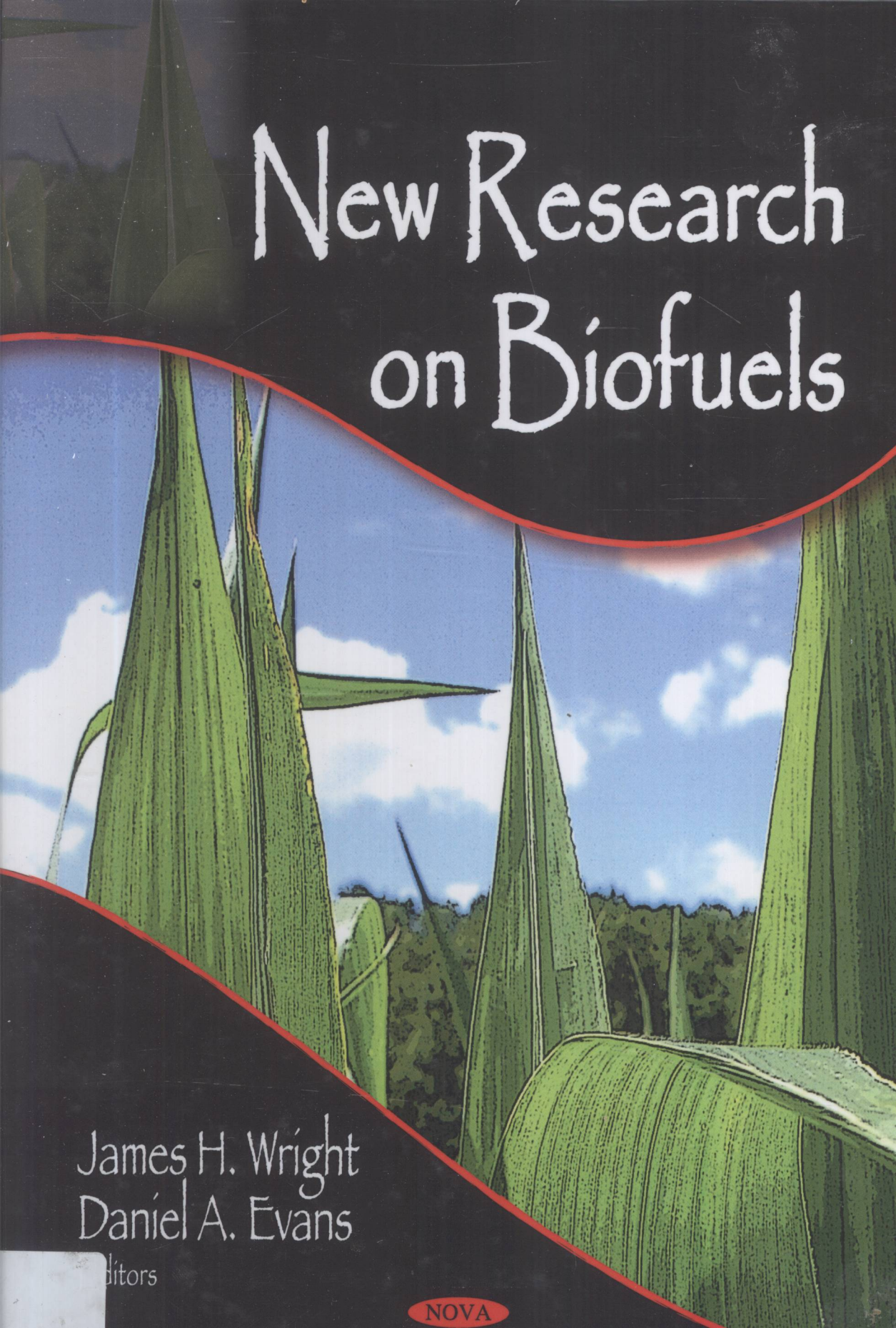
New Research on Biofuels



James H. Wright
Daniel A. Evans
Editors

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JAMES H. WRIGHT AND DANIEL A. EVANS
EDITORS

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NEW RESEARCH ON BIOFUELS

PREFACE

This book provides new research on biofuels from around the globe.

Chapter 1 - The production of ethanol by yeast is a key technology in ethanol fermentation. However, ethanol is toxic to yeast cells. It causes misfolding of proteins, an increase in membrane fluidity, changes in mRNA export from the nucleus, and activation of various stress signaling pathways, including the protein kinase A pathway. Ultimately, it causes cell death. Ethanol-induced death in yeast occurs during the fermentation of several alcoholic beverages such as sake (Japanese rice wine) and wine. In contrast, during most of the fermentation of bioethanol yeast cells do not die, either because, the ethanol concentration does not reach a high enough level, or because the fermentation period is not long enough to cause the yeast to die. However, future bioethanol production technology may require more severe fermentation conditions. Such technology could include the production of ethanol to a higher ethanol concentration, repetitive fermentation by fixed yeast cells, or fermentation at high temperature. Thus, ethanol-induced death during fermentation may also be a limiting factor in the future production of bioethanol. Therefore, elucidation of the mechanism of ethanol-induced death merits further research. This knowledge could be used for the development of technology to enhance yeast productivity in the fermentation of alcoholic beverages and bioethanol.

Cell death can be grouped into two categories—necrosis and apoptosis. While necrosis is a catastrophic and passive death, apoptosis is a programmed death that is of benefit to the organism as a whole. Historically, apoptosis was first identified as an altruistic death in the cells of multicellular organisms such as mammals. Although yeast is a unicellular organism, many apoptotic factors have recently been identified in yeast, and several stimuli have been reported to induce mitochondria-mediated apoptosis in this organism. Ethanol-induced death in yeast also exhibits features of apoptosis mediated by the mitochondrial fission pathway under certain conditions. Whether yeast causes apoptosis during fermentation remains to be determined. However, it appears that manipulation of apoptotic factors in yeast has the potential to increase fermentation efficiency and to ensure the survival of yeast during storage.

Chapter 2 - Two of the main plants currently being considered as potential biofuel feedstocks in the U.S. are switchgrass (*Panicum virgatum* L.) and maize (*Zea mays* L.). Recent expanded production of both has raised serious questions about natural resource utilization, notably, soil carbon, soil nutrients, and water. Water is often the limiting resource for crop and grass productivity. The objective of this study was to calculate and compare water use and water use efficiency of maize with current growth characteristics, switchgrass

with current growth characteristics, and switchgrass with characteristics improved by normal plant breeding selection techniques. We used the calibrated and validated ALMANAC model for five sites representing the southern Great Plains (Stephenville, TX), the northern Great Plains (Mead, NE), and two locations in the Corn Belt (Ames, IA and Columbia, MO). Ten years of historical weather data were used. Mean values for water use and water use efficiency were calculated for maize, switchgrass with currently growth characteristics, and switchgrass with anticipated improved growth characteristics. These results show the relative impact of expanded maize production, expanded switchgrass production, and use of improved switchgrass varieties, on the water balance in these regions. The water use efficiency (WUE) of four switchgrass types showed means ranging from 3 to 5 mg g^{-1}. Switchgrass WUE values were much greater than WUE of maize grain, but such was not always the case when compared to WUE of maize plants. Changes in switchgrass light extinction coefficients (k) and in switchgrass radiation use efficiency (RUE) showed the expected trends. Increased RUE caused increases in dry matter yield and in WUE, but not usually as great as the percentage increase in RUE. Results from this simulation work will give guidance to policy planners, producers, and economists.

Chapter 3 - Energy remains the mainstay for the entire civilized world. The concept dates back to 1885 when Rudolf Diesel built the first diesel engine with the full intention of running it on vegetative source. The Kyoto protocol has prompted resurgence in the use of biodiesel throughout the world. There is a growing interest in *Jatropha curcas* as a biodiesel 'miracle tree' to help alleviate the energy crisis, reduce the countries dependence on foreign oil imports and generate income in rural areas of developing countries. It is becoming a poster child amongst some proponents of renewable energy. *J.curcas* also called the physic nut is used to produce the non-edible *Jatropha* oil and the estimates of the oil content in seeds range from 35-40% and in the kernels 55-60%. India is the 5th largest energy consumer in the world. The country imported 90MT of crude oil by 2003-04 that was only 70 % of the requirement. By 2030 the estimated consumption is to scale up to 5.6 m barrels/day of which 95% is to be met by import. The ever-increasing demand can only be met by an alternative source as biofuel. Normal conventional propagation of *Jatropha curcas* has several drawbacks like poor seed viability, low germination, scanty and delayed rooting of seedlings, etc. The yield of nuts is unsure and unsustainable, coupled with unknown genetic potential. The plants propagated by cuttings show a lower longevity and possess a lower drought and disease resistance. Therefore, the need of the hour is to shake hands with biotechnological processes to overcome the hindrances. This chapter is just a hand forward to uniform, genetically stable, quality planting material yield, intricately interwoven with sustainability in energy. Plant biotechnology serves as a powerful tool for fast and quality plant production, and regerneration of plants in *J.curcas* has been successfully applied. Protocol development is the prerequisite for creating genetically improved crops, and three protocols have been successfully developed that will serve as an ideal system for future transgenic research and can act as a powerful tool for genetic improvement of the plant species. Various authors have reported *in vitro* micropropagation in *J.curcas*, using different explants. This chapter will highlight an up-to-date overview of the present and future trends of research in *Jatropha curcas* along with the work done in the author's laboratory.

The authors have reported micropropagation through nodal meristem culture from field grown plant. This is the first report of complete plant regeneration through somatic embryogenesis in this species. Somatic embryogenesis is the focus of all applied research and

it is now considered as the gateway to many more technologies. Plant propagation by somatic embryogenesis not only helps to obtain a large number of plants year round, but also can act as a powerful tool for further transgenic research. In this review we are reporting complete plant production from excised immature zygotic embryos. This method can serve as a support system to conventional and modern agriculture for the genetic improvement of the crop.

Chapter 4 - On an experimental field in eastern Germany 10 haulm-type and woody crop species which are adequate for combustion and gasification have been cultivated under practical conditions on a sandy brown soil for 14 years now. Each crop received 4 different levels of fertilization, from 0 kg N ha^{-1} to 150 kg N ha^{-1} combined with straw and wood ashes. The measuring program includes yields, energy gain and environmentally relevant substances in plants and soil, as well as fertilizer-induced emissions. Measured long-term yields are between 7 and 10 t ha^{-1} y^{-1} for all crops with the exception of topinambur and a special poplar variety. In contrast to the yields of haulm-type crops which decrease along with reduced fertilization, the woody crops such as poplar and willow show an increase. Energy yields average out between 100 and 160 GJ ha^{-1} y^{-1}, while the energy demand for cultivation and harvesting only accounts for 1% to 13% of these yields.

The nitrogen content (N_t) varies between the crops in a range from 0.1% to 3.3% and depends on the fertilization level. With 0.2 to 1.2% N_t, poplars and willows have only half the N_t of whole crop cereals and hemp, and approximately only one third that of cocksfoot grass. Therefore, these species cause less NO_x emissions during combustion. Nitrous oxide (dinitrogen oxide: N_2O) flux measurements, carried out over nine years, show that the mean nitrogen conversion factor, which describes the N_2O greenhouse burden of fertilization, is about 0.8±0.1 % and thus slightly lower than the default value of 1% for N_2O inventories. Therefore, the production of lingo-cellulosic energy crop species considered here will not lose its CO_2 advantage by nitrogen fertilizing as long as fertilizing results in an adequately higher biomass yield.

Soil organic carbon (C_{org}) stocks are likely to be improved by a land-use change from annual to woody crops. Twelve years after establishment of the plantation, a comparison of C_{org} stocks under annual crops with C_{org} under short rotation willow and poplar revealed an increase of soil C_{org} under the trees of 1300 kg ha^{-1} y^{-1}. By comparison, fertilization only accounts for a difference in soil C_{org} between fertilized and non-fertilized tree blocks of 250 kg ha^{-1} y^{-1}.

Chapter 5 - Biomass is a key feedstock to produce renewable biofuels such as Fischer-Tropsch hydrocarbons, methanol, and hydrogen. However, limitation of land and water, and competition with food production reduce potential significance of biomass as a renewable energy source. The development of efficient conversion technologies, which are able to compete with fossil fuels, is a key challenge for biomass-based systems.

Production of biofuels is traditionally analyzed by energetic analysis based on the First Law of Thermodynamics. However, this type of analysis shows only the mass and energy flows and does not take into account how the quality of the energy and material streams degrades through the process. In this chapter the exergy analysis, which is based on the Second Law of Thermodynamics, is used to analyze the conversion of biomass to biofuels.

The most promising biomass-to-biofuel route is a two-stage process involving production of syngas from biomass gasification, followed by synthesis of transportation fuels. Several overall technological chains biomass-to-biofuels are evaluated, including Fischer-Tropsch

hydrocarbons, hydrogen, and methanol. It is shown that that exergetic efficiency of production of biofuels is lower than that for fossil fuels.

Biomass gasification shows the largest exergy losses in the overall chain biomass-to-biofuels. Modification of process conditions to improve efficiency of gasification is discussed. It is shown that the optimal gasification conditions correspond to the carbon boundary point where all carbon present in biomass is gasified. The efficiency of biomass gasification can be also improved in a thermal pretreatment called torrefaction.

Chapter 6 - In this study energy balance and fuel properties of biodiesel has been calculated. Accordingly, the cost of 1 liter of oil is calculated 0.32 € after the income from the seed meal is deduced. Finally, the cost of per unit of biodiesel (1 liter) was calculated as 0.55 €, after deduction of the income provided by the sales of glycerin for use in soap and cosmetic industry.

The energy equivalent of total output was calculated 147605.50 MJ per hectare. The net energy gain (refined oil) was found as 15105.63 MJ per hectare (The net energy ratio 11.031) according to yield and inputs values.

The viscosity values of vegetable oils vary between 27.2 and 53.6 mm^2/s whereas those of vegetable oil methyl esters between 3.59 and 4.63 mm^2/s. The flash point values of vegetable oil methyl esters are highly lower than those of vegetable oils. The flash point values of vegetable oil methyl esters are highly lower than those of vegetable oils. An increase in density from 860 to 885 kg/m^3 for vegetable oil methyl esters or biodiesel increases the viscosity from 3.59 to 4.63 mm^2/s and the increases are highly regular. There is high regression between density and viscosity values vegetable oil methyl esters. The relationships between viscosity and flash point for vegetable oil methyl esters are irregular. An increase in density from 860 to 885 kg/m^3 for vegetable oil methyl esters increases the flash point from 401 to 453 K and the increases are slightly regular.

The LHV values of vegetable oils methyl ester vary between 35.74 and 39.16 MJ/kg.

Expert Commentary - Large scale production and consumption of biofuel is being promoted by many nations because of its potential for economic, political, and environmental benefits. Biofuels are partially renewable and substituting them for petroleum fuels may reduce emissions of greenhouse gases and other pollutants, and dependency on imported oil. Of special interest is the possibility of widespread use of biofuels such as biodiesel in the transportation sector, especially if integrated with existing and new hybrid and plug-in technologies and emission control technologies such as particle traps.

CONTENTS

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Chapter 1

THE MECHANISM OF ETHANOL-INDUCED DEATH IN YEAST AND ITS POTENTIAL APPLICATION FOR THE FERMENTATION INDUSTRY

Hiroshi Kitagaki
National Research Institute of Brewing, Senior Researcher
Hiroshima Prefecture, JAPAN

ABSTRACT

The production of ethanol by yeast is a key technology in ethanol fermentation. However, ethanol is toxic to yeast cells. It causes misfolding of proteins, an increase in membrane fluidity, changes in mRNA export from the nucleus, and activation of various stress signaling pathways, including the protein kinase A pathway. Ultimately, it causes cell death. Ethanol-induced death in yeast occurs during the fermentation of several alcoholic beverages such as sake (Japanese rice wine) and wine. In contrast, during most of the fermentation of bioethanol yeast cells do not die, either because, the ethanol concentration does not reach a high enough level, or because the fermentation period is not long enough to cause the yeast to die. However, future bioethanol production technology may require more severe fermentation conditions. Such technology could include the production of ethanol to a higher ethanol concentration, repetitive fermentation by fixed yeast cells, or fermentation at high temperature. Thus, ethanol-induced death during fermentation may also be a limiting factor in the future production of bioethanol. Therefore, elucidation of the mechanism of ethanol-induced death merits further research. This knowledge could be used for the development of technology to enhance yeast productivity in the fermentation of alcoholic beverages and bioethanol.

Cell death can be grouped into two categories—necrosis and apoptosis. While necrosis is a catastrophic and passive death, apoptosis is a programmed death that is of benefit to the organism as a whole. Historically, apoptosis was first identified as an altruistic death in the cells of multicellular organisms such as mammals. Although yeast is a unicellular organism, many apoptotic factors have recently been identified in yeast, and several stimuli have been reported to induce mitochondria-mediated apoptosis in this organism. Ethanol-induced death in yeast also exhibits features of apoptosis mediated by

the mitochondrial fission pathway under certain conditions. Whether yeast causes apoptosis during fermentation remains to be determined. However, it appears that manipulation of apoptotic factors in yeast has the potential to increase fermentation efficiency and to ensure the survival of yeast during storage.

INTRODUCTION

Many fermentation industries utilize the ability of the yeast *Saccharomyces* to ferment and to produce ethanol. However, ethanol itself accumulates in yeast cells (D'Amore et al., 1988; Dombek and Ingram, 1996), is toxic, and ultimately causes the death of the yeast cells during some forms of ethanol fermentation. One such example is in the brewing of sake, Japanese rice wine. During sake brewing, the concentration of ethanol reaches as high as 21% volume per volume, which causes the yeast to die. During the production of bioethanol, the concentration of ethanol also reaches a toxic level for yeast cells. However, in many cases, the concentration of ethanol is not high enough, or the fermentation period is not long enough to cause death. However, future advances in fermentation technology of bioethanol might require more severe growth conditions for yeast cells. For example, if a higher temperature was used for faster fermentation, or if repeated fermentation was used in order to bypass propagation process of yeast cells. Therefore, yeast cell death during fermentation of bioethanol could be a problem in the future. For all of the above reasons, elucidation of the pathway by which ethanol induces cell death is potentially useful for enhancing fermentation productivity. This pathway could be used as a basis for the design of strategies to prevent the ethanol-induced death of yeast during industrial fermentation.

CELLULAR TARGETS OF ETHANOL TOXICITY IN YEAST

Ethanol has various deleterious effects on yeast cells, which are summarized in Figure 1. Although the precise mechanism by which ethanol induces cells death is complex and remains undefined, the primary targets of ethanol stress appear to be protein structure and membrane lipids. Examples of proteins whose structures are modulated by ethanol include GABA (A) and NMDA receptors of neurons (Peoples and Weight, 1999). Moreover, ethanol inhibits enzymes such as hexokinase (Augustin et al., 1986) and dehydrogenases (Nagodawithana and Steinkraus, 1976). One of the heat shock proteins, Hsp104, which can refold and reactivate aggregated proteins, has been shown to play a critical role in ethanol tolerance (Fahrenkrog et al., 2004), further suggesting a critical role for protein structure in the action of ethanol. Secondly, cell membranes are considered to be one of the primary targets of ethanol stress (Dombek and Ingram, 1984). Ethanol is believed to increase the membrane fluidity of yeast cells and induce oxygen-derived free radical attack of membrane lipids (Beaven et al., 1982; Alexandre et al., 1994a; Alexandre et al., 1994b; Chi and Arneborg, 1999; Ingram, 1976; Kajiwara et al., 1996; Mishra and Prasad, 1989; Swan and Watson, 1999). Consistent with this hypothesis, when yeast cells are supplemented with fatty acids, oleic acid ($C_{18:1}$) is the most potent in endowing cells with ethanol tolerance, while linoleic ($C_{18:2}$) or linolenic ($C_{18:3}$) acids have weaker effects on ethanol tolerance (Swan and

Watson, 1999). Moreover, You et al. demonstrated that ethanol tolerance is dependent on oleic acid content. They showed that a desaturase-deficient *ole1* knockout strain is deficient in growth in the presence of ethanol but the same cells, when supplemented with oleic acids, are rescued from this growth deficiency in ethanol-containing medium (You et al., 2003). From these data, they concluded that ethanol tolerance in yeast results from incorporation of oleic acid into lipid membranes, which leads to a compensatory decrease in membrane fluidity that counteracts the fluidity-increasing effect of ethanol. Other studies also confirm the role of fatty acids in ethanol resistance. Overexpression of *OLE1*, a desaturase gene (Kajiwara et al., 2000), or exogenous *FAD2*, a delta-12 fatty acid desaturase gene (Kajiwara et al., 1996), renders yeast cells tolerant to ethanol (*S. cerevisiae* does not encode *FAD2* in its genome). In addition, growth in a culture medium rich in palmitic acid ($C_{16:0}$) renders yeast cells ethanol tolerant (Ohta and Hayashida, 1983). Ergosterol, a rigid structural component of membrane lipids, also confers ethanol tolerance to yeast cells (Novotný et al., 1992; Inoue et al., 2000; Alexandre et al., 1994). Hsp12 is reported to be induced upon ethanol treatment, and to protect cell membranes (Sales et al., 2000). In support of this role of Hsp12, cells deleted in *HSP12* were extremely sensitive to ethanol (Sales et al., 2000). Growing yeast in a media containing lipids such as a mixture of a surfactant Tween 80, ergosterol and monoolein, or phosphatidylcholine (Hayashida et al., 1974; Hayashida et al., 1975; Hayashida et al., 1976; Hayashida and Ohta, 1978; Hayashida and Ohta, 1980; Ohta and Hayashida, 1983) increased the final ethanol concentration obtained following fermentation of sake yeast strains, indicating a critical role of these lipids in ethanol fermentation ability. From the observation that yeast cells enriched with phosphatidylserine had greater ethanol tolerance, it was suggested that the anion:zwitterion ratio of phospholipids may be one of the important determinants of ethanol tolerance in *S. cerevisiae* (Mishra and Prasad, 1998). The ratio of unsaturated fatty acids has been shown to increase at later stages of ethanol fermentation (Gil et al., 1990). There is also a study which reports that yeast cells adapted to increased concentrations of produced ethanol contain increased amount of ergosterol and decreased amount of lanosterol, increased amount of phosphatidylinositol and decreased amount of phosphatidylcholine, and increased amount of C18:0 fatty acids in total phospholipids and decreased amount of C16:0 fatty acids (Arneborg et al., 1995). It is also reported that palmitoyl-CoA pool and incorporation of exogenously added palmitic acid by Faa4 is critical for growth in the existence of ethanol (Nozawa et al., 2002). Several studies have reported that ethanol damages mitochondrial DNA (Castrejón et al., 2002; Ibeas and Jimenez, 1997). Moreover, ethanol treatment of yeast cells has been reported to increase reactive oxygen species, most of which reside in the mitochondria (Du and Takagi, 2007). Together with the observation that cells deficient in superoxide dismutase are sensitive to ethanol (Costa et al., 1997), these facts suggest a critical role of reactive oxygen species in the ethanol-response of yeast. More recently, it has been shown that leakage of reactive oxygen species is caused by membrane damage to the mitochondria caused by ethanol (Chi and Arneborg, 1999). The combined results are consistent with the hypothesis that ethanol causes membrane damage by enhancing membrane fluidity and that a reduction in membrane fluidity renders yeast cells tolerant to ethanol.

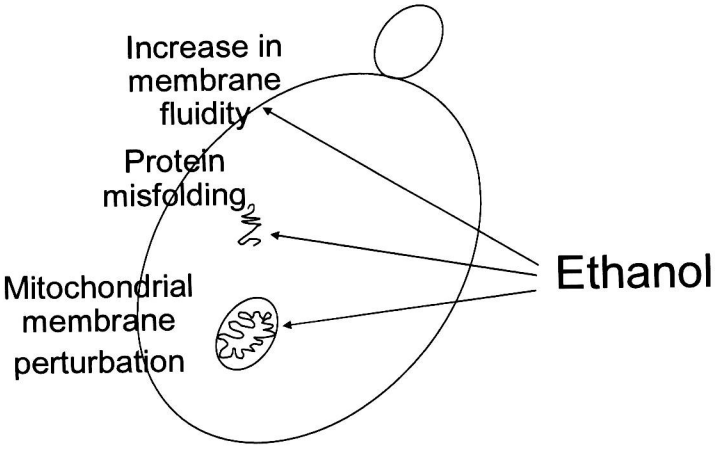

Figure 1. Cellular targets of ethanol toxicity in yeast.

PHYSIOLOGICAL RESPONSES OF YEAST CELLS CHALLENGED WITH ETHANOL

As a result of the toxic effects of ethanol on yeast cells described above, yeast cells induce several physiological responses (Figure 2). These responses include activation of cell signaling pathways and induction of cell-protective molecules such as heat shock proteins and trehalose. Signaling pathways activated by ethanol included the stress-responsive protein kinase A-Msn2 pathway (van Voorst et al., 2006; Martínez-Pastor et al., 1996). Ethanol treatment induces the translocation of a GFP-tagged-Msn2 into the nucleus (van Voorst et al., 2006; Watanabe et al., 2007). Stress response elements (STRE)-regulated genes such as *CTT1*, *DDR2* and *HSP12*, are also upregulated upon ethanol treatment (Martínez-Pastor et al., 1996; Schüller et al., 1994). It has been reported that one family of cell integrity and stress response sensors localized on the plasma membrane plays critical roles in the ethanol response of yeast (Zu et al., 2001). Yeast cells disrupted in these sensors (*wsc1-3*△) were sensitive to, and showed insufficient adaptation to ethanol. Wsc1-3 have been reported to activate the signaling cascade of Rho1, MAPK cascade (Bck1, Mkk1/2, Mpk1), Pkc1 and Rlm1 (Philip et al., 2001). Consistent with the involvement of this signaling pathway with ethanol tolerance, Takahashi reported that *BEM2* and *ROM2*, which encode a GAP (GTPase activating protein for Rho1) and a GEF (GDP-GTP exchange factor for Rho1), respectively, are required for the growth of yeast in ethanol (Takahashi et al., 2001). The combined data indicate that the Wsc1-3-Rho1-MAPK cascade-Pkc1-Rlm1 signaling pathway plays a critical role in ethanol tolerance.

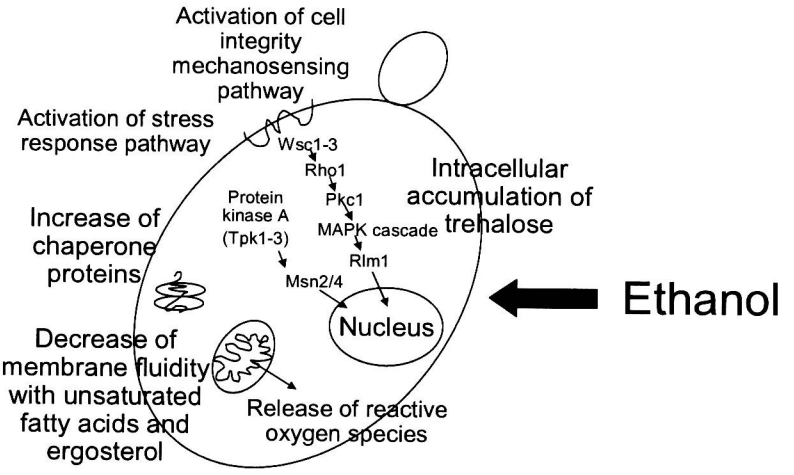

Figure 2. Physiological response of yeast cells challenged with ethanol.

Several metabolic constituents within yeast cells are required for ethanol tolerance. For example, intracellular trehalose protects yeast cells against ethanol inhibition of endocytosis (Lucero et al., 2000), its cellular level is increased upon ethanol stress (Sharma, 1997), and the trehalose biosynthesis genes are required for growth in the presence of ethanol (van Voorst et al., 2006; Kubota et al., 2004), although clear correlation between the trehalose content and viability could not be observed (Alexandre et al., 1998). Also, normal tryptophan biosynthesis is required for ethanol tolerance, since many yeast mutants that are deficient in tryptophan biosynthesis are ethanol sensitive, based on DNA microarray data analysis (Hirasawa et al., 2007). Inositol levels also influence ethanol tolerance, since increasing the inositol levels of yeast in static culture results in high ethanol tolerance, probably because of the resulting increase in phosphatidylinositol (Furukawa et al., 2004). Disruption of the genes *URA7* and *GAL6* also improves ethanol tolerance and the fermentation capacity of yeast, probably through modification of cell wall integrity and membrane fluidity (Yazawa et al., 2007).

Ethanol induces the transcription of specific sets of genes including genes involved in ionic homeostasis, heat protection, trehalose synthesis and antioxidant defense and energy metabolism (Alexandre et al., 2001). Moreover, ethanol stress also induces the accumulation of bulk polyA$^+$ mRNAs in the nucleus in a similar manner to that induced by heat shock (Takemura et al., 2004). However, the mRNAs of heat shock proteins are selectively exported from the nucleus whereas export of the DEAD box helicase Rat8p from the nucleus is observed only in ethanol-treated cells, but not in heat-shocked cells. This suggests that a mechanism of nuclear export exists that can distinguish between heat shock and ethanol shock. When yeast are grown on wine must and synthetic media the Rat8p accumulates in the nucleus as fermentation proceeds. However, during sake brewing, this protein only accumulates in the nucleus of the yeast until the ethanol concentration of the mash reaches approximately 12%. At a later stage of sake brewing, Rat8p re-localizes to the cytosol (Izawa et al., 2005a). The accumulation of bulk polyA$^+$ mRNAs in the nucleus has also been observed during wine brewing (Izawa et al., 2005b). These results suggest that selective export of polyA$^+$ mRNAs has a physiological role in wine fermentation and sake brewing.

Ethanol also enhances the formation of P-bodies, in which mRNAs, for example mRNAs that are not translated, are stored and degraded (Izawa et al., 2007). The formation of P-bodies was enhanced upon ethanol treatment and during fermentation of wine. In contrast, during fermentation of sake, the number and size of P-bodies began to increase gradually only after the ethanol concentration exceeded 13%.

Asr1, which is a ring/PHD protein that constitutively shuttles between the nucleus and the cytoplasm but accumulates in the nucleus upon exposure to ethanol, has been reported to be involved in ethanol sensing of yeast cells (Betz et al., 2004). The hypersensitivity of Asr1-deficient mutants to alcohol and sodium dodecyl sulfate could not be confirmed (Izawa et al., 2006). There is another study that reports the normal sensitivity of asr1 disruptant to 8% ethanol but the translocation of GFP-tagged Asr1 to the nucleus upon treatment with 6% ethanol (van Voorst et al., 2006). Therefore, the significance of Asr1 in ethanol sensing remains open to discussion.

APOPTOSIS AND ITS MECHANISM IN YEAST

There are two forms of cell death, necrosis and apoptosis. Necrosis is a catastrophic and passive death. Apoptosis is an altruistic cell suicide program first identified in the cells of multicellular organisms such as mammals. In multicellular organisms, the clear purpose of apoptosis is to sacrifice some cells for the benefit of the entire organism. Therefore, the reason why unicellular organisms might have an apoptotic program is not immediately apparent. One hypothesis is that unicellular organisms execute apoptosis in order to benefit siblings with similar genomes by release of nutrients from the apoptotic cells. However, nutrients emitted from apoptotic cells do not necessarily reach their siblings. A second hypothesis is based on the fact that apoptotic cells degrade their genomes (Ribeiro et al., 2006). Thus, it has been suggested that apoptotic cells release DNA fragments and that these DNA fragments cause homologous recombination in the neighboring cells. As a result, genes are transmitted from the apoptotic cells to their siblings. However, this hypothesis remains to be proved.

Many, but not all, of the known mammalian apoptotic factors are conserved in yeast. For example, caspase, which is the key protease of apoptosis in mammalian cells, is conserved in yeast and plays a critical role in apoptosis induced by H_2O_2 (Madeo et al., 2002), chronological aging (Herker et al., 2004), hyperosmotic stress (Silva et al., 2005) and viruses (Ivanovska et al., 2005) in yeast. However, it is clearly not involved in DNA damage-(Wysocki et al., 2004) or ethanol-induced apoptosis (Kitagaki et al., 2007a) in yeast. The enzymatic activity of the yeast caspase homolog was experimentally confirmed upon treatment of yeast with hydrogen peroxide (Madeo et al., 2002). *AIF1*, which encodes apoptosis-inducing factor, also regulates H_2O_2-induced apoptosis (Wissing et al., 2004). Aif1p was shown to translocate from the mitochondria to the nucleus upon treatment with an apoptotic stimulus and its in vitro DNA degradation activity has been demonstrated. Bir1p protein, the inhibitor of apoptosis protein, is conserved in yeast, is a substrate of the human pro-apoptotic serine protease Omi/HtrA2, and has been show to play a role in apoptotic events in yeast (Walter et al., 2006). Apoptotic nuclease, Tat-D, is also conserved in yeast and has been suggested to play a role in the yeast apoptotic process (Qiu et al., 2005). Nuclear

serine protease HtrA-like protein was recently identified in yeast as Nma111p and was reported to function in apoptosis upon heat treatment and hydrogen peroxide treatment in yeast (Fahrenkrog et al., 2004). Endonuclease G, that is normally localized in the mitochondria, but translocates to the nucleus in diseased states in mammalian cells, is encoded by *NUC1* in yeast and has been shown to function in a manner similar to that in mammalian cells (Buttner et al., 2007). Nuc1p executes apoptosis independently of metacaspase, but its apoptotic activity is dependent on the permeability transition pore protein, karyopherin Kap123p, as well as on histone H2B. Histone chaperone *ASF1/CIA1* also plays a role in apoptosis in yeast (Yamaki et al., 2001). The release of cytochrome c from mitochondria is also considered to play a key role in acetic acid- and pheromone-induced apoptosis in yeast as it does in mammalian cells (Ludovico et al., 2002; Zhang et al., 2006;Yamaki et al., 2001). In spite of these components of apoptosis that are highly conserved between yeast and mammals, some mammalian apoptotic factors are not encoded by the yeast genome. These apoptotic genes not encoded for by yeast include BH3-only proteins that regulate the permeability of the outer membrane of mitochondria. However, to our surprise, some of these proteins, such as, Bax, Bcl-2, tBid, Bad and Puma, when transfected into yeast do function in apoptosis in yeast, although others, including BNip3, BNip3L and Noxa, do not (Kissová et al., 2006; Yang et al., 2006; Zheng et al., 2007; Gonzalvez et al., 2005; Li et al., 2005; Guscetti et al., 2005; Polcic et al., 2003;Tao et al., 1997). These BH3-only proteins appear to regulate reactive oxygen species, since Bcl-2 reverses survival defects in yeast caused by disruption of superoxide dismutase (Longo et al., 1997). It is possible that the Fis1 protein, that is a yeast mitochondrial fission protein, can function in a similar manner to Bcl-2 (Fannjiang et al., 2004). It has been reported that yeast cells lacking the Fis1 protein contain an increased amount of reactive oxygen species, indicating that Fis1 regulates the release of reactive oxygen from the mitochondria (Kitagaki et al., 2007a). Neither of the apoptotic proteins Smac or Diablo are conserved in yeast (Guscetti et al., 2005). These results imply that common and specific mechanisms underlie apoptosis in yeast and mammalian cells.

APOPTOTIC FEATURES IN ETHANOL-INDUCED DEATH IN YEAST

Several apoptotic-inducing stimuli have been described for yeast. These include aging (Laun et al., 2001), hydrogen peroxide (Madeo et al., 1999), acetic acid (Guaragnella et al., 2006; Ludovico et al., 2001), high osmotic stress (Silva et al., 2005), heat stress (Lee et al., 2007), NaCl (Wadskog et al., 2004), UV (Del Carratore et al., 2002), nitric oxide (Almeida et al., 2007), ammonia (Váchová et al., 2005), pheromones (Zhang et al., 2006; Pozniakovsky et al., 2004), aspirin (Sapienza et al., 2005), killer toxins (Reiter et al., 2005), a mutation in *CDC48* (Braun et al., 2006), an N-glycosylation defect (Hauptmann et al., 2006), decapping of mRNA (Mazzoni et al., 2003; Mazzoni et al., 2005), the formation of F-actin and hyperactivated Ras signaling (Gourlay and Ayscough, 2005a; Gourlay and Ayscough, 2005a; Gourlay and Ayscough, 2006; Gourlay et al., 2004) and defects in the initiation of DNA replication(Weinberger et al., 2005).

Recently, it has been shown that ethanol-induced death of yeast exhibits features of mitochondrial fission pathway-mediated apoptosis (Kitagaki et al., 2007a). Ethanol-killed

yeast cells exhibited chromatin aggregation and fragmentation, DNA degradation, suppression of death by inhibition of de-novo protein synthesis as revealed by DAPI staining, TUNEL (terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling) labeling of the nucleus, pulse field electrophoresis and cycloheximide treatment. Consistent with this result, reactive oxygen species have been shown to increase upon exposure to ethanol (Du and Takagi, 2007).The Yca1 or Aif1 is not apparently involved in ethanol-induced death, although inhibition of caspase activity rescued the ethanol-induced death of *fis1*△ cells. When this data was combined with a comparison of the number of PI-positive and TUNEL-positive cells, the results suggested that not all of the cells die of apoptosis in ethanol-induced death, but that some of the cells die of necrosis. If all of the research on ethanol toxicity in yeast is combined, the following model is suggested. Ethanol treatment perturbs the mitochondrial membrane and, together with the modulation of protein structure, and the increase in membrane fluidity of other cellular membranes, induces yeast death by both apoptosis as well as necrosis. The ratio of apoptosis to necrosis appears to depend on the physiological state of the yeast cells at the time they are exposed to ethanol stress. These results suggest that inhibition of apoptosis during ethanol fermentation has the potential to at least partially enhance the survival of yeast cells challenged with ethanol, and, thereby to increase the productivity of ethanol fermentation to a certain extent.

Another study has shown that mitochondrial structure is present throughout the alcohol fermentation process and to fragment during the process (Figure 3, Kitagaki et al., 2007b). Since mitochondrial proteins have been reported to regulate (either accelerate or inhibit) apoptosis, this study suggests that introduction or manipulation of mitochondrial apoptotic factors such as BH3 family proteins (Ligr et al., 1998), cytochrome c (Ludovico et al., 2002), apoptosis-inducing factor (*AIF1*) (Wissing et al., 2004), Smac and Diablo (Anguiano-Hernandez et al., 2007) through genetic engineering techniques has the potential to endow yeast cells with the ability to encounter ethanol stress (Figure 4).

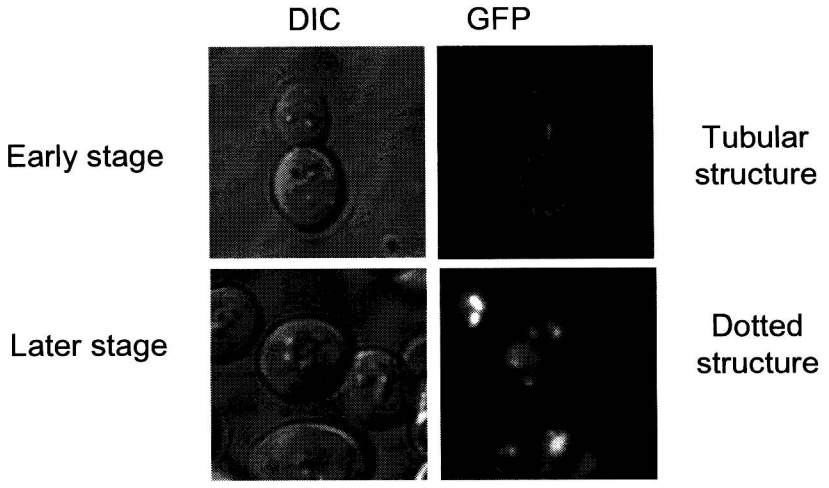

Figure 3. Mitochondrial morphology of yeast during alcohol fermentation.