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Effects of Potassium on the Ethanol Production Rate of *Saccharomyces cerevisiae* Carrying the Plasmid pCYG4 Related with Ammonia Assimilation

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ABSTRACT

The influence of potassium on ethanol production by *Saccharomyces cerevisiae* wild type and AR5 cells carrying the plasmid pCYG4 was investigated. This plasmid carries the glutamate dehydrogenase gene conferring an 11-fold higher level of expressed enzyme activity over the wild type cells. All experiments were carried out in batch culture with medium supplemented to different potassium concentrations up to 180 m*M*. Maximum ethanol production rate was observed in the AR5 cells grown in medium supplemented with 3.5 m*M* of potassium ions. Glucose uptake rate increased with increasing potassium up to 60 m*M*, but higher concentrations depressed glucose uptake rate in both strains. Furthermore, the wild type cells showed higher growth rate, ethanol production, and glucose consumption rate than the AR5 cells. These lower rates in the AR5 cells could be explained by repression of potassium uptake by an enhancement of ammonium feeding, and greater energy requirements by these cells due the presence of the plasmid.

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Index Entries: *Saccharomyces cerevisiae;* ethanol; glucose; potassium; plasmid.

INTRODUCTION

Ethanol is the main and end product of glycolysis in *Saccharomyces cerevisiae* when it is growing under anaerobic conditions. In high concentration, ethanol inhibits sugar fermentation and causes other unfavorable effects in yeasts cells, including decreasing the growth rate and viability (*2*). These effects are probably caused by inhibitory effects on glucose and ammonium uptake, related to an ethanol-carrier interaction (*18*). This interaction causes modification of membrane permeability that can result in proton and ion extrusion, especially potassium (*1–3,4,6*). Potassium influx in the cells facilitates glucose assimilation (*7,8*) and has a stoichiometrical relationship with ammonium assimilation. Ammonia can be assimilated into the cells following two different pathways (*9–11*). The first is catalyzed by glutamine synthetase (GS) an ATP-requiring enzyme, together with glutamine 2-oxo-glutarate aminotransferase (GOGAT), which is strongly induced under nitrogen limitation (*5*). The second is catalyzed by NADP-linked glutamate dehydrogenase (NADP-GDH), which is usually regarded as a low affinity enzyme (with high Km), mainly functional under unlimited ammonia supply (*13*). In organisms having both aminating pathways, glutamine synthetase is drastically decreased when the ammonia supply is increased, and amination proceeds via glutamate dehydrogenase (*13*).

This work is concerned with the study of the influence of potassium on ethanol production by *Saccharomyces cerevisiae* AR5, cells transformed with glutamate dehydrogenase (NADP-GDH) gene on a 2-μ bared vector (pCYG4) promoting an 11-fold increase in enzyme activity compared to wild type cells and also an increase on ammonium assimilation. This is based on the fact that potassium influx in the cells facilitates glucose assimilation and because it has a stoichoimetric relationship with ammonium assimilation. Consequently, these cells might increase biomass yield and ethanol production growing in presence of potassium and ammonium.

MATERIALS AND METHODS

Microorganism, Media, and Inoculum Preparation

Saccharomyces cerevisiae wild type Σ1278b and AR5 (genetically engineered strain (GOGAT$^-$ [glu4-9] gdh-1$^-$ [plasmid gdh-pCYG4]) obtained from J. R. Kinghorn (University of St. Andrews, Scotland, UK). They

were maintained at 4°C on following medium: D(+) glucose 1%, ammonium sulphate 0.4%, Difco (E. Molesley, Surrey, UK) yeast nitrogen base (YNB) w/o amino acids and ammonium sulphate 0.17% and agar 1.5%. The liquid medium used was the same as described above, except that glucose concentration was 5% and the pH was adjusted to 5.0.

Inoculum Preparation and Experimental Work

Initially the strains were grown on agar slants for 48 h at 30°C. A slant was then washed under manual stirring with 10 mL of liquid medium (as described above—cells concentrations of 10^7) and added to 50 mL of the same medium. The cells were grown for 18 h under stirring (200 rpm) at 30°C. After this period the cells were added to 450 mL of fresh medium in the experimental flasks (working capacity of 2 L). Experiments were carried out for about 48 h at 30°C under aerobic conditions with agitation at 200 rpm.

BIOCHEMICAL ANALYSES

Preparation of Samples

A 4.5 mL sample of fermentation broth was centrifuged at 3000*g* for 2 min. The supernatant was used for the analysis of glucose and ethanol.

Determination of Biomass

Biomass was determined by optical density measurements at 650 nm and referenced to a standard graphical relationship.

Metabolite Analysis

Glucose measurements were carried out by method of Bernfield (*14*). Ethanol concentrations were determined by gas chromatography (*15*), using CG-200 (model 3537-D) Chromatograph equipped with a Carbowax 20*M* column. 1-propanol (Merck, Germany) was used as an internal standard (1 mg/mL). The measurements were carried out under the following conditions: column at 80°C, detector 198°C, and injector 150°C.

RESULTS AND DISCUSSION

Figures 1a and 1b show the cell growth curves of *Saccharomyces cerevisiae* wild type and AR5 cells, growing in such conditions in which the excess of nitrogen inhibits the GOGAT and NAD-GDH activities, but does not interfere with NADP(H)-GDH activity (*12*). Previously Navarro et al. (*16*) have reported that NH_4^+ and Na^+ compete with K^+ for transport. In

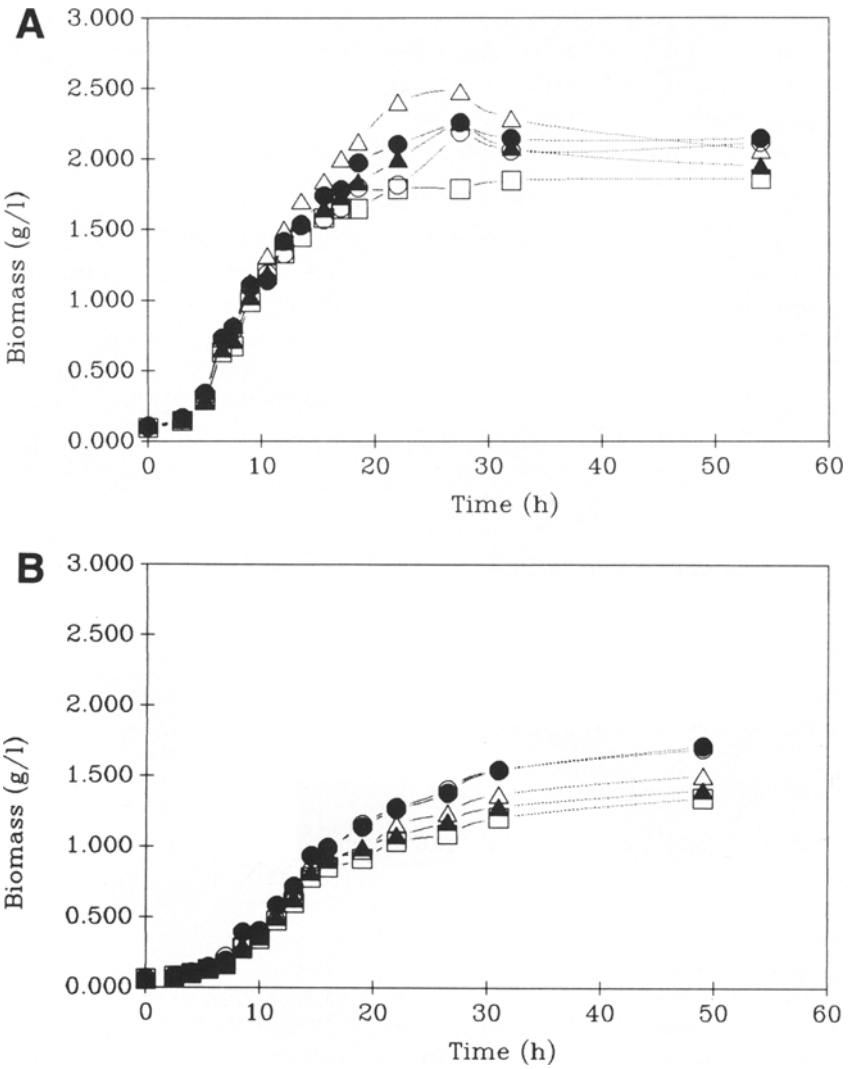

Fig. 1. (a) The growth curve of *Saccharomyces cerevisiae* wild type Σ1278b; (b) The growth curve of *Saccharomyces cerevisiae* AR5. The growth conditions were the same for both strains; 30°C, medium with glucose 1%, ammonium sulphate 0.4%, and yeast nitrogen base (YNB) w/o amino acids and ammonium sulphate, supplemented with different concentrations of KCl, under aeration. ○····○ Control; ●····● 3.5 m*M* KCl; △····△ 60 m*M* KCl; ▲····▲ 120 m*M* KCl; □····□ 180 m*M* KCl.

the presence of NH_4^+ the cells require a much larger concentration of K^+ to obtain a similar growth rate. This behavior could be explained by the lower growth rate observed with AR5 cells compared with wild type cells. A slight decrease in the growth rate after 10 h in both strains is probably caused by oxygen limitation. After 20 h of growth (end of exponential phase), the wild type cells concentration started to increase, suggesting a

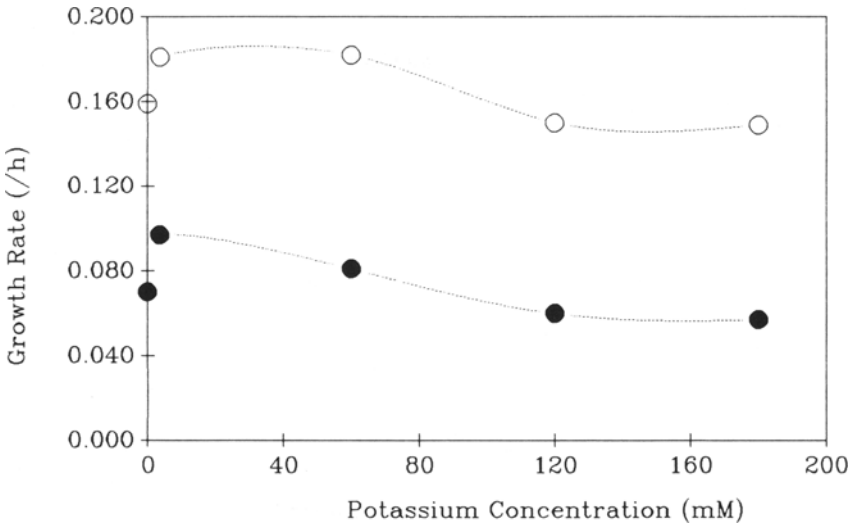

Fig. 2. Growth rate related with different concentrations of KCl in both strains of *Saccharomyces cerevisiae.* ○····○ Wild type cells; ●····● AR5 cells.

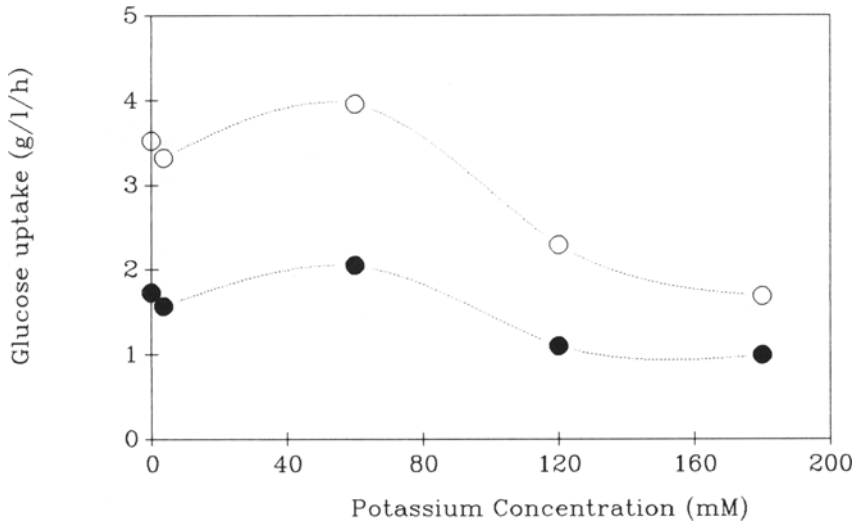

Fig. 3. Glucose consumption rate related with KCl concentration in both strains of *Saccharomyces cerevisiae.* ○····○ Wild type cells; ●····● AR5 cells.

diauxie growth phase by these cells. Glucose was not detectable (Figure 4a) and the ethanol concentration started to decrease, suggesting diauxie growth on ethanol was occurring (Fig. 5a).

Nevertheless, the reduced activity of potassium activated catabolic enzymes, with particular importance to the ratio of the fermentative to oxidative glucose catabolism rate, as described by Wimpalmann et al. (7), could explain the higher ethanol production rate in the AR5 cells in all

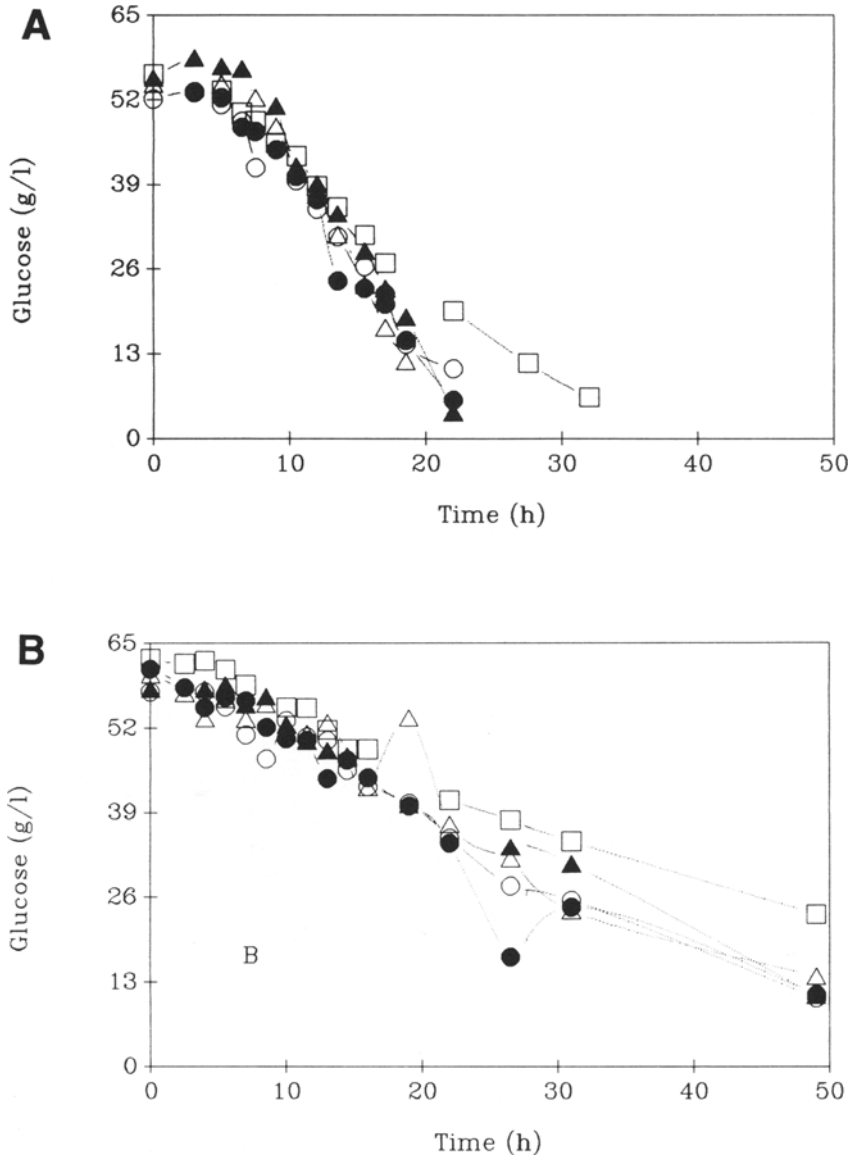

Fig. 4. (a) Glucose consumption rate curve of *Saccharomyces cerevisiae* wild type Σ1278b; (b) Glucose consumption rate curve of *Saccharomyces cerevisiae* AR5. The growth conditions were the same for both strains: 30°C, medium with glucose 1%, ammonium sulphate 0.4%, and yeast nitrogen base (YNB) w/o amino acids and ammonium sulphate, supplemented with different concentrations of KCl, under aeration. ○····○ Control; ●····● 3.5 m*M* KCl; △····△ 60 m*M* KCl; ▲····▲ 120 m*M* KCl; □····□ 180 m*M* KCl.

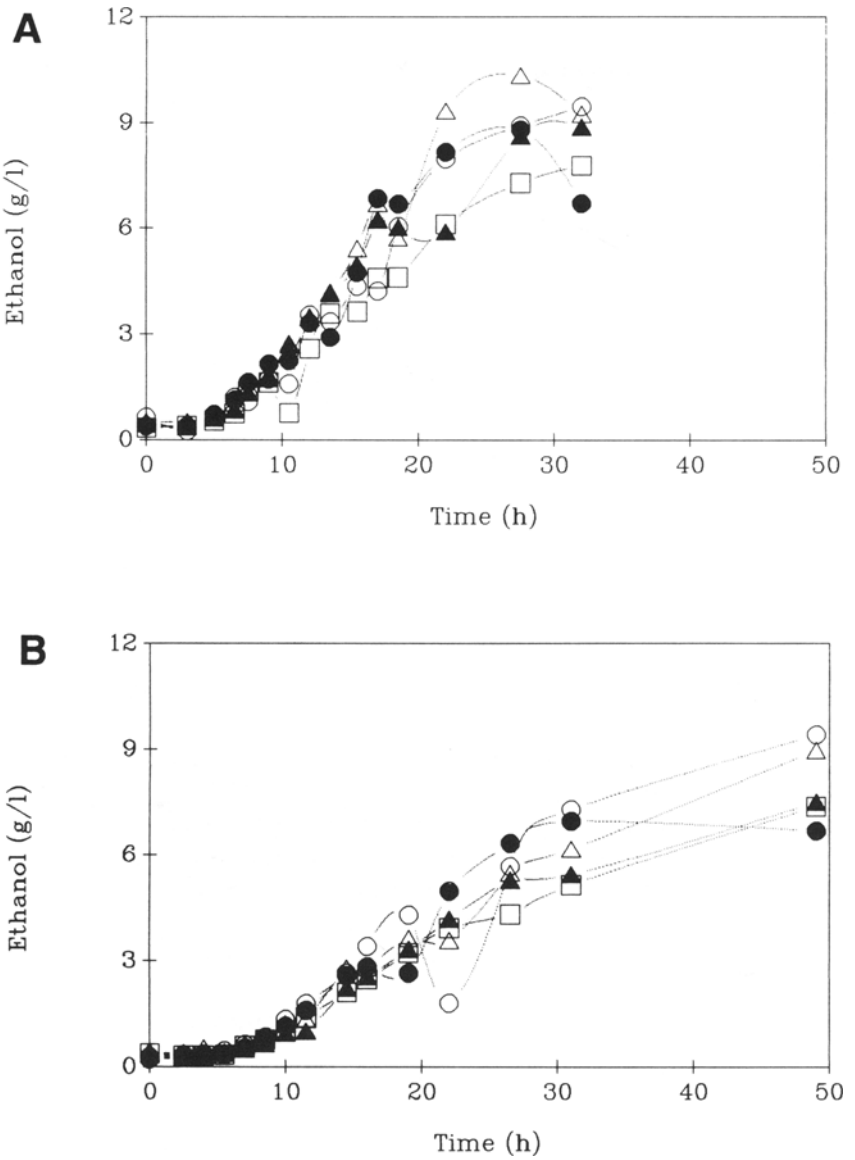

Fig. 5. (a) Ethanol production rate curve of *Saccharomyces cerevisiae* wild type Σ1278b; (b) Ethanol production rate curve of *Saccharomyces cerevisiae* AR5. The growth conditions were the same for both strains: 30°C, medium with glucose 1%, ammonium sulphate 0.4%, and yeast nitrogen base (YNB) w/o amino acids and ammonium sulphate, supplemented with different concentrations of KCl, under aeration. ○····○ Control; ●····● 3.5 m*M* KCl; △····△ 60 m*M* KCl; ▲····▲ 120 m*M* KCl; □····□ 180 m*M* KCl.

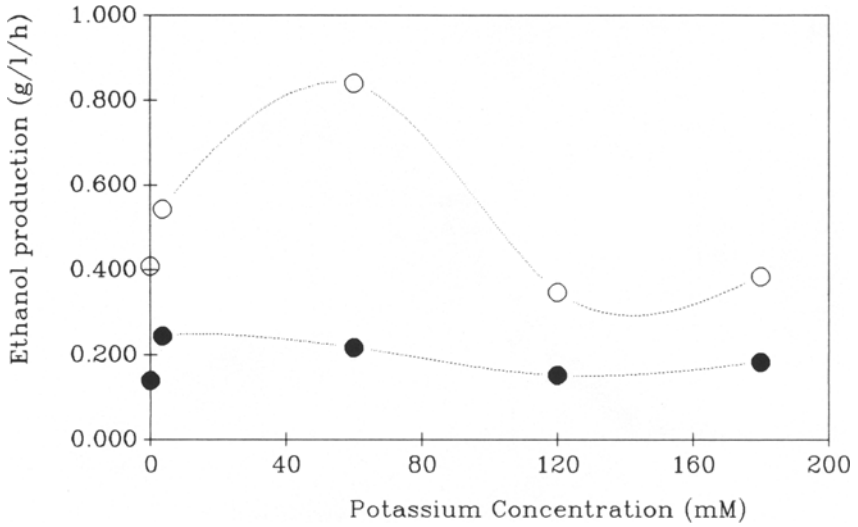

Fig. 6. Ethanol production rate related with different concentrations of KCl in both strains of *Saccharomyces cerevisiae.* ○····○ Wild type cells; ●····● AR5 cells.

potassium-containing media. The highest ethanol production rate was obtained with 3.5 m*M* of potassium (Fig. 6). It was found that after 25 h of growth, the ethanol concentration still slightly increased in the AR5 strain (Figs. 5a,b). Nevertheless, in all growth experiments with wild type cells, the rate of ethanol production was 2.7 times greater than with AR5 cells.

The lower ethanol production rate by the transformed cells could be influenced by the lower growth rate and glucose uptake rate, as a consequence of potassium presence during growth. Both strains showed higher growth and glucose uptake rates in media supplemented with KCl at concentrations up to 60 m*M*. However, media supplemented with KCl above 60 m*M* showed decreased growth rates (Fig. 2). These results are in agreement with Pena et al. (*10*), who observed that the potassium content in culture medium influences the growth rate of *Saccharomyces cerevisiae.* They also observed an upper limit, above which growth did not respond further to K^+ increases. Below the lower limit of 60 m*M*, yeast viability was affected (*10*). Navarro and Ramos (*16*) also reported that NH_4^+ and Na^+ compete with K^+ for transport, where in the presence of NH_4^+ the cells require a much larger concentration of K^+ to obtain a similar growth rate (*16*). This behavior was observed in our experiments, where the cells required a much larger concentration of K^+ to obtain a similar growth rate (*16*). Similar behavior was observed with the cells that carry the plasmid transformed with the NADP-GDH gene, because their ammonia uptake rate is potentially higher than that of the wild type (*17*). Consequently, using the same concentration of potassium they can grow slower than wild type cells. Additionally, Pena et al. (*19*) have shown that potassium uptake leads to a decrease in cell ATP.

It is known that in yeast, some sugars, ammonium ion, amino acids, and K^+ are cotransported with protons. This suggests that K^+ ions can facilitate glucose assimilation. Accordingly, the results presented in Fig. 3 show glucose uptake was stimulated by up to 60 m*M* potassium ion. Above this concentration, glucose uptake was depressed in both strains. In the case of the AR5, repression was twice that of the wild cells. Therefore, the final glucose concentration in the AR5 media during the first 30 h of growth was greater than with the wild type (Figs. 4a,b). This behavior is probably caused by the influence of ammonium in the medium that competes with potassium.

In conclusion, it was observed that in transformed cells the lower ethanol production rate was related to the slower growth and glucose uptake rates. This could be because of a larger rate of ammonium uptake, stimulating the growth of cells.

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