Research Article

Optimization of Fermentation Temperature for Very High Gravity Ethanol Production using Industrial Strain of *Saccharomyces cerevisiae* SC90

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Abstract

Very high gravity (VHG) ethanol fermentation has been remarkably developed in order to significantly enhance productivity through higher ethanol concentration in fermentation broth. This technique is helpful for saving water and energy consumption, reducing bacterial contamination, and decreasing distillate discharge treatment from the distillation system. However, this technology causes of yeast cell suffers according to several stresses including osmotic stress, anaerobic shift, accumulation of ethanol concentration, and nutrient limitation under VHG conditions. *Saccharomyces cerevisiae* produces heat during the process of ethanol production. Consequently, a cooling system is recommended during fermentation to disperse heat. There are many advantages including the increased capacity during the fermentation process at high temperatures, the reduction in the cooling cost, and the economic benefits from the increased productivity during fermentation and distillation. Even though VHG ethanol fermentation has several advantages, it causes the fermentation process to become stuck or sluggish, resulting in a prolonged attenuation period and an increase in the amount of unfermentable sugar. Consequently, the efficiency of ethanol production decreases. The basic background of yeast strain on stress tolerance including osmotic pressure, temperature, and ethanol is therefore very important to overcome this challenge. The current research aimed to identify the temperature range (30, 35 and 40°C) within which industrial ethanologenic strain of *Saccharomyces cerevisiae* SC90 would ferment effectively under VHG conditions. It also investigated the effect of fermentation temperature on cell growth and kinetic parameters of ethanol production from VHG medium (YPD containing 34% (w/v) glucose). The results showed that optimum fermentation temperature for ethanol production using SC90 was 35°C with the maximum amount of ethanol content at 97.03 g/l and volumetric productivity at 2.70 g/l.h. It could be summarized that the remaining percentage viability after complete fermentation indicates the ability of yeast in ethanol and temperature tolerance.

Keywords: Very high gravity fermentation; *Saccharomyces cerevisiae*; osmotic pressure; ethanol; temperature.

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1. Introduction

Bioethanol, a clean and renewable biofule, is reckoned as one of the best alternatives [1]. Several reasons have made it become a highly attractive fuel energy resource, including reduction in air pollution, CO_2 [2], and CO generation reduction in the emission of CO [3]. In addition, it can be blended with gasoline, currently 10% and 22% in the US and Brazil, respectively [2]. Bioethanol is produced from several kinds of substrates such as fermentable sugar and starch [4]. More recently, lignocelluloses have also been well known as a feedstock for bioethanol production [5]. The difference in ethanol production process depends on kinds of raw materials [3, 6]. Fermentable sugars in molasses or sugar cane are able to ferment to ethanol directly using yeast or bacteria [6]. Ethanol production from starch has to be started with digestion of starch using enzyme to fermentable sugars. Then, yeast can utilize sugars to produce ethanol [7]. It has been reported that to achieve the optimum conversion of sugar to ethanol this process requires a yeast strain capable of high ethanol tolerance because ethanol inhibits growth and fermentation [8]. *Saccharomyces cerevisiae* is one of several species of interest for bioethanol production in regards to a variety of substrate utilization, high growth and fermentation rates under severe conditions of ethanol production including low pH, high temperature as well as high ethanol concentration [8].

Very high gravity (VHG) fermentation is able to significantly improve ethanol productivity by enhancing ethanol concentration in media. A number of advantages of this technique have been reported including reduced energy, labor, cleaning, effluent costs, and an increase in the amount of ethanol per unit of fermentable broth because the reduction of yeast growth increase the glucose flux to ethanol production [9-11]. Normal gravity fermentation containing an initial sugar concentration of 150 to 200 g/l is fermented to produce ethanol concentration of only 7.5 to 10% (v/v) [12]. To enhance ethanol level, initial sugar concentrations higher than 200 g/l are required. However, this technology has been considered impractical because some problems adversely affect yeast fermentation performance [13, 14]. Initial high sugar content in the fermentation medium induces an increase in osmotic pressure which can have a deleterious effect on yeast cells [15]. In addition, elevated ethanol levels can cause an increase in the stress to yeast cells leading to stuck fermentation [16].

Temperature has been considered one of the most important environmental factors positively and negatively affecting yeast growth and ethanol production efficiency [17-19]. Increasing temperature induces a rate of yeast growth and fermentation. However, it also impairs ethanol tolerance due to the synergistic inhibition of ethanol and the high temperature results in stuck fermentation [12, 20, 21]. The optimum temperature for yeast cell growth and ethanol formation have been reported at range 30-32°C but it is strain dependent to be more tolerate above 35°C [22]. The rapid reduction of yeast growth rate has been observed after optimum point of temperature due to alteration of the cellular composition of yeasts under O_2 limited conditions [23], enzyme denaturation, and cellular membranes damage [24]. Even though high temperature has a deleterious effect on yeast fermentation performance, it offers the benefits of a minimized bacterial contamination, a reduction of cooling costs and therefore lower costs of energy consumption [19, 25]. Yeast strains for ethanol production should therefore be able to tolerate high temperature because ethanol fermentation cannot be operated under low temperature in summer for most tropical and

subtropical regions without a cooling system [21].

There is no report delineating stress tolerance and optimum temperature for VHG ethanol fermentation using industrial strain *S. cerevisiae* SC90. In the current research we aimed to investigate the effect of osmotic pressure induced by sorbitol, temperature, ethanol, and the synergistic effects between temperature and ethanol. According to several advantages of VHG fermentation and high temperature, this research identified the temperature range and investigated impact of temperature on very high gravity ethanol production using industrial yeast strain of *Saccharomyces cerevisiae* SC90. The results would be useful for tropical climates where a day time maximum temperature is 32°C. The data set might provide more understanding on stress occurrence during ethanol production and might be applied to improve stress tolerance in yeast under VHG fermentation.

2. Materials and Methods

2.1 Microorganism and Inoculum Preparation

S. cerevisiae SC90 was obtained from Department of Biotechnology, Faculty of Agro-Industry, Kasetsart University, Bangkok. The strain was pre-grown into a 250 ml Erlenmeyer flask containing 100 ml of YPD (20 g/l neutralised bacteriological peptone, 10 g/l yeast extract, 20 g/l glucose) media. The flask was incubated on an orbital shaker at 30°C and 120 rpm for 48 h. In order to increase cell concentration, we transfer the yeast into a 1000 ml Erlenmeyer flask containing 500 ml of YPD. The flasks were further incubated under the same conditions. After 48 h, this was used as an inoculum for ethanol production.

2.2 Spot Plate for Stresses Assessment

The culture was diluted to a concentration of 3×10^7 cells/ml with sterile distilled water in sterile microcentrifuge tubes. Four subsequent 1:10 dilutions of cell suspensions were performed. A volume of 10 µl from each dilution was spotted onto the surface of the plate. Cells were spotted in duplicates on each plate. Serial dilutions of the yeast strain were spotted onto YPD agar supplemented with different concentrations of sorbitol (0, 5, 10, 15, 20, 25 and 30% (w/v)). Plate cultures were incubated in an inverted position at 30°C under aerobic conditions for 2 days. For assessment of ethanol and temperature tolerance, serial dilutions of the yeast strain were spotted onto YPD agar supplemented with different concentrations of ethanol (0, 5, 10, 15, 20 and 25% (v/v)) and plates were incubated in an inverted position at various temperatures (30, 35 and 40°C). Growth was recorded using a camera.

2.3 Effects of Fermentation Temperature on Mini Laboratory Batch Ethanol Fermentation

The fermentation profile of yeast was studied by using mini laboratory scale fermentations. *S. cerevisiae* SC90 was inoculated into a 250 ml Erlenmeyer flask comprising 50 ml of YPD medium (340 g/l glucose concentration, 20 g/l neutralised bacteriological peptone and 10 g/l yeast extract) and adjusted to a pH optimized for yeast growth. The media were sterilized at 121°C for 15 min. The medium was inoculated with *S. cerevisiae* SC90 to achieve an initial cell concentration of 3×10^7cells/ml. Parafilm was used to cover the top of a flask. A bunsen valve was inserted through the parafilm to prevent pressure build up. The bunsen valve was constructed using a sterile needle and a Durham tube (10 mm diameter) connected via a section of silicone tubing (10 mm diameter) with a narrow slit cut into it to allow release of CO_2 but no ingress of air. Bag ties were used to fasten the tubing to the needle and Durham tube. The fermentation was conducted in batch mode

in an orbital shaker at 120 rpm without pH control. The fermentation temperatures were conducted at 30, 35 and 40°C. Cells grown under YPD containing 20 g/l of glucose and incubated at 30°C were set as a control. Samples were collected to investigate growth, sugar utilization and ethanol contents. The data set was analyzed and presented with respect to the corresponding kinetic parameters.

2.4 Fermentation Analysis

Weight Loss: The progression of the fermentation was monitored by measuring sugar utilization in terms of weight loss over time [26, 27].

Viable Cell Count: Cell suspensions were diluted to an appropriate concentration, and density was measured using a counting chamber and a standard light microscope. Viability was determined by mixing a volume of cell suspension with an equal volume of methylene blue solution (methylene blue dissolved in sodium citrate solution (2% w/v) to a final concentration of 0.01% (w/v)). Unstained cells are assumed to be viable. The viability of the sample was expressed as a percentage [28].

Glucose Utilization: The reduction in sugar concentration was analyzed using 3,5-dinitrosalicylic acid reagent as described by Miller [29]. The fermentation data set was analyzed and presented with respect to the glucose utilization rate with units grams of glucose consumed per hour.

Ethanol: Ethanol concentration was determined via gas chromatography (SHIMADZU, GCMS-QP2010 Ultra, Japan). Isopropanal was used as the internal standard. Data was presented with respect to a set of kinetic parameters (rate of glucose utilization, ethanol yield, volumetric productivity and fermentation efficiency).

Data Analysis: The mean and standard deviation of a data set were calculated using the AVERAGE and STDEV functions of Microsoft® Excel 2003. The presented results were expressed as mean ± STDEV obtained from three independent experiments and error bars corresponding to STDEV. The statistical analysis of the data was conducted using analysis of variance (ANOVA): single factor at 95% confidence limits.

3. Results and Discussion

3.1 Stress Tolerance of *S. cerevisiae* SC90

3.1.1 Osmotic Stress

At the beginning of fermentation, yeast is faced with osmotic stress which is one of the first environmental stresses. Under this condition, yeast cells have to resist this stress in order to start growing and to carry out the alcoholic fermentation [30]. High initial sugar concentration causes a loss in sugar transport activity, consequently resulting in less ethanol [31]. In this study, we investigated the range of osmotic tolerance induced by sorbitol in *S. cerevisiae* SC90. Spot plate technique was applied on YPD containing various concentrations of sorbitol (0, 5, 10, 15, 20, 25 and 30% (w/v)). Then the plates were incubated at 30°C. The results were presented in Fig. 1.

According to Fig. 1, increased concentrations of sorbitol induced higher osmotic pressure and resulted in a reduction of cell growth and therefore a decrease in form a colony. However, *S. cerevisiae* SC90 was able to recover and grow well on YPD even under the sorbitol concentration of up to 30% (w/v).

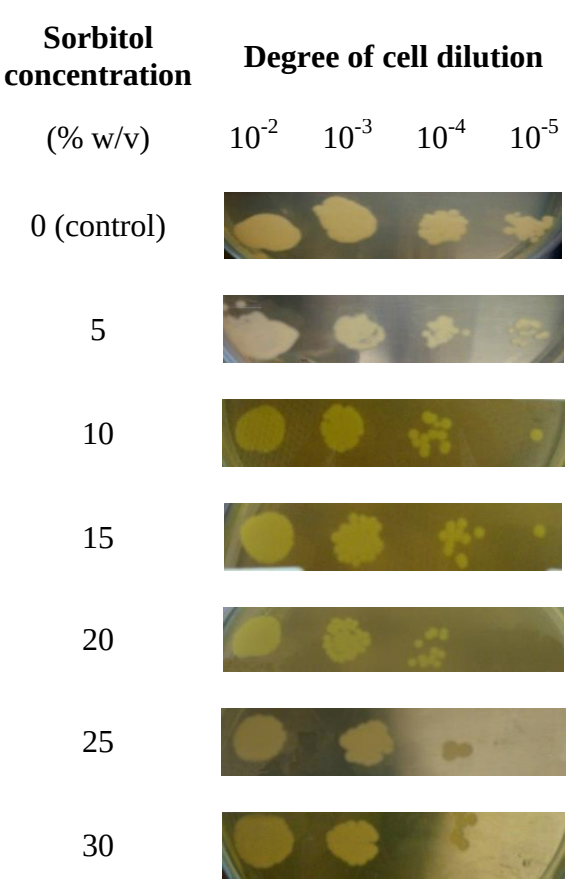

Fig.1. Effect of osmotic stress induced by sorbitol on yeast cell growth. Serial dilutions of *S. cerevisiae* SC90 were spotted onto YPD and then incubated at 30°C under aerobic conditions for 2 days.

3.1.2 Temperature Stress

Exothermic process occurs during ethanol production carried out by the yeasts during their growth. High temperature might be harmful to growth and fermentation potential of cells. Therefore, a cooling system has to be included in the process for temperature control. This current study aimed to identify the temperature range that yeast could grow. Yeast cell cultures were diluted as required and spotted on to YPD agar plates. The plates were aerobically incubated under various temperatures (30°C, 35°C and 40°C). The results were shown in Fig. 2.

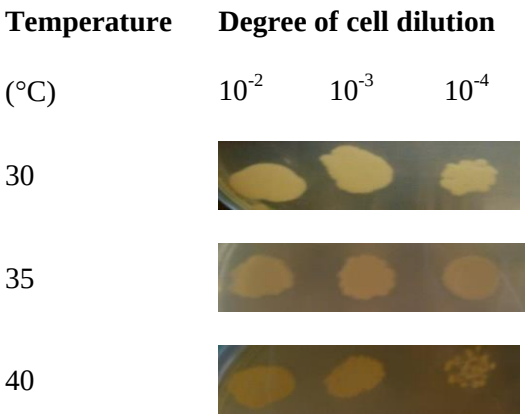

Fig.2. The impact of temperature on the growth of *S. cerevisiae* SC90 aerobically incubated for 2 days under different temperatures.

Strong growth was observed when cells were incubated under 30 and 35°C. However, increasing incubation temperature to 40°C resulted in lower growth. Our results suggested that this strain could be effective in ethanol production for a tropical climate as that of Thailand. The cost for a cooling system would be saved. Consequently, ethanol production would become cheaper.

3.1.3 Synergistic Effect of Ethanol and Temperature on Cell Growth

Apart from common stress as osmotic and temperature stress, ethanol stress is also considered a chemical stress that occurs during ethanol production. Ethanologenic yeast should therefore be tolerant to ethanol. It has been reported that both ethanol and temperature stresses result in deleterious synergistic effect on yeast cell growth. The purpose of this study was to assess the ability of SC90 on a combination of ethanol and high temperatures stress. Yeast cell cultures were diluted as required and spotted onto YPD agar plates containing various concentrations of ethanol (0, 5, 10, 15, 20 and 25% (v/v)). These plates were

incubated aerobically at various temperatures (30, 35 and 40°C).

As presented in Fig. 3, increasing ethanol concentration resulted in a reduction of cell growth. Cells incubated in 30°C were able to grow at 15% (v/v) ethanol concentration. When ethanol concentration was increased to 20 and 25% (v/v), it became necessary to prolong the incubation period for cell repair and recovery (data not shown). The result in this study corresponded to a study on the topic of osmotic stress that showed that the ability of osmotic tolerance of SC90 was 30% (w/v). At 30% (w/v) sugar concentration, yeast could theoretically produce 15% (v/v) of ethanol.

In an experiment with an incubation at 35°C for 2 days, cells exposed to ethanol concentrations between 0 and 10% (v/v) were observed. A small growth was observed at ethanol concentration of 15% (v/v). Cells demonstrated no growth in the presence of 20 and 25% (v/v) ethanol. The impact on growth at the temperature of 40°C and various level of ethanol stress was shown in Fig. 3. Colony formation of cells incubated between 0 and 5% (v/v) ethanol was apparent within 2 days. No growth was observed when cells were incubated in ethanol concentration above 5% (v/v).

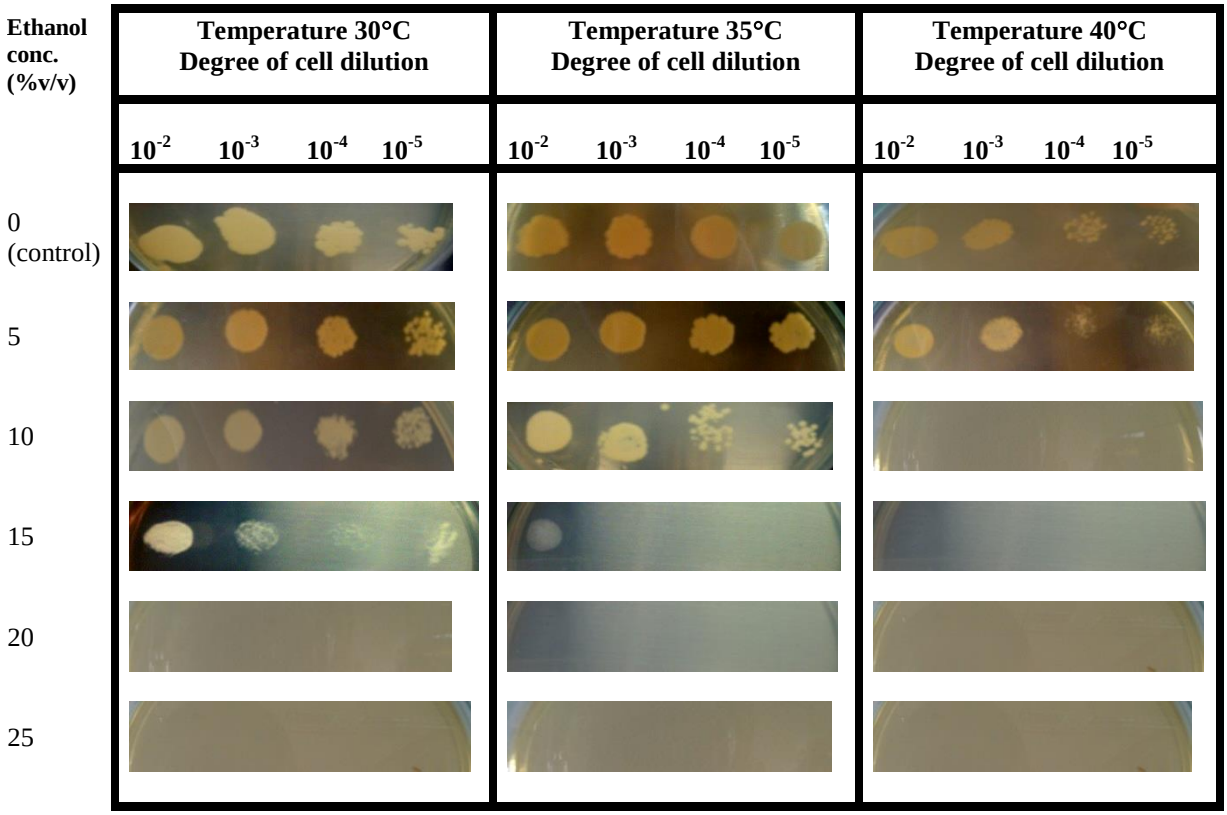

Fig.3. Impact of ethanol and temperature on growth of *S. cerevisiae* SC90 on YPD aerobically incubated for 2 days.

Fig. 3 suggested that the synergistic effect of ethanol and temperature played an important role on yeast cell growth. At a similar concentration of ethanol, increasing temperature could retard cell repair and recovery. According to our results, it appears that a high gravity ethanol production should maintain temperature no higher than 35°C. Several studies reported that a fermentation temperature above 35°C causes stuck fermentation and consequently low ethanol yield [23, 32].

3.2 Impact of Fermentation Temperature on Very High Gravity Ethanol Production

To assess the combined effects of fermentation temperature and initial substrate concentration on ethanol fermentation, YPD containing glucose concentration approximately 340 g/l conducts with yeast industrial strain SC90 at temperatures between 30 and 40°C. The results were reported as follows.

3.2.1 Progression in Weight Loss

CO_2 is one of the major fermentation products of sugar utilization by *S. cerevisiae*. Evolution of CO_2 leads to a progressive weight loss during fermentation which is a rapid and convenient method as well as represents an accurate assessment of fermentation rate instead of ethanol production [33]. A study on the impact of temperature on utilization of a very high glucose concentration (340 g/l) was therefore assessed by observing the progression of weight loss for *S. cerevisiae* SC90. The result was demonstrated in Fig. 4.

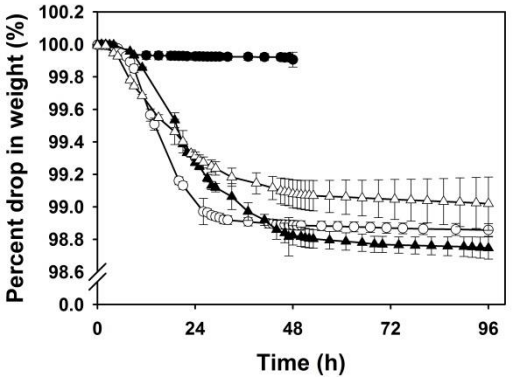

Fig.4. Impact of temperature on residual weight of *S. cerevisiae* SC90 in YPD containing approximately 340 g/l of glucose. Flasks were incubated in an orbital shaker at 120 rpm and different temperatures (30°C (○), 35°C (▲) and 40°C (△). Yeast grown in normal YPD and incubated at 30°C (●) was carried out as a control. Values represent the means of three independent measurements. Error bars indicate the standard deviations of the means.

A sample with the glucose concentration of 20 g/l incubated at 30°C was set as a control to compare with samples with a higher concentration of glucose (340 g/l) under various fermentation temperatures (30, 35 and 40°C). The lower concentration of glucose presented a shorter attenuation time at 8 h. Under VHG, fermentation at 30°C reached attenuation point at 36 h. Increasing temperature to 35 and 40°C permitted attenuation to be achieved at more than 48 h. Even though the initial rate of weight loss at 40°C appeared to be quite rapid during the first 10 h, a small amount of glucose content after 48 h was observed. The results suggested that the rate of weight loss was dependent on the concentration of glucose and the fermentation temperature. Using attenuation time and rate of progression in weight loss as comparative markers of fermentation, we can assume that VHG ethanol production at

30°C incubation temperature reach the maximum ethanol content in the shortest amount of time compared to the incubation temperatures of 35 and 40°C. We hypothesized that 35°C of fermentation temperature may present in the highest amount of ethanol according to the lowest residual weight. To prove this hypothesis, we present the reduction of glucose content and ethanol produced in Fig. 5 and Fig. 6, respectively.

3.2.2 Glucose Utilization

To investigate the effect of fermentation temperature on glucose uptake rate, we conducted four batch experiments. Glucose utilization was monitored using the DNS method, and the fermentation data was illustrated in Fig. 5. As the initial sugar content was increased, the time required to complete sugar utilization increased. However, increasing content of glucose from 20 to 340 g/l appeared to enhance the rate of glucose utilization.

At a high initial concentration of glucose, the fermentation temperature at 30°C demonstrated the most rapid rate of reduction in glucose during the first 4 h. Increasing fermentation temperature to 35 and 40°C slowed down the glucose utilization rate. During the first 24 h, cells incubated at 40°C appeared to utilize glucose faster than those incubated at 35°C. However, after 30 h fermentation at 35°C demonstrated a more rapid rate of glucose utilization. It has been reported by Gibson *et al*. [34] that, due to the rapid growth of yeast cells, initial rapid reduction of glucose and the rapid conversion of sugar to ethanol using glycolysis pathway were observed. At the end of fermentation (96 h), the glucose content remaining at 30, 35 and 40°C were 1.87, 5.15 and 5.10 g/l, respectively.

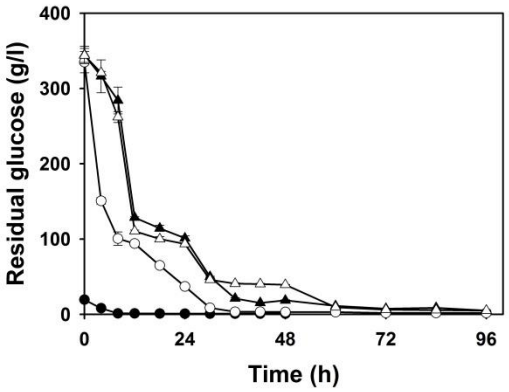

Fig.5. Impact of temperature on glucose utilization of *S. cerevisiae* SC90 in YPD containing approximately 340 g/l of glucose. Flasks were incubated in an orbital shaker at 120 rpm and different temperatures (30°C (○), 35°C (▲); and 40°C (△). Yeast grown in normal YPD and incubated at 30°C (●) was carried out as a control. Values represent the means of three independent measurements. Error bars indicate the standard deviations of the means.

3.2.3 Ethanol Production

The major fermentation products of sugar utilization by *S. cerevisiae* are ethanol and CO_2. To determine the optimum temperature for ethanol production, we assessed ethanol content by GC as presented in Fig. 6.

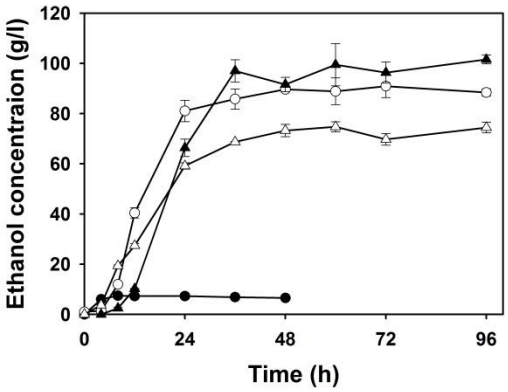

Fig.6. Ethanol production of *S. cerevisiae* SC 90. Cells were grown in 340 g/l of glucose and incubated under different temperatures; 30°C (○), 35°C (▲) and 40°C (△) with 120 rpm in orbital shaker. Yeast grown in normal YPD and incubated at 30°C (●) was carried out as control. Values represent the means of three independent measurements. Error bars indicate the standard deviations of the means.

Ethanol, a primary metabolite, is produced in close association with the growth of yeast cells during fermentation. The decline in glucose was inversely related to an increase in ethanol content. The rate of ethanol production during the first 8 h of fermentation was dependent on the fermentation temperature, the highest being 40°C the ethanol production rate was the highest. However, after this point, a reduction in the rate of ethanol formation was observed. The reason for this is likely to be a result of a more efficient metabolic pathway, which can be induced by high temperature [23]. After the incubation period of 8 h the temperatures in decreasing order of ethanol production rates were 30, 35 and 40°C. The highest amount of ethanol formation from approximately 340 g/l of glucose by SC90 was observed at 35°C with 99.50 ± 8.40 g/l of ethanol in 60 h, followed by 30°C with 89.64 g/l in 48 h, and then at 40°C with 74.72 ± 1.99 g/l in 60 h. Our data suggested that fermentation temperature above 35°C might lead to an adverse effect on ethanol fermentation. This was supported by the work of Damore *et al.* [35], which reported that maximum ethanol fermentation by yeast required temperatures between 30 and 35°C. However, the results from this study were in disagreement with Jones and Ingledew [36], which reported that a peak of sugar utilization is observed at 20 and 25°C. In addition, an optimal temperature for VHG wheat mashes ethanol production supplemented with urea has been suggested at 27-30°C to obtain above 20% (v/v) ethanol within 55 h. The different results might be due to yeast strain dependence and the differences in fermentation media.

3.2.4 Cell Growth and Viability

Viable cell density of SC90 was shown in Fig. 7A. All fermentations were conducted with viable cell density of 3×10^7 cells/ml. The initial viability of SC90 was high at approximately 99%. A rapid increase in viable cell density during the first 8 h was observed for all conditions. However, cells grown at 35°C with the initial glucose concentration of 340 g/l appeared to have a longer lag period. Interestingly, the fermentation temperature of 40°C resulted in the highest growth rate after 4 h of incubation. The only exception was the sample with 20 g/l of glucose (control) incubated at 30°C for which the viable cell density remained static whereas the rate of VHG ethanol production at 30, 35, and 40°C incubation temperatures rapidly declined after 42, 16, and 4 h, respectively. It is worth noting that the cell viability for the four batch differencing fermentation temperatures was significantly different. The maximal viability was obtained at the initial glucose concentration of 20 g/l at 30°C with 98.88 ± 1.16% when the maximum of ethanol level was reached

(Fig. 7B). The viability of VHG fermentation remaining at 30, 35, and 40°C were 80.18 ± 4.43, 40.49 ± 3.19, and 25.81 ± 1.14 cells/ml, respectively (Fig. 7B). A shorter period of fermentation presented higher viability (data not shown) because less ethanol toxicity was exerted on yeast cell. The results indicated that high temperature had a negative impact on yeast cell viability. The impact was probably due to the damage to hydrogen bonding and hydrophobic interaction [37]. Furthermore temperature higher than 20°C induced a rapid reduction of cell viability at the end of fermentation, enzyme activity and membrane function might be disrupted under high temperature [38].

Our results suggested that a low initial concentration of glucose provided the highest viability (Fig. 7B), but lowest ethanol content was observed (Fig. 6). Under VHG at 30°C, high viability of yeast cells (80.18 ± 4.43%) was found and was accompanied by a high amount of ethanol content (85.79 ± 4.04 g/l), indicating the possibility that the yeast cells were reused for a consecutive batch fermentation process. The VHG ethanol fermentation in this current study indicated that the reduction in viability seemed to depend on fermentation temperature and ethanol concentration (Fig. 6). It was therefore indicated that two or more stress factors can occur simultaneously or in succession during fermentation. However, some stresses occur during fermentation in sequence rather than combination [16]. As in this study, the occurrence of high osmotic pressure from the substrate sugar at the beginning of fermentation steadily declines after a consequence of ethanol accumulation. D'Amore [39] reported that using high gravity substrate increased external osmotic pressure leading to cell dehydration and damage to plasma membrane ion gradients which result in a deterioration of viability, growth and fermentation performance. However, there was no substrate inhibition in our study because the fermentation started with high viability of yeast cells, and they utilized glucose rapidly (Fig. 5).

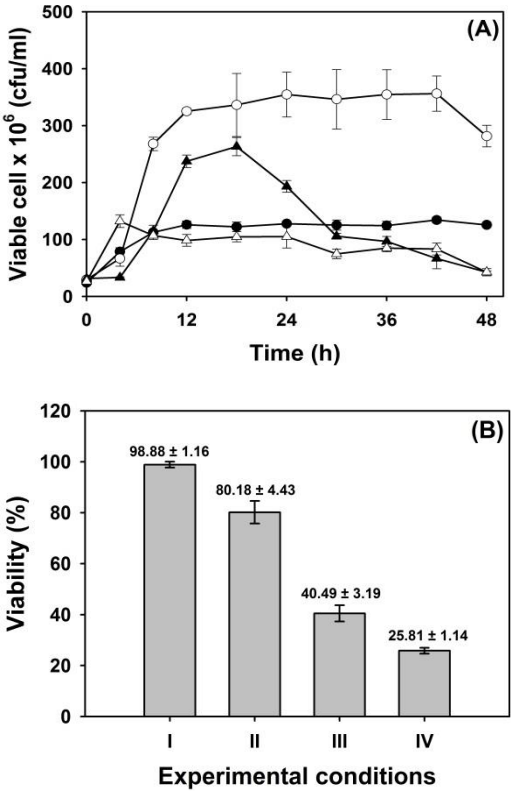

Fig.7. Viable cell count (A) and viability (B) of SC90 in YPD containing 340 g/l of glucose. Fermentations were initiated with a viable cell density of 3×10^7 cells/ml. Batch fermentations were conducted in 100 ml conical flasks and incubated in orbital shaker at 120 rpm and different fermentation temperatures (30°C (○), 35°C (▲) and 40°C (△)). The control (●) was YPD containing 20 g/l of glucose and incubated at 30°C in orbital shaker. Viability was calculated at time taken to reach maximum ethanol (Table 1). Values represent the means of three independent measurements. Error bars indicate the standard deviations of the means. Experimental condition I: 20 g/l glucose at 30°C; II: 340 g/l glucose at 30°C; III: 340 g/l glucose at 35°C; IV: 340 g/l glucose at 40°C.

Ethanol is normally generated and accumulated in the broth after osmotic stress decreases. The data demonstrated that the accumulation of ethanol content of up to 85.79 g/l at 30°C had no significant effect on cell viability during 48 h. It might be implied that the industrial yeast strain SC90 is able to tolerate an ethanol level of up to 85.79 g/l at 30°C. Our result is in agreement with Deesuth *et al*. [40] who reported that *S. cerevisiae* NP01 can withstand up to 93 g/l of ethanol at 30°C. However, a reduction in cell viability of this study occurred during fermentations conducted at high temperature (35 and 40°C) with ethanol content exceeding 70 g/l (Fig. 6 and 7B).

Ethanol, a toxic metabolite, exerts in cells at cellular levels by modifying the fluidity of plasma membrane, destroying various membrane structures of yeast cells [41] as well as denaturing proteins [42]. This results in a strong inhibition on yeast cell growth and no more than 13% (v/v) ethanol production [43]. Several authors stated that the reduction in yeast viability is due to a massive leakage of intracellular metabolites when ethanol levels in the broth reach 10% (v/v) or more [44, 45]. This level of ethanol not only impairs plasma membrane integrity but also induces the formation of heat shock proteins (hsps) [46, 47].

Apart from osmotic stress and ethanol toxicity, temperature, a physical stress, is considered to be another crucial stress that can affect cell growth and fermentation performance in terms of viability, the length of lag period, enzyme activity, membrane function, and the rate of ethanol production [38, 48]. During ethanol production, yeast also generates heat [49]. Therefore, it confronts dual stresses of ethanol and temperature as shown in Fig. 7B. Our results of spot plate and fermentation test indicated that both ethanol and temperature induced a reduction in yeast cell viability. It can be said that ethanol and temperature have a synergistic effect [50] resulting in similar effects on yeast cells, such as an inhibition of the glycolysis pathway, an increase in membrane permeability, a reduction of proton motive force and intracellular pH, consequently leading to a reduction in fermentation rates [51]. In addition, Jones and Ingledew [36] concluded that increasing temperature exacerbates the toxicity of ethanol on cell lysis.

3.2.5 Kinetic Parameters of Ethanol Fermentation

Fermentation temperature is one of several factors that have been proposed to influence the efficiency of ethanol production. The effect of temperature on kinetic parameters associated with ethanol production was assessed. The maximum rate of glucose utilization, the ethanol yield, the volumetric productivity, and the fermentation efficiency were calculated and were presented in Table 1.

Increasing concentrations of glucose resulted in a higher rate of glucose utilization, ethanol concentrations, and volumetric productivity. However, ethanol yield and fermentation efficiency decreased inversely with increasing glucose concentration in the media. Our result has demonstrated that ethanol content was enhanced with increasing fermentation temperature providing this did not exceed 35°C. Furthermore, at temperature higher than 35°C, ethanol levels decreased thus showing negative effects on fermentation efficiency with the reduction of almost all kinetic parameters.

Table1. Kinetic parameters for SC90 under different fermentation temperatures.

Experimental conditions	Time taken to reach maximum ethanol (h)	Rate of glucose utilization (g/l.h)	Residual glucose (g/l)	Ethanol concentration (g/l)	Ethanol yield ($Y_{P/S}$ g/g)	Volumetric productivity (g_p/l.h)	Fermentation efficiency (%)
I	8	2.29 ± 0.04^{a}	1.24±0.05	7.42±0.36	0.41 ± 0.02^{c}	0.93 ± 0.05^{a}	79.49 ± 4.75^{c}
II	36	9.22 ± 0.39^{c}	3.30±0.09	85.79±4.04	0.26 ± 0.01^{a}	2.38 ± 0.11^{c}	50.61 ± 1.18^{a}
III	36	8.99 ± 0.30^{c}	21.13±1.18	97.03±4.45	0.30 ± 0.02^{b}	2.70 ± 0.12^{c}	58.77 ± 4.25^{b}
IV	48	6.35 ± 0.19^{b}	39.35±0.39	73.26±2.45	0.24 ± 0.01^{a}	1.53 ± 0.05^{b}	47.07 ± 1.51^{a}

Mean values in each column with different letters indicate significant differences ($p\leq0.05$). All values are expressed as means ± standard deviation (n=3). Residue glucose was calculated at the end of fermentation. Experimental condition I: 20 g/l of glucose concentration at 30°C; II: 340 g/l of glucose concentration at 30°C; III: 340 g/l of glucose concentration at 35°C; IV: 340 g/l of glucose concentration at 40°C.

Under VHG, attenuation time which is time taken to reach a peak level of ethanol might depend on fermentation temperature and ethanol concentration. Fermentation temperature at 40°C presented more residual glucose compared to lower temperatures. Ethanol yield depended on the sugar feed in the fermentation system. The value of 0.24-0.41 g/g for four batch fermentations were achieved which is equivalent to 47.07-79.49% of the theoretical concentration of ethanol in stoichiometric yields of 0.511 [52]. The impact of fermentation temperature under VHG conditions in this study implied that the maximal concentration of ethanol was seen at 35°C at 97.03 ± 4.45 g/l and volumetric productivity of 2.70 g/l.h while the corresponding low viability was 40.49 ± 3.19% (Fig. 9B). Other optimum temperatures for bioethanol production were reported. Slaa *et al*. [53] produced ethanol from 18% of D-glucose by baker's yeast with the optimum temperature at 35°C. Liu and Shen [33] reported that the optimum temperature for ethanol fermentation from stalk juice of sweet sorghum using immobilized *S. cerevisiae* CICC1308 was 37°C with an ethanol yield of 89.89%. Deesuth *et al*. [40] concluded that ethanol formation was dependent on temperature, strain, and medium for fermentation. Normally, industrial bioethanol product applies reused yeast cells for a consecutive batch fermentation process. Therefore, high viability of yeast cells with high ethanol content has been considered. According to this current research, it was suggested that fermentation temperature should be at 30°C in order to prevent drops in viability due to the ethanol induced lethality of increasing amounts of ethanol produced by the yeast cells. Laluce *et al*. [54] stated that the lowering of the process temperature is therefore recommended in order to minimize cell mortality and maintain high levels of ethanol production when the temperature is increasing in an industrial reactor. However, a low fermentation temperature may result in a longer fermentation period and an increase in cooling cost.

Apart from ethanol and CO_2, main products, yeast cells assimilate sugar to produce cell biomass, organic acids, glycerol, higher alcohols, esters, acetaldehyde and volatile sulphur compounds [55, 56]. Therefore percentage fermentation efficiency of ethanol production in Table 1 is less than 100%. In addition, some of the glucose might be taken to produce storage carbohydrates such as glycogen and trehalose. The former provides carbon and energy for cellular maintenance functions [57, 58] whereas the

later serves as a cell protectant [59-61]. However, in this study the amount of glycogen and trehalose has been not examined. Further study will therefore consider both of these storage carbohydrates.

4. Conclusion

Increasing fermentation temperature is one of the promising methods to improve yeast fermentation performance under very high gravity fermentation. However, our results revealed that the temperature should not be above 35°C. Keeping temperature at this level appeared to enhance fermentation rate but also reduced the capacity of the cells to tolerate ethanol. Loss of cell viability was due to the negative effect of osmotic stress at the beginning of fermentation and was a consequence of ethanol accumulation, which together with temperature had a synergistic effect. In terms of kinetic parameters (we used ethanol yield, volumetric productivity, and fermentation efficiency as selective markers), the optimum temperature for VHG ethanol production using SC90 was 35°C. After maximum ethanol content was obtained, fermentations conducted at above 30°C presented a lower remaining percentage viability. Therefore, the remaining percentage viability after complete fermentation was indicated the ability of yeast to ethanol and high temperature tolerance. Further study on VHG ethanol production process need to be explored to improve the fermentation efficiency similar to that in normal gravity ethanol production including the fed-batch fermentation process [62], nutrient supplementations [40, 45, 63-65], the use of high cell densities [54, 64, 66], and yeast adaptation for stress tolerance [67].

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