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Full Length Research Paper

Effects of heat and ethanol on fermentable yeast cells

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This study investigated the effect of heat and ethanol on fermentable yeast cells. Fermentable yeast cells (*Saccharomyces cerevisiae* strains and non-saccharomyces species) were subjected to varying temperature (37, 40 and 44°C) and ethanol concentration (8, 10, 14 and 18%) respectively. In the research, 55.35% of the fermentable yeasts were highly thermotolerant, moderately thermotolerant (42.86%), slightly thermotolerant (1.79%) while the industrial strain was non-thermotolerant. With respect to ethanol - osmotic shock effect, 41.07% were highly tolerant, moderately tolerant (58.93%) while none was slightly or non-tolerant. Thus, there was significant difference in biomass yield obtained at different temperatures and ethanol concentrations. Furthermore, the flocculation analysis showed that 80% of the yeast cells were highly flocculant, slightly flocculant (13%) and non-flocculant (7%) respectively. Summarily, exposure of yeast cells to elevated temperatures and ethanol concentrations increase ability of cells to survive exposure to lethal heat and ethanol osmotic shock during ethanol production.

Key words: Fermentable yeasts, heat shock, ethanol, flocculation.

INTRODUCTION

Thermotolerance occurred as a result of both increased protection of cellular components at extreme temperatures and increased reactivation and/or degradation of heat-inactivated components. The major components of cells, such as proteins and plasma membrane are affected when cells are exposed to elevated temperatures (Beney and Gervais, 2001). Thus, under defined conditions, most microorganisms acquire certain degree of thermotolerance to protect their components (Morozov et al., 1997). Yeasts exposed to a mild preconditioning heat treatment acquired certain degree of thermotolerance related to heat shock factor and stress response element pathways that regulate the synthesis of heat shock proteins (Hsps) which are major factors in the acquisition of thermotolerance (Morano et al., 1998). The expression of several Hsps, (including Hsp104) induced in cells recovering from severe heat shock (delayed upregulation) is responsible for the refolding of heat-denatured proteins and survival (Seppä and Makarow, 2005).

Studies based on detailed physiological and molecular analyses have contributed to the understanding of the processes underlying ethanol stress tolerance aiming to increase ethanol productivity and successful engineering of yeast transcription machinery (Alper et al., 2006). Factors that affect ethanol tolerance include the proportion of ergosterol in the cellular membranes, phospholipid biosynthesis, degree of unsaturation of membrane fatty acids, temperature, the activity of plasma membrane ATPase, superoxide dismutase, and the capacity of a strain to produce trehalose (Alexandre et al., 1994). Heat and ethanol stress cause similar changes to plasma membrane protein composition, reducing the levels of plasma membrane H+-ATPase protein and inducing the plasma membrane-associated Hsp30. Yeast responses to ethanol-induced stress include changes in

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Table 1. Range of yeast biomass yield at different temperature.

Isolate	Temperature (°C)		
	37	40	44
Wild strains (lowest biomass yield/OD)	0.747 ± 0.001	0.375 ± 0.003	0.016 ± 0.001
Wild strains (highest biomass yield/OD)	1.999 ± 0.001	1.532 ± 0.001	1.367 ± 0.001
Industrial strain biomass yield/OD	0.361 ± 0.001	0.211 ± 0.003	0.128 ± 0.001

Initial biomass yield at 0 h = 0.512 OD.
*Results are arithmetic means and standard deviation of triplicate analysis

the levels and composition of membrane phospholipids and ergosterol which affects the level of plasma membrane organization and function leading to intracellular acidification (You et al., 2006). In response to this effect, yeast exhibits increased plasma membrane H+-ATPase activity, which is important to maintain the intracellular pH and secondary transport mechanisms, which are dependent on the proton gradient across the plasma membrane (Aguilera et al., 2006). Thus, the expression of ethanol tolerance determinants is highly variable depending on the strain and growth condition (Miguel et al., 2009). The aim of this study is to determine the effect of elevated temperature and ethanol concentration on yeast biomass yield.

METHODOLOGY

Yeast cell required for analysis

Fermentable yeast cells used for the analysis were isolated from locally fermented beverages comprising different strains of *Saccharomyces cerevisiae* and non-saccharomyces species such as *Rhodotorula mucilaginosa, Rhodotorula glutinis*, *Candida colliculosa, Candida utilis*, *Candida magnolia*, *Candida pelliculosa, Trichosporon asahii* and *Cryptococcus albidus* respectively, while an industrial strain (*Saccharomyces cerevisiae IS-2*) was used as control.

Ethanol-osmotic effect

Sterile yeast-peptone broth containing graded concentrations of 10-18% ethanol inoculated with fresh yeast cells at 1.5×10^8 cells/150 ml media medium was used. The initial optical density of each inoculated medium was determined using spectrophotometer at 650 nm against the medium as blank (Osho, 2005). The inoculated medium was kept on shaker set at 150 rpm at 30°C for 48-72 h and increase in optical density was recorded as evidence of growth.

Exposure to elevated temperature

Yeast cells were grown in yeast-peptone-dextrose broth containing yeast extract, 10 g/l; peptone, 20 g/l and 10 g/l at 28°C for 24 h respectively. YPD medium was further inoculated with 0.1 ml of 5×10^5 cells/ml suspension of each isolate and cell cultures were exposed to a heat gradient of 37, 40 and 44°C respectively. Turbidity was determined using CECIL CE 1020 spectrophotometer at 615 nm subsequently at 24 h interval for 72 h. Cultures were slowly cooled from the different elevated temperatures to 25°C for 5 min in a water bath and plated immediately after the appropriate dilutions on YPD plates. Viability was recorded after 72 h of incubation at 28°C. All experiments were repeated independently (separate cultures at different times) in triplicate.

Flocculation rate determination

The yeast cells were harvested from 24 h old malt yeast extract-glucose-peptone medium by high-speed centrifugation at 1600 rpm for 10 min. A standard cell suspension of 1.5×10^8 cells/ml in 0.9% NaCl solution was prepared and utilized for measuring decrease in optical density reading over a period of 2 h at 650 nm (Nwachukwu et al., 2006); thus, the flocculation rate was expressed as follows:

$$\% \text{ Flocculation} = \frac{\text{Total decrease in optical density reading} \times 100\%}{\text{Colorimeter reading at 0 h}}$$

RESULTS

Thermotolerance of the yeast isolates

Thermotolerance exhibited by the various yeast strains at different temperatures are shown in Table 1. Initial biomass yield (absorbance) at 0 h was 0.512 OD (5×10^5 cells/ml) at 615 nm thus, yeast strains that had values greater than 0.512 OD at 37, 40 and 44°C were categorized as highly thermotolerant, moderately thermotolerant (greater than 0.512 OD at 37 and 40°C), slightly thermotolerant (greater than 0.512 OD at 37°C) and non-thermotolerant (less than 0.512 OD at all temperature). Based on these criteria, 31 strains (55.35%) were highly thermotolerant, 24 strains (42.86%) were moderately thermotolerant, 1 strain (1.79%) was slightly thermotolerant, while none was non-tolerant except the industrial strain (Figure 1). Furthermore, there was growth on all the agar plates subjected to viability

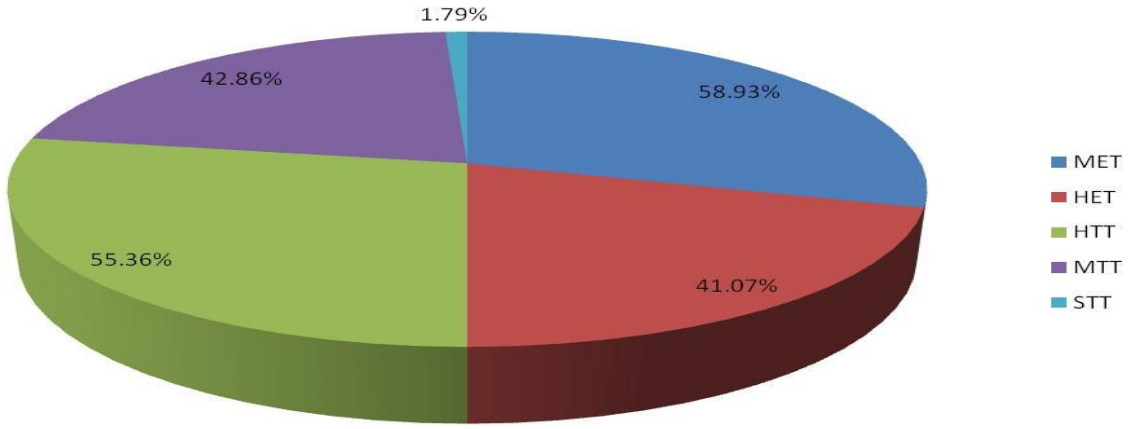

Figure 1. Yeast tolerance pattern to heat and ethanol stress. Key: MET = Moderately ethanol tolerant; HET = Highly ethanol tolerant; HTT = Highly thermotolerant; MTT = Moderately thermotolerant; STT = Slightly thermotolerant.

Table 2. Range of yeast biomass yield at various ethanol concentrations.

Isolate	Ethanol concentration (%)			
	10	14	16	18
Wild strains (lowest biomass yield/OD)	0.746 ± 0.033	0.619 ± 0.031	0.451 ± 0.016	0.119 ± 0.012
Wild strains (highest biomass yield/OD)	2.861 ± 0.025	1.562 ± 0.016	1.259 ± 0.083	0.933 ± 0.016
Industrial strain biomass yield/OD	0.842 ± 0.046	0.791 ± 0.083	0.589 ± 0.018	0.315 ± 0.062

Initial turbidity rate at 0 h = 0.451 OD. *Results are arithmetic means and standard deviation of triplicate analysis.

test except the industrial strain agar plate. This implied that all the isolates were viable and tolerant to heat shock at different temperature while the industrial strain was not viable after being subjected to the heat shock. There was significant difference in the biomass yield obtained at different temperatures using ANOVA test (P=0.05).

Ethanol tolerance of yeast isolates

The ethanol tolerance of wild yeasts revealed a wide range of tolerance levels between 10 and 18% (v/v). The initial turbidity rate at 0 h was 0.451 OD (1.5×10^8 cells/150 ml) at 650 nm, thus isolates that had greater than 0.451 OD at 10, 14, 16 and 18% ethanol concentrations were categorized as highly ethanol-tolerant strains, moderately tolerant (greater than 0.451 OD at 10, 14 and 16%), slightly tolerant (greater than 0.451 OD at 10 and 14%) and non-tolerant strains (less than 0.451 OD at all ethanol concentrations) respectively (Table 2). In this research, 23 (41.07%) isolates were highly tolerant, 33 strains (58.93%) were moderately tolerant while none were slightly or non-tolerant respectively. The industrial strain *Saccharomyces cerevisiae 2 –IS2* also tolerated 10 to 16% ethanol concentrations, thus it was categorized as moderately ethanol-tolerant strain (Figure 2). Statistically, there was significant difference in the biomass yield obtained at different ethanol concentrations using ANOVA test (P=0.05).

Flocculation rate

Among the yeast strains analyzed, 80% were highly flocculant, 13% slightly flocculant and 7% non-flocculant respectively. The yeast strain *Saccharomyces cerevisiae 2-PQ28* (89.78%) displayed the highest flocculation rate while the lowest flocculation rate was observed in *Candida colliculosa PB12* (26.82%). The flocculation rate of the brewing strain of *Saccharomyces cerevisiae 2 IS-2* was 66.89% after 60 h (Figure 2). Statistically, all isolates were not susceptible to flocculation using chi square test (P=0.025).

DISCUSSION

Heat shock proteins (Hsps) function as molecular chaperones in the synthesis, folding, trafficking, maturation and degradation of proteins (Burnie et al., 2006). They are rapidly induced when cells are subjected to elevated temperatures (Westerheide and Morimoto, 2005). Thus, the high thermotolerance pattern of some wild yeast strains in this study (Table 1 and Figure 1) may be due to the expression of heat shock proteins (Hsps).

The decrease in optical density of some yeast cells

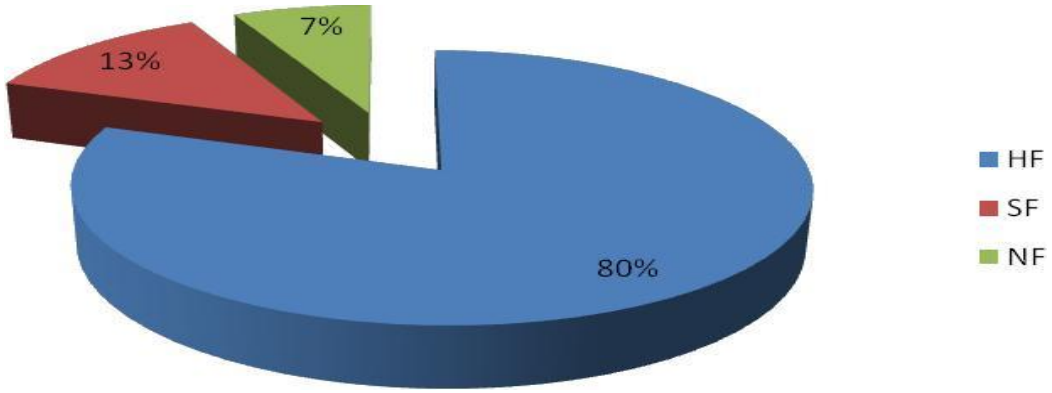

Figure 2. Flocculation rate of yeast isolates. Key: HF = Highly flocculant; SF = Slightly flocculant; NF = Non flocculant.

obtained may also be due to the possible scenario for the cellular response of cell to heat shock which involves an increase in membrane fluidity leading to an increase in cellular volume; these induce membrane compression because of the presence of the cell wall which eventually induces membrane permeabilization, ion and water leakage, and a decrease in cell volume leading to cell death according to Gervais et al. (2003).

The increased fraction of survival of wild yeast cells grown at physiological temperature (room temperature) and then subjected to mild heat shock (37°C) and lethal heat shock (40 and 44°C) agrees with the report of Piper (1993) that prior induction of the heat shock response enables cells to survive subsequent exposure to lethal high temperatures. These variations may be due to the presence of protein Hsp104 which is essential in the acquisition of thermotolerance by wild-type *S. cerevisiae* compared to the industrial strain. Lindquist and Kim (1996) reported similar pattern of survival levels by wild-type *S. cerevisiae* grown at 50°C. Partially redundant transcription factors Msn2 and Msn4 (Msn2/4), regulate the general stress response induced by heat shock, osmotic shock, oxidative stress, low pH, and glucose starvation (Estruch, 2000), thus, the non-tolerant capacity of the industrial strain and some wild yeast (*Saccharomyces cerevisiae* and non-saccharomyces) to various temperatures (40 and 44°C) is due to lack of Msn2/4 because cells lacking Msn2/4 do not exhibit temperature sensitivity at 38°C but exhibit thermotolerance defect (Martínez-Pastor et al., 1996). Statistical analysis using ANOVA showed that there was significant difference in the biomass yield obtained at different temperature and ethanol concentration.

Ethanol is well known as an inhibitor of microorganism growth and its toxic effects on yeast cells involve loss of cell viability and inhibition of both yeast growth and different transport systems such as the general amino acid permease and the glucose transport system (Alexandre and Charpentier, 1998). The variations in the ethanol tolerance level of the different yeast strains obtained from the fermented beverages (Table 2) agrees with the report of Gibson et al. (2007) that the stress induced by increasing amounts of ethanol accumulated to toxic concentrations during ethanolic fermentation which is the major factor responsible for reduced ethanol production and stuck fermentations. The increase in optical density of highly ethanol-tolerant strains (Table 2) may also be as a result of the presence of sterols especially ergosterol which promote growth and ethanol tolerance by providing rigidity to the cell membrane (Zinser et al., 1991). The variation in the optical cell density with respect to the ethanol concentration is due to variation in ergosterol to phospholipid ratio of the yeast cell composition according to the report of Chi et al. (1999) that a highly ethanol-tolerant strain of *S. cerevisiae* had a higher ergosterol to phospholipid ratio, a higher incorporation of long chain fatty acids in total phospholipids and a slightly higher proportion of unsaturated fatty acids in total phospholipids than a slightly or moderately ethanol-tolerant strain.

The decrease in optical density (growth) of some yeast strains isolated with respect to increase in ethanol concentration agrees with the report of Piper (1995) which stated that the rising ethanol level during batch fermentation acts initially to reduce growth and fermentation rates and adversely affects cell viability. The ability of some yeast strains obtained in this research to withstand 18% v/v ethanol concentration (Figure 1) is also due to the effect of an increase in fatty acid unsaturation in cellular membranes which increases ethanol tolerance (Alexandre et al., 1994). The yeast strains (*Saccharomyces cerevisiae* and the non-saccharomyces) that are slightly tolerant and non-tolerant to ethanol are due to the effect of the ethanol on the physiological and genetic make-up of the strains. Miguel et al. (2009) reported that ethanol interferes with membrane lipid organization, affecting its function as a matrix for enzymes, perturbing the conformation and

function of membrane transporters, increasing the nonspecific plasma membrane permeability, and leading to the dissipation of transmembrane electrochemical potential. Statistical analysis using ANOVA (P=0.05) showed that there was significant difference in the biomass yield obtained at different ethanol concentrations.

Yeast flocculation rate analyzed in this research showed that flocculation is characterized by intense interactions between yeast cells which result in the formation of large cell aggregates or flocs (Figure 2). Variation in the flocculation rate agrees with the report of Baryly et al. (2005) which stated that flocculation frequently occurs during late-logarithmic or stationary phases of growth upon depletion of the carbon source and this might be partly due to increased transcription of flocculin genes at the post-diauxic shift. Some processes have replaced centrifugation by the natural ability of certain yeast strains to flocculate (Kondo et al., 2002), or by the introduction of flocculation genes into non-flocculant strains (Watari et al., 1990), but recently cell separation is normally obtained by natural cell sedimentation (Schwan et al., 2001). The variation in yeast flocculation rate is due to the process employed by natural yeast strains presenting the *FLO* genes, in which the flocculins are inhibited by sugars, leading to sedimentation at the end of fermentation (Cunha et al., 2006). The highly flocculant yeast strains obtained in this research can be useful at an industrial scale because flocculating yeast cells have been employed only in the later phase of primary beer fermentation to separate biomass from the fermented broth (Domingues et al., 2001). Furthermore, the non-flocculant strains can be useful in biotechnology because the possibility of inducing controlled flocculation in a naturally non-flocculant strain provides great potential for improving brewing, wine-making, baking and ethanol-producing yeast (Dequin, 2001). The flocculation rate of the wild yeast isolates which occurs spontaneously compared with the industrial strain showed that flocculation of yeast cells can be used for separating yeast cells from beer in the brewing industry (Verstrepen et al., 2003). Statistical analysis using CHI square (P = 0.025) showed that all isolates are not susceptible to flocculation, that is, all isolates are not flocculant.

CONCLUSION AND RECOMMENDATION

Ethanol tolerance significantly improves when yeast cells are self-flocculated and form the flocs compared with the free yeast cells. The process is simpler and economically competitive compared with the yeast cell immobilization by supporting materials because no supporting material is consumed and it also completely eliminates the potential contamination to the quality of the product by the supporting materials. The yeast flocks during fermentation can be recovered by sedimentation rather than by centrifugation which is widely used in the recovery of free yeast cells, saving the capital investment for centrifuges as well as the energy consumption for centrifuge operation. Highly ethanol-tolerant yeast strains are recommended in order to overcome the problem with inhibition during fermentation because the amounts and types of inhibitory compounds vary strongly between different raw materials. Ethanol tolerance profile is also recommended in ethanol production rather than applying a fed-batch mode of substrate addition with proper feed protocol and control variables in which the levels of inhibitors are kept at an acceptable level.

Thermotolerance is important for simultaneous saccharification and fermentation (SSF) in order to allow fermentation at temperatures closer to the optimal temperature for the enzymes because in SSF, a compromise between the optimal temperatures for the cellulolytic enzymes and the yeast is required. Since some strains of *S. cerevisiae* are tolerant at 44°C and the optimal temperature for cellulolytic enzyme activity is 45-50°C, thus, fermentation at elevated temperature is recommended.

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