嘉南藥理科技大學專題研究計畫成果報告

葡萄糖濃度對釀酒酵母菌（*Saccharomyce cerevisiae*）之存活率及酒精產量的影響

The Effects of Glucose on the Cell Viability and Ethanol Production of *Saccharomyce cerevisiae*

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中文摘要

在此研究中我們發現酵母菌的存活率隨著葡萄糖的濃度增加而下降，而酵母菌所利用之葡萄糖用來發酵製造酒精的比例則隨著培養時間的增長(自 0 到第 9 小時)而增加，如果有更多的葡萄糖被酵母菌利用來製造酒精，則影響到酵母菌的繁殖進而降低了其存活率。從此實驗的結果建議在發酵的過程中，如果葡萄糖的濃度大於 4％，酵母菌利用葡萄糖行呼吸作用的步驟會受抑制，而無氧發酵將成為酵母菌利用葡萄糖的主要途徑，也因為酵母菌無氧發酵一分子葡萄糖只能產生 2 分子的 ATP 及 1 個 NAD+因此無法提供足夠的能量來繁殖新細胞和維持生命所用。

關鍵詞：存活率、發酵、葡萄糖濃度

Abstract

During the same incubation period, the yeast cell viability was decreased as the glucose concentration increased. The percentage of the relative carbon flow of glucose to ethanol production was increased with the increasing of incubation time from 0 hr to 9 hr. The more glucose used by yeast cells to produce ethanol, the less yeast cells were reproduced and thus the cell viability was decreased. These results suggest that if the glucose concentration in the growth media were exceeded 4 %, the respiration process will be inhibited. Fermentation become the predominate catabolic route for yeast cells to use glucose. The two ATP and one NAD^+ generate inside the yeast cells during fermentation was not enough for yeast cells to reproduce and maintain their viability.

Keywords ： cell viability 、 fermentation、glucose concentration

Introduction

For thousands of years have been cultivated by man as a source of food, drink, and preservative thus a close relationship exists between mankind and yeast (Rose and Harrison, 1987). Today, over1.3million tons of yeasts are produced per year. They are used for the production of potable and fuel alcohols, leavened bread, biomass (single cell protein), flavor enhancers, as a source of vitamins, and enzymes (Matthews and Webb, 1990).

Among yeasts, *Saccharomyce*

cerevisiae strains are the most important strain not only for commercial purposes but also as a model for investigating the biochemical activities of eucaryotic organisms (Tuite and Oliver, 1990). *S. cerevisiae* is able to utilize a wide variety of sugars as a source of carbon and energy. Among these sugars, glucose is the easiest sugar for yeast to metabolize (Berry and Brown, 1987). Although glucose is easy for yeast to use, it may cause problems such as catabolite repression in utilizing of other sugars, or the Crabtree effect (Maatthew and Webb, 1990). Several enzymes for utilization of particular sugar in yeast are subjected to repression by glucose (Mahler et al., 1981). This phenomenon has been called catabolite repression (Magasanik, 1961). It affects expression of many genes and the mechanism of catabolite repression in yeast is not fully understood (Gottschalk, 1979). *S. cerevisiae* is sensitive to glucose, and when the glucose concentration is high, respiration is repressed. This affect of glucose is known as the Crabtree effect (Richard and Hogan, 1978). Under such conditions, fermentation is the major catabolic route for utilization of glucose even in the presence of oxygen (Polakis and Bartley, 1965; De Deken, 1968; Lagunas, 1979). During fermentation, controlling of the correct number of viable yeast cells is critical consistent fermentation performance (Cahill et al., 1999). Lower cell viability may cause loss of bitterness filtration problems and increased the risk of yeast autolysis (Boyd et al., 2003). Glucose uptake is the principal phenomenon closely related to metabolic activity (Flier et al., 1987; Venama and Palmgren, 1995), and thus considered a more accurate indicator of cell viability than indicators of membrane permeability or of a particular enzyme activity (Oh and Mastsuoka., 2002).

This study was designed to examine the relationships of yeast cell viability with the growth medium containing different glucose concentrations. The relationship between cell viability and ethanol production was also studied.

Materials and methods：

Yeast strain

All experiments were conducted with the brewing yeast, *Saccharomyces cerevisiae*, WY 2007, which was obtained from WYEAST laboratories （Mt. Hood, OR）. After grown in 50ml of YEPD medium in a 500 ml flask at 28 ℃ and agitation at 200rpm in a controlled environment incubator shaker for 9hrs（late log phase）, 7.5ml（15％）sterilized glycerol was added to this culture, mixed to homogeneity; and 1.8 ml of this suspension were transferred aseptically to each of twenty five 2ml cryogenic vials. This stock cultures were preserved in a -80℃ freezer.

Media

YEPD medium, 1 % yeast extract （wt/vol.）, 2 % peptone（wt/vol.）, and 2 % glucose（wt/vol.）, was used for stock culture and inoculum preparations.

2%, 4 %, and 6 % Glucose Complete Synthetic Media（GCSM）（Klig et al, 1985）were used in all fermentation experiments. Following are the constituents of this medium per liter: Glucose 20 or 40g, yeast nitrogen base w/o amino acid 6.7g（with vitamins）, DL lysine 0.04g, argining 0.02g, leucine 0.06g, methionine 0.02g, DL hisidine 0.03g, L-threonine 0.2g, L-tryptophan 0.2g, adenine 0.02g, uracil 0.04g, and myo-inositol 0.013g.

Inocula preparations and experimental conditions

A 1.8ml frozen stock was transferred to a 50 ml YEPD broth in a 500ml flask and grown at 28℃ with shaking at 200 rpm in a controlled environment incubator shaker for 9 hrs. After 9 hrs inoculation the culture was centrifuged at 3000 x g for 15min., the supernatant was poured off and the yeast cells were resuspended in 15 ml 0.85 % saline and centrifuged at 3000xg for 15min again. The supernatant was poured off and the washed yeast cells were resuspended in 0.85 % saline to a final optical density of 0.6 at 450 nm . A 1 volume yeast cell suspension was used to seed 9 volumes of glucose complete synthetic medium in a 500 ml flask and grown at 28 ℃ with shaking at 200 rpm in a controlled environment incubator shaker for 9 hrs.

Sample preparation

A 2 ml sample was removed from the fermentation broth every hour the GCSM medium. One ml was filtered through a preweighed membrane （0.2mm, Nuclepore Co. inc.）. The membrane was washed with 5ml deionized water, dried in a desiccator at 30 ℃ for 72 hrs, and reweighed. The filtrate was used for ethanol and glucose concentration determinations. The other one ml of the sample was used for total cell and viable cell number determination.

Analytical methods

Optical density: The optical density was measured by a spectrophotometer wavelength set at 650 nm.

Viable cell counts: a one ml sample was diluted using 8.5ml of 0.85 % saline, and mixed with 0.5 ml methylene blue and placed in a hemoacytometer, cells in 25 squares were counted to provide an accurate direct count. Since these 25 squares covering an area of $1mm^2$ and the chamber is 0.1mm deep, therefore, cell number per mm^3 = the sum of cell number in 25 squares times 10. The number of cell per cm^3 is multiplied by 10^3 so that the final number of cells/cm^3 are expressed. A tally was made of the number of live cells and of dead cells （dead cells take up methlene blue）, and multiplied by the proper factor

（10,000）and dilution factor（10）to provide total cells per ml and viable cells per ml from where the percentage of the total population which were non-viable cells could be calculated.

Substrate and product analyses:

The ethanol concentration was determined by gas chromatographic analysis using a Hewlett-Packard 5730A with an FID detector, 2meter x 2mm glass column packed with 80/120 Carbopack BAW/6.6 % PEG 20M, using a nitrogen carrier gas at a flow rate of 23ml/min. The column temperature was held isothermal at 90℃ for 4min. The data was processed via a Shimadzu C-R3A processor with integration of the peak area against external standards. Residual glucose concentration was determined by the glucose oxidase method in a YSI 23A glucose analyzer.

Data analyses:

The yield of cell mass was calculated on the basis of g/l of cells dry weight produced divided by g/l of glucose consumed by yeast.

The yield of ethanol was calculated by mmole of ethanol produced by yeast cell divided by mmole of glucose consumed by yeast cell.

The relative flows of carbon to yield of ethanol：

Theoretically, 1 mole of glucose can be converted to 2 mole of ethanol and two mole of carbon dioxide by yeast which is based on the following equation:

$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2$

Based on this equation, we can calculate the relative percentage of carbon flow to ethanol, cell mass and cell maintenance. For example, if x moles of glucose were used by yeast and y moles of ethanol were produced by yeast, the relative carbon flow to ethanol production was y/2x times 100 % . To see the relationship of oxygen and relative carbon flow, 2 %, 4 %, and 6 % GCSM were used. The effects of oxygen on cell mass, specific ethanol production rate, specific growth rate, amount of glucose uptake, and cell viability were determined in this study.

Results

The growth curve of *S. cerevisiae* 2007 in 2 % GCSM, 4 % GCSM, and 6 % GCSM at 28 ^{0}C, 200 rpm was shown on Fig 1. In 2 % GCSM, the growth curve of *S. cerevisiae* 2007 reached the stationary phase at 10 hrs of incubation time. For the 4 % and 6 % GCSM, the incubation time for *S. cerevisiae* 2007 to reach the stationary phase was greatly delayed (Fig. 1). The changes in total cell number, viable cell number, cell viability, cell mass, amount of glucose consumed, and amount of ethanol produced during the 12 hrs incubation period were shown in Table 1 to Table 3. In 2 % GCSM, the cell viabilities were all above 90 % from 0 to 12 hr incubation time (Table 1). While in 4 % and 6 % GCSM, the cell viabilities

were decreased as the incubation time increased. There were higher biomass yield in 4 % and 6 % GCSM then in the 2 % GCSM, but the cell viabilities in 4 % and 6 % GCSM were significant lower then the 2 % GCSM in the same incubation periods (Table 1-3). The amount of glucose consumed was increased with the increasing of incubation time from 0 to 9 hr. In the incubation period of 0-3 hr, the highest glucose uptake rate was found in the 4 % GCSM. Same as the amount of glucose consumed, the yield of ethanol was increased with the increasing of incubation time from 0 to 9 hr. In the incubation period of 0-3 hr, the highest yield of ethanol was found in the 6 % GCSM.

Discussions

Fermentation is the major catabolic route for *S. cerevisiae* to metabolize glucose (Polakis and Bartley, 1965; De Deken, 1968; Lagunas, 1979). The first regulatory step in the utilization of glucose by *S. cerevisiae* is the uptake of glucose. Glucose uptake is a highly regulated process and is thought be the rate limiting step of glucose catabolism during fermentative growth of *S. cerevisiae* (den Hollander et al., 1985). Three mechanisms for the transport of glucose into yeast cell have been described; simple diffusion, facilitated diffusion and active transport. Since the rate of simple diffusion is so slow, simple diffusion is not very important in assimilation of carbohydrates (Fiechter et al., 1981), whereas, both facilitated diffusion and active transport are the major routes for transport of glucose into cell and both require permease and are regulated by glucose itself (Cirillo, 1989). Two transport systems for glucose have been reported, one is a low-affinity transport system consisting of nonconstitutive facilitated diffusion process with a high K_m of approximately 20 mM (50 mM for fructose) and another is a kinase-dependent, glucose-repressible high-affinity transport system with a low K_m value of approximately 1 mM (5 mM for fructose) (Bisson and Fraenkel, 1983). The affinity of these two glucose permeases is decreased in the presence of low glucose concentration (Cirillo, 1981). It may be stated that the only reason for fermentation to occur under anaerobic conditions is for the regeneration of NAD^+ which allows glycolysis to continue. Under these conditions, glucose is used by yeast for the production of two net ATP, one NAD^+, and ethanol as a fermentation product. Thus, the yield of cell mass and viability are low. If pyruvate is directed into the TCA cycle, yeast cells will have enough energy, essential metabolites, and reducing power to synthesize cell materials and to reproduce. Based on the material balance, the biomass and cell number will increase significantly while the amount of ethanol produced from glucose will decrease. On the other hand,

if the majority of pyruvate is used to produce ethanol, the biomass and cell number will decrease (Grosz et al., 1989).

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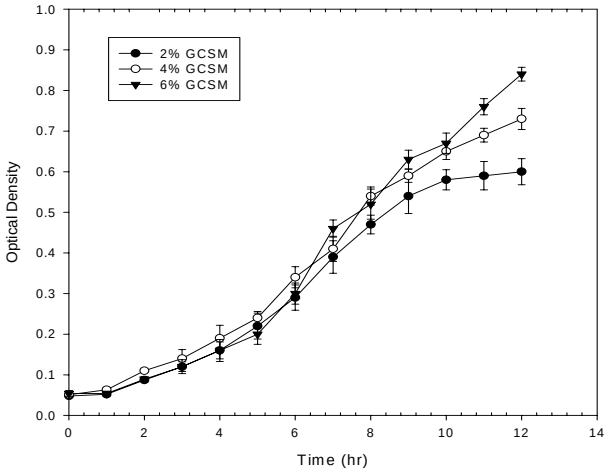

Fig. 1. Growth curve of Saccharomyce cerevisiae in 2%, 4%, and 6% GCSM at 28^{0}C, 200 rpm.

Table 1. The changes of total cell number, viable cell number, dry weight, amount of glucose uptake, and amount of ethanol production at phase of accelerating growth (0-3 hr), early log phase (3-5 hr), log phase (5-9 hr), and stationary phase (9-12 hr)in 2% glucose complete synthetic medium at 28^0C, 200 rpm.

	0-3 hr	3-5 hr	5-9 hr	9-12 hr
Total cell number (cells/ml)	$1.03 \pm 0.21 \times 10^7$	$1.63 \pm 0.14 \times 10^7$	$2.47 \pm 0.17 \times 10^7$	$0.52 \pm 0.04 \times 10^7$
Viable cell number (cells/ml)	$1.04 \pm 0.25 \times 10^7$	$1.53 \pm 0.15 \times 10^7$	$2.30 \pm 0.20 \times 10^7$	$0.38 \pm 0.16 \times 10^7$
Cell Viability	100 %	93.86 %	93.11 %	94.44 %
Cell mass (dry wt. g/l)	0.26 ± 0.05	0.39 ± 0.07	1.24 ± 0.16	0.25 ± 0.03
Glucose consumed (mmole)	5.56 ± 0.88	10.56 ± 1.54	44.44 ± 4.48	18.75 ± 5.62
Ethanol production (mmole)	8.70 ± 1.36	17.39 ± 5.85	75.22 ± 7.46	33.49± 10.32
% of Relative carbon flow to yield of ethanol	78 %	82 %	88 %	89 %

Table 2. The changes of total cell number, viable cell number, dry weight, amount of glucose uptake, and amount of ethanol production at phase of accelerating growth (0-3 hr), early log phase (3-5 hr) and log phase (5-9 hr) in 4% glucose complete synthetic medium at 28^0C, 200 rpm.

	0-3 hr	3-5 hr	5-9 hr	9-12 hr
Total cell number (cells/ml)	$9.70 \pm 0.24 \times 10^6$	$1.46 \pm 0.28 \times 10^7$	$2.97 \pm 0.37 \times 10^7$	$1.11\pm 0.23 \times 10^7$
Viable cell number (cells/ml)	$9.59 \pm 0.35 \times 10^7$	$1.39 \pm 0.32 \times 10^7$	$2.76 \pm 0.39 \times 10^7$	$0.97 \pm 0.07 \times 10^7$
Cell Viability	98.87 %	95.21 %	92.93 %	87.39 %
Cell mass (dry wt. g/l)	0.28 ± 0.05	0.41 ± 0.11	1.23 ± 0.24	0.43 ± 0.04
Glucose consumed (mmole)	13.89 ± 0.12	13.00 ± 3.23	54.54 ± 5.61	27.28 ± 8.12
Ethanol production (mmole)	10.96 ± 1.36	21.91 ± 3.73	96.04 ± 6.58	47.35 ± 8.61
% of Relative carbon flow to yield of ethanol	78 %	84 %	88 %	87 %

Table 3. The changes of total cell number, viable cell number, dry weight, amount of glucose uptake, and amount of ethanol production at phase of accelerating growth (0-3 hr), early log phase (3-5 hr) and log phase (5-9 hr) in 6% glucose complete synthetic medium at 28^0C, 200 rpm.

	0-3 hr	3-5 hr	5-9 hr	9-12 hr
Total cell number (cells/ml)	$1.03 \pm 0.22 \times 10^7$	$1.66 \pm 0.37 \times 10^7$	$3.27 \pm 0.54 \times 10^7$	$1.47 \pm 0.17 \times 10^7$
Viable cell number (cells/ml)	$9.89 \pm 0.34 \times 10^6$	$1.48 \pm 0.32 \times 10^7$	$2.76 \pm 0.73 \times 10^7$	$0.82 \pm 0.04 \times 10^7$
Cell Viability	96.02 %	89.16 %	84.40 %	72.59 %
Cell mass (dry wt. g/l)	0.26 ± 0.05	0.38 ± 0.17	1.53 ± 0.19	0.51 ± 0.04
Glucose consumed (mmole)	10.23 ± 0.68	16.89 ± 4.55	62.37 ± 7.34	31.36 ± 5.42
Ethanol production (mmole)	17.31 ± 1.82	29.47 ± 6.33	117.76 ± 6.65	56.58 ± 6.73
% of Relative carbon flow to yield of ethanol	85 %	87 %	94 %	90 %