THE EFFECT OF MEDIUM COMPOSITION AND ETHANOL TOXICITY ON THE GROWTH OF *SACCHAROMYCES CEREVISIAE* STRAIN W303-1A(a).

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ABSTRACT

The growth of *Saccharomyces cerevisiae* strain W303-1A(a) was evaluated in complex and chemically defined media. The use of chemically defined medium allowed the complete utilisation of glucose within 20 h, as well as all of the produced ethanol within 45 h. Maximum specific growth rates (μ_{max}) were increased from 0.28 h^{-1} to 0.42 h^{-1} and the volumetric rate of ethanol production increased from 0.204 g l^{-1} h^{-1} to 0.597 g l^{-1} h^{-1}. However, when the ethanol concentration exceeded a threshold value of 10 g l^{-1}, the μ_{max} value was significantly decreased. These observations suggest that ethanol metabolism related growth experiments for the relevant strain should be carried out in chemically defined medium with ethanol concentrations below 10 g l^{-1}.

Keywords: *Saccharomyces cerevisiae*, ethanol toxicity, media composition

1. INTRODUCTION

In especially physiological studies of microorganisms, it is important to ensure that a well formulated culture medium is used so as not to impose an unknown nutrient limitation. Unaccounted for nutrient limitations can result in misinterpretation of experimental data (Lagunas, 1986). Most yeast species produce satisfactory biomass on YPD medium containing yeast extract, peptone and glucose as carbon source. Yeast extract originates from lysed yeast cells and consists primarily of oxidised amino acids, but also includes nutrients such as vitamins (especially B vitamins) and minerals. Peptone is a protein derivative and is a broadly used component of yeast culture media because it is a rich source of amino acids and nitrogen (Martinez *et al*., 2006). Magnesium is an essential cofactor for many of the glycolytic enzymes and has been identified as a limiting nutrient in fermentation broth containing peptone and yeast extract (Dombek & Ingram, 1986; Dombek & Ingram, 1987).

Several groups have reported improvements in alcoholic fermentation and final ethanol concentration by broth supplementation with additives such as lipids, proteins and vitamins (Casey *et al*., 1983; Casey *et al*., 1984; 1987; Janssens *et al*., 1983). The addition of inositol to the culture medium improved the ethanol yield during fermentation and phospholipid synthesis, with the yeast cells producing more saturated fatty acids (Chi *et al*., 1999).

Ethanol exerts different and separable effects on the specific growth rate of the yeast population, its viability and its specific rate of fermentation (D'Amore & Stewart, 1987). The amount of ethanol able to cause a decrease in growth rate is highly dependent on the yeast strain and medium composition, where values ranging from 10 to 35 g ethanol l^{-1} are cited in the literature (Casey *et al.*, 1984). When growing *S. cerevisiae* on sugars as carbon substrate, the inhibitory effect of the produced ethanol should also be considered.

The aim of this investigation was to evaluate the effect of the culture medium composition on the growth parameters of *Saccharomyces cerevisiae* strain W303-1A(a) in batch cultures. The ethanol tolerance of this strain was also determined to avoid severe inhibitory effects of ethanol, since it is essential that the culture medium allow complete utilisation of ethanol produced from glucose, or when added as sole carbon source.

2. MATERIALS AND METHODS

2.1. Strains and media

Growth experiments were conducted with *S. cerevisiae* strain W303-1A(a) (W303-1A, *MATa, his3, leu2, trp1, ura3) (Thomas & Rothstein, 1989)*. Chemically defined medium contained (per liter) citric acid (0.5 g), NaCl (0.1 g), $(NH_4)_2SO_4$ (5 g), $MgSO_4 \cdot 7H_2O$ (0.4 g), $CaCl_2 \cdot 2H_2O$ (0.02 g), KH_2PO_4 (5 g), trace elements [35 mg $FeSO_4.7\ H_2O$, 7 mg $MnSO_4.\ H_2O$, 11 mg $ZnSO_4.7\ H_2O$, 1 mg $CuSO_4.5\ H_2O$, 2 mg $CoCl_2$, 1.3 mg $Na_2MoO_4.2\ H_2O$, 2 mg H_3BO_3, 0.35 mg KI, 0.5 mg $Al_2(SO_4)_3$], vitamin solution (1 mg calcium panthotenate, 1 mg nicotinic acid, 0.2 mg *p*-aminobenzoic acid, 1 mg pyrodoxine HCl, 1 mg thiamine HCl, 25 mg *m*-inositol, 0.05 mg *d*-biotin), amino acids (50 mg each of alanine, arginine, asparagine, aspartic acid, glutamic acid, glutamine, glycine, histidine, isoleucine, lysine, methionine, phenylalanin, proline, serine, threonine, tryptophan, tyrosine, valine and cysteine, 400 mg leucine, 50 mg adenine, and 50 mg uracil. Complex media contained (per liter) yeast extract (2 g), peptone (4 g), $MgSO_4.\ 7H_2O$ (0.4 g), $CaCl_2\ 2H_2O$ (0.02 g), KH_2PO_4 (2 g) and trace elements (as described for the chemically defined medium). Glucose or ethanol was added as carbon source.

2.2. Cultivation conditions

Evaluating the effects of medium components. Aerobic cultivations were carried out in 500 ml Erlenmeyer flasks with stoppers of non-absorbent cotton wool, each containing 200 ml of the appropriate medium and either glucose or ethanol as carbon source. The glucose concentrations ranged from 11.3 g l^{-1} to 37.6 g l^{-1} and the ethanol concentrations were either 5 g l^{-1} or 20 g l^{-1}. All shake flask cultivations were carried out at an initial pH of 5.5 at 30°C on a rotary shaker at 200 r min^{-1}.

For experiments using glucose as carbon substrate, an inoculum was prepared in a 250 ml shake flask containing 50 ml of the relevant medium with 10 g glucose l^{-1} and grown for 9 to 12 h. Similar inocula were prepared for experiments using ethanol as carbon substrate, but were grown for three more hours after the glucose had been depleted (derepressed conditions) before being transferred to the 500 ml flasks. These were inoculated with the pre-inocula to give an initial OD_{600} of *ca.* 0.2.

Determining ethanol toxicity levels. The cells were grown in a temperature gradient incubator (Scientific Industries Inc., New York) at 25ºC. Each of the L-shaped 30 ml test tubes contained 11 ml chemically defined medium and were located in 30 equidistant wells in the aluminium bar, which rocked gently to provide agitation. Oscillation was gradually increased from 25 to 50 per min over a period of 10 h. The appropriate volume of ethanol was added to each set of tubes and the concentrations determined by gas chromatography. Each tube was inoculated from an actively growing shake flask culture to an initial OD_{600} value of *ca.* 0.04. Growth was considered to have occurred where the maximum specific growth rate exceeded 0.03 h^{-1}.

2.3. Analyses

Culture turbidity was monitored at 600 nm with a BioPhotometer (Eppendorf, Germany) and converted to dry biomass using a standard curve. Cells were harvested during late exponential phase, cooled on ice, centrifuged at 3 000 r min^{-1} for 5 min, washed twice with distilled water and dried at 105ºC overnight prior to gravimetric determination of the dry biomass concentration. The ethanol concentrations in the supernatant were determined with a 2010 gas chromatograph (Shimadzu, Japan) equipped with an SGE PBX-70 column (30 m x 0.25 ID) at an over temperature of 180ºC, with 30 ml nitrogen carrier gas min^{-1}. Glucose concentrations were determined with a Sugar Analyser I high-performance liquid chromatograph equipped with a refractive index detector and a Waters Sugarpack I column (Waters Associates, US) operating at 85ºC with an eluent (degassed water) flow rate of 0.5 ml min^{-1}.

3. RESULTS AND DISCUSSION

Initially strain W303-1A(a) was grown in the complex medium as described by du Preez *et al.* (2001). Different glucose concentrations were added as carbon source and growth in shake flasks was monitored over a period of 45 h. The μ_{max} value was not significantly influenced by the initial glucose concentration (Table 1, Fig. 1A). The glucose was completely consumed in the culture medium with an initial glucose concentration of 22.9 g l^{-1} or less, while some residual glucose was still detected after 45 h in the medium with and initial glucose concentration of 37.6 g l^{-1} (Fig. 1B). Ethanol was produced at similar volumetric rates (Table 1), but no significant ethanol assimilation was apparent even after 45 h (Fig. 1C).

Strain W303-1A(a) was able to grow on ethanol as added carbon source in complex medium in shake flasks (Fig. 2A). At an initial ethanol concentration of 5 g l^{-1} the ethanol was consumed at a volumetric rate of 0.086 g l^{-1} h^{-1}, but residual ethanol was still detected after 70 h (Table 1, Fig. 2A). The effect of the culture medium composition on the poor ethanol utilisation was investigated by the omission of certain constituents. In complex medium without peptone the maximum specific growth rate was 0.08 h^{-1} compared to 0.17 h^{-1}in the complete complex medium. Similarly, the rate of ethanol utilisation was only 0.029 g l^{-1} h^{-1} compared to 0.086 g l^{-1} h^{-1} in the complete complex medium, which resulted in a considerable amount of residual ethanol after 70 h (Fig. 2B). The exclusion of yeast extract had no discernable influence on the growth parameters (Fig. 2C). This apparent requirement for peptone can probably be attributed to the auxotrophic nature of the yeast strain.

Table 1. Growth parameters of *S. cerevisiae* strain W303-1A(a) in shake flasks on different glucose and ethanol concentrations in a complex and a chemically defined medium.

			Growth parameters		
Medium	**Substrate**	**gl^{-1}**	**μ_{max}, h^{-1}**	**Qs^{max}, g l^{-1} h^{-1}**	**Qp^{max}, g l^{-1} h^{-1}**
Complex	Glucose	11.3	0.28	0.6	0.204
		22.9	0.3	0.712	0.296
		37.6	0.27	0.918	0.259
	Ethanol	5	0.17	0.086	
Chemically defined	Glucose	10	0.42	0.974	0.597
	Ethanol	5	0.19	0.176	

Growth rates (μ_{max}) were calculated by linear regression analysis of the exponential growth data. Qs and Qp were calculated from the maximum slopes of the glucose and ethanol concentration curves respectively.

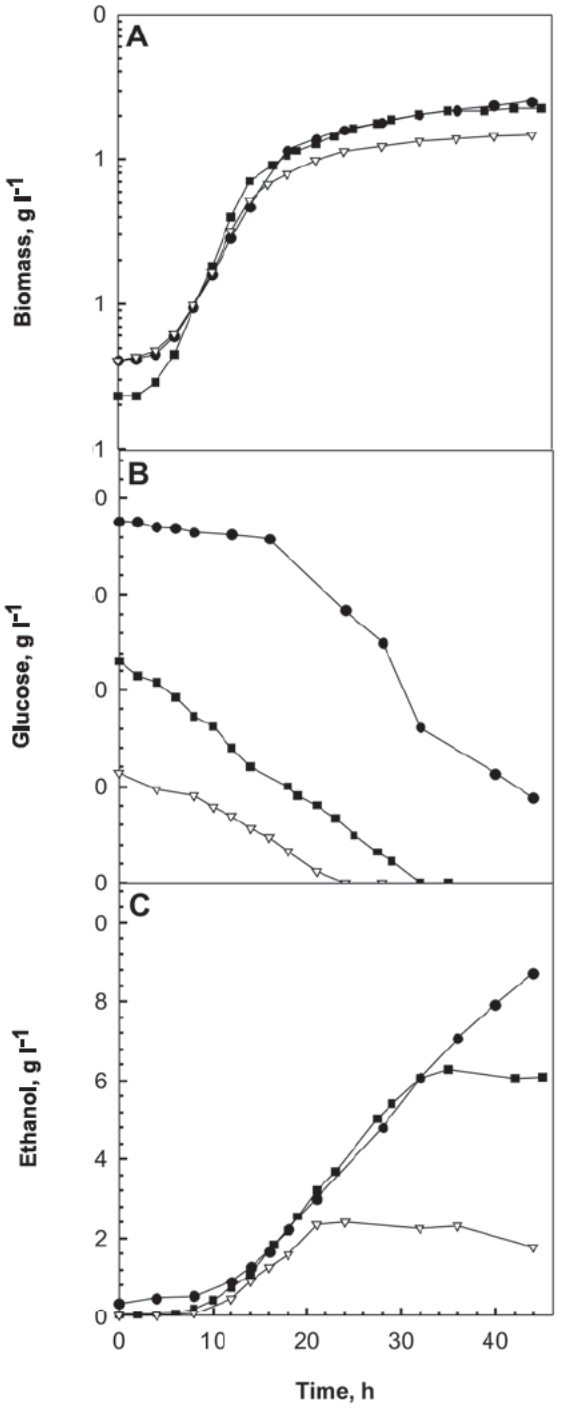

Figure 1.

Growth profile of *S.cerevisiae* strain W303 1A(a) in complex medium in shake flasks containing different glucose concentrations, 37.6 g l^{-1} (●), 22.9 g l^{-1} (■), 11.3 g l^{-1}(▽)

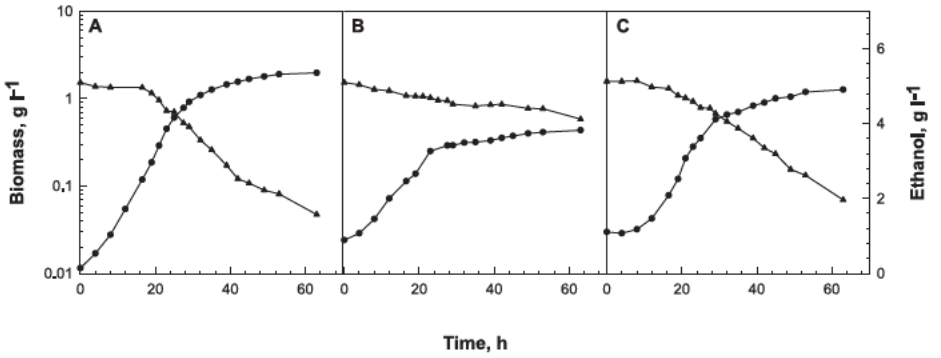

Figure 2.

Growth profiles of *S. cerevisiae* W303-1A(a) in complex medium in shake flasks with 5 g ethanol l^{-1} as carbon source (A). Growth profiles where peptone (B) and yeast extract (C) were excluded from the medium are also shown. Biomass (●) and ethanol (▲) concentrations were measured over a period of 70 h.

Since studying the ethanol metabolism of this specific yeast strain requires a well formulated medium, it is essential to avoid unknown nutrient limitations and, therefore, facilitate maximal utilisation of ethanol. Subsequently, a chemically defined medium was formulated based on the media used by various other researchers for a number of different yeast strains. Amino acid concentrations were derived from the media of de Kock *et al.* (2000) and Flikweert *et al.* (1996), vitamin components from Schulze *et al.* (1995), trace element concentrations as described by du Preez & van der Walt (1983) and macro elements derived from media described by Verduyn *et al.* (1990) and du Preez *et al.* (2001). Uracil, adenine, tryptophan, histidine and leucine were added to satisfy the auxotrophic requirements of strain W303-1A(a) (Cakar *et al.*, 1999; Pronk, 2002).

In the chemically defined medium with glucose as carbon source, strain W303-1A(a) consumed all of the glucose during the first 20 h, as well as all of the produced ethanol within 45 h (Fig. 3). The other growth parameters were also considerably greater, compared to growth in the complex medium (Table 1) and resembled values available in literature (Boubekeur *et al.*, 2001). The strain was also able to utilise all the available ethanol within 45 hours when grown in the chemically defined medium containing 5 g ethanol l^{-1} as carbon source (Fig. 4).

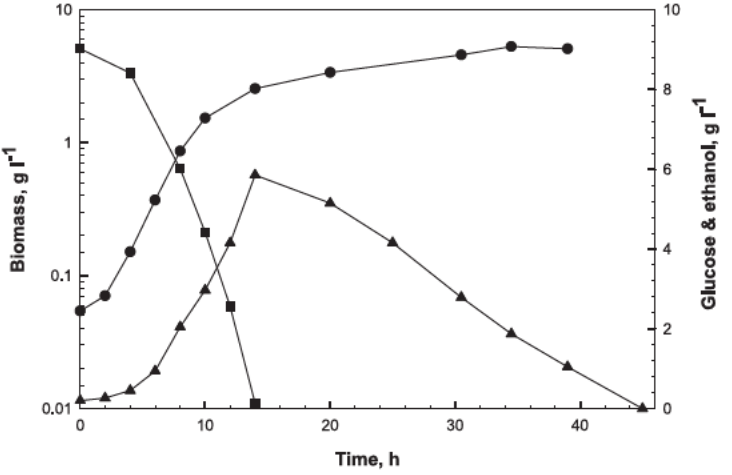

Figure 3.

Growth profile of strain W303-1A(a) on 10 g glucose l^{-1} in a chemically defined medium in shake flasks. Biomass (●), glucose (■) and ethanol (▲) concentrations are indicated in the graph.

Investigation of the effect of externally added ethanol showed that at concentrations of between 0 to 10 g l^{-1} less than 10% inhibition of the μ_{max} value occurred (Fig. 5). However, at ethanol concentrations greater than 10 g l^{-1} an inverse linear correlation between the ethanol concentration and the μ_{max} value was found (R^2 = 0.9931), with a concentration of 72 g l^{-1} completely inhibiting growth. Therefore, for studies investigating ethanol utilisation; the externally added ethanol concentrations should be kept below 10 g l^{-1}.

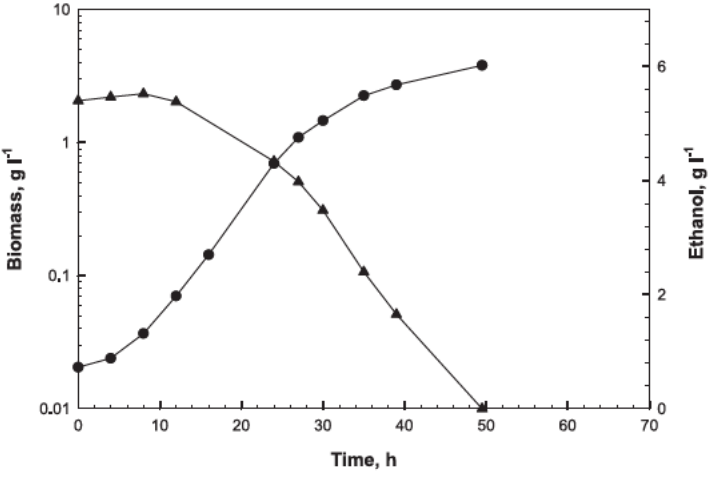

Figure 4.

Growth profile of strain W303-1A(a) on 5 g ethanol l^{-1} in a chemically defined medium in shake flasks. Biomass (●) and ethanol (▲) concentrations are indicated in the graph.

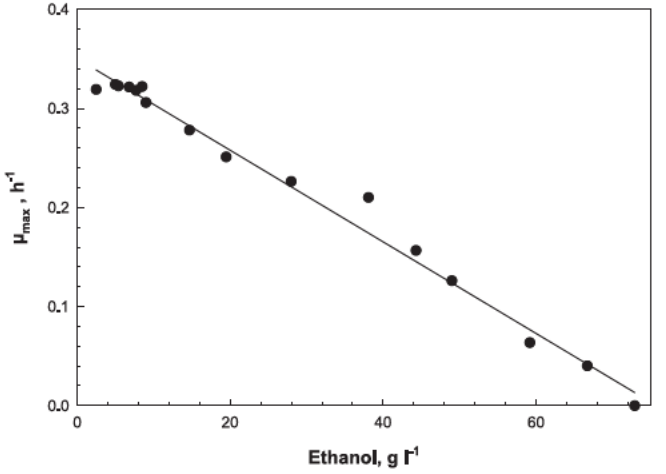

Figure 5.

Effect of the exogenous ethanol concentration on the maximum specific growth rate (μmax) of W303-1A(a) grown at 25ºC in chemically defined medium.

4. CONCLUSIONS

S. cerevisiae strain W303-1A(a) utilised produced or externally added ethanol at a low rate when grown in complex medium. Apparently the complex medium failed to satisfy the demanding nutritional requirements of this auxotrophic yeast strain, judging from the growth parameters shown in Table 1. The use of a chemically defined medium resulted in a higher μ_{max} value of 0.19 h^{-1} compared to 0.17 h^{-1} in the complex medium. The rate of ethanol utilisation also increased from 0.087 in the complex medium to 0.176 g l^{-1} h^{-1} in the chemically defined medium and all of the ethanol was consumed within 50 h. Ethanol inhibited growth and fermentation of strain W303-1A(a) and when the ethanol concentration exceeded a value of 10 g l^{-1} the growth rate was significantly inhibited. In conclusion, the results clearly indicate that chemically defined medium should be used for quantitative growth studies involving the ethanol metabolism of *S. cerevisiae* strain W303-1A(a) and the inhibitory effect of ethanol concentrations in excess of 10 g l^{-1} should be considered in the experimental design.

5. LITERATURE CITED

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