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Kinetics of Asparaginase II Fermentation in *Saccharomyces cerevisiae ure2dal80* Mutant

Effect of Nitrogen Nutrition and pH

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Abstract

Although the quality of nitrogen source affects fermentation product formation, it has been managed empirically, to a large extent, in industrial scale. Laboratory-scale experiments successfully use the high-cost proline as a nonrepressive source. We evaluated urea as a substitute for proline in *Saccharomyces cerevisiae ure2dal80* fermentations for asparaginase II production as a model system for nitrogen-regulated external enzymes. Maximum asparaginase II levels of 265 IU/L were observed in early stationary-phase cells grown on either proline or urea, whereas in ammonium cells, the maximum enzyme level was 157 IU/L. In all cases, enzyme stability was higher in buffered cultures with an initial pH of 6.5.

Index Entries: *Saccharomyces cerevisiae; ure2dal80* mutants; nitrogen sources; asparaginase II; fermentation kinetics.

Introduction

The quality of nitrogen source in a growth medium regulates a variety of bacteria, yeast, and fungus genes. This phenomenon, known as nitrogen regulation *(1)*, besides affecting the synthesis of permeases and metabolic enzymes, plays an important role in the fermentation of industrial bioproducts such as enzymes *(2)*.

Nitrogen regulation is also important for the production of a range of products derived from the secondary metabolism since it regulates the

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induction or repression of the genes responsible for enzymes involved in the relevant metabolic pathways. Although medium optimization, including the quality and concentration of nitrogen source, has been of paramount importance in industrial fermentations, it has been managed empirically, to a large extent, with only a few studies on product formation by changing the nitrogen source. The knowledge that has been accumulated so far on nitrogen regulation allows the use, in laboratory-scale experiments, of proline as a nonrepressive source. However, its high cost precludes its use in industrial scale. Therefore, in light of its nonrepressive role stepwise studies to better characterize other less expensive sources are relevant to process yields and economical viability. Within this framework, we have been studying nitrogen regulation of asparaginase II in *Saccharomyces cerevisiae* as a model system aiming at the acquisition of valuable information to be used in the optimization of medium composition for external enzyme production *(3–5)*.

The synthesis of the *S. cerevisiae* periplasmic asparaginase II, encoded by the gene *ASP3*, is not repressed by carbon or induced by asparagine, being solely regulated by nitrogen *(6)*. Preferred sources, such as ammonium, glutamine and asparagine, repress its production, whereas nonpreferred sources, such as proline, or nitrogen starvation allow higher enzyme levels. Nitrogen regulation of asparaginase II synthesis operates via the GATA activators Gln3p and Nil1p and the GATA repressors Nil2p and Dal80p. The protein Ure2p, which is involved in the signaling pathway in response to preferential nitrogen sources, down-regulates Gln3p and Nil1p *(3,4,5,7,8)*. Nitrogen regulation studies of this enzyme using *S. cerevisiae* mutant strains have shown that fresh mid-log *ure2dal80* cells present enzyme levels 4-fold higher when grown in ammonium and 16-fold higher when grown in proline, in comparison with their wild-type counterparts. The $mRNA_{ASP3}$ levels were in accordance with the enzyme activity data *(5)*. Reported data have also shown that enzyme levels are affected by the pH of the growth medium and the cell's growth phase *(9,10)*.

In the present work, we studied the effect of repressive and nonrepressive nitrogen sources and different pH conditions on the kinetics of asparaginase II fermentations using an *S. cerevisiae ure2dal80* mutant strain. The profiles of cell growth, substrate consumption, asparaginase II accumulation, and changes in pH were monitored in cultures having glucose as carbon source and proline, urea, or ammonium sulfate as nitrogen source. Cultures were carried out under buffered and nonbuffered conditions in the pH range of 4.5–6.5.

Materials and Methods

Microorganism and Fermentation Conditions

S. cerevisiae ure2dal80 mutant strain YAE3R-D12 *(5)* was used. Fermentations were carried out in 1000-mL flasks containing 100 mL of growth medium (20 g/L of glucose; 1.6 g/L of ammonium sulfate, 2.0 g/L of pro-

line, or 0.5 g/L of urea; plus 2.0 g/L of yeast nitrogen base without amino acids and ammonium sulfate).

All media were supplemented with 0.1 g/L of adenine, 0.2 g/L of uracil, 0.1 g/L of histidine, and 0.1 g/L of leucine and presented a C/N ratio of 37. Whenever necessary, the initial pH of the medium was adjusted using an NaOH solution. Potassium phosphate buffer (20 m*M*) was used in buffered cultures. Fermentations were carried out at 29°C and 180 rpm in an Innova 4340 incubator shaker (New Brunswick Scientific).

Analytical Procedures

The culture supernatant was used for determination of glucose concentration by the dinitrosalicylic acid method *(11)* and pH measurement. Cell growth was monitored by the determination of optical density (OD) at 600 nm in a spectrophotometer (Shimadzu-UV 1601). One OD_{600} unity was found to be equivalent to 0.46 mg dry cell weight (DCW). Asparaginase II activity was assayed using fresh cells as previously described *(3,4)*.

Results and Discussion

The effect of initial pH values of 4.5 (pH of the medium without adjustment), 5.5, and 6.5 was evaluated in a first round of proline fermentations using nonbuffered medium. According to the data presented in Fig. 1, irrespective of the medium initial pH, a specific growth rate of 0.32 h^{-1} was measured. After a short lag phase, exponential growth was observed for 7 h, followed by a subsequent decline in the growth rate. Fermentations were completed within 18 h on glucose depletion (data not shown). Asparaginase was detected in late exponential phase cells and peaked (100–120 IU/g of DCW) within 11 to 12 h of fermentation. Although enzyme production per unit of dry cell weight decreased sharply in the cultures with an initial pH of 4.5 and 5.5, in cultures with an initial pH of 6.5 half of the peak enzyme level was still present after 20 h of fermentation. Different from the other cultures, the minimum pH value in this case did not fall bellow 3.3, protecting the enzyme from full inactivation. In accordance with these results, Kim and Roon *(12)* reported a correlation between asparaginase II inactivation and the culture pH: when the pH was maintained at 3.4 or above, a significant retention of asparaginase II activity was observed, whereas when the pH fell below 3.2, the enzyme became inactivated.

Considering the detrimental effects of low pH on the periplasmic asparaginase, phosphate buffer (pH 4.5, 5.5, and 6.5) was used in the subsequent proline fermentations. In these fermentations, the measured minimum pH values were higher than the threshold inactivating pH of 3.2 (Fig. 2). Although maximum enzyme levels were similar to the ones observed in the nonbuffered cultures, the use of buffer reflected positively on the enzyme stability, since a plateau rather than a peak of maximum levels was observed. The use of an initial pH of 6.5 resulted in the highest enzyme level (106 IU/g of DCW) and also in the highest volumetric enzyme concentra-

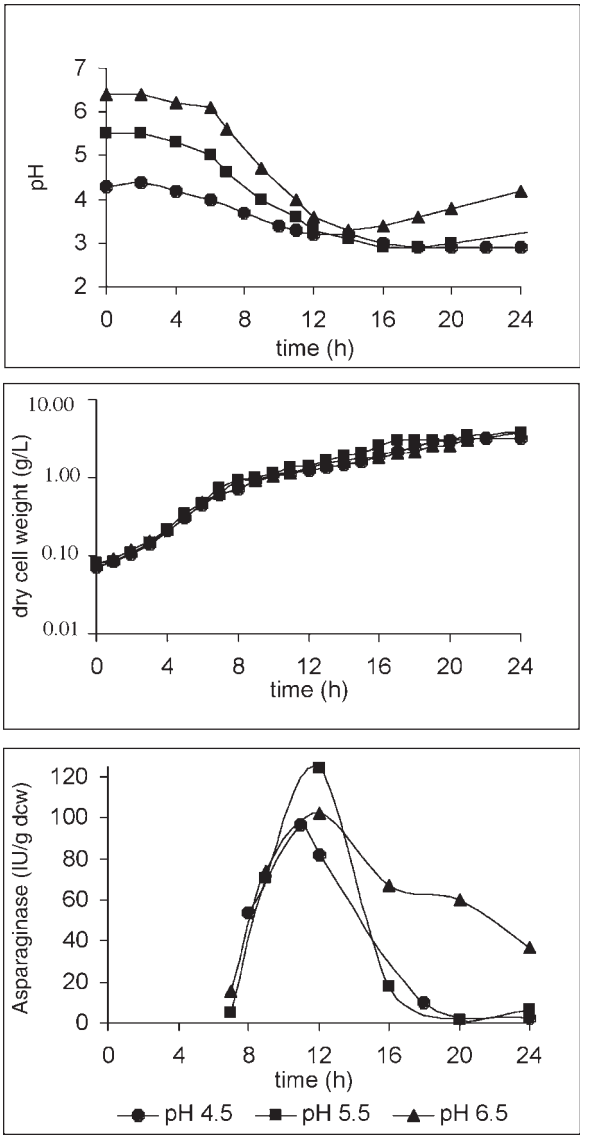

Fig. 1. Time course of *S. cerevisiae ure2dal80* mutant strain culture in nonbuffered medium containing proline as nitrogen source as function of initial pH.

tion (265 IU/L) within 15 h of culture. As such, enzyme levels increased 2.5-fold in comparison to the nonbuffered proline culture with an initial pH of 4.5. Cell growth rates (0.31 h^{-1}) and glucose consumption profiles (data not shown) were not significantly affected by the differences in the initial pH values of the culture nor by the use of buffer.

Accordingly, the subsequent urea and ammonium fermentations were carried out in buffered medium, pH 6.5. Figure 3 compares the cell growth,

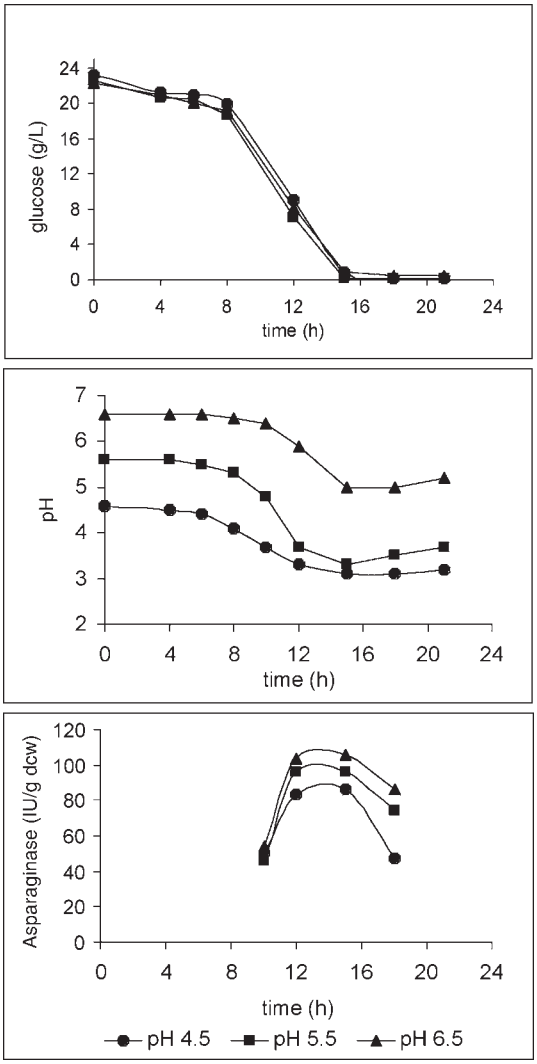

Fig. 2. Time course of *S. cerevisiae ure2dal80* mutant strain culture in buffered medium containing proline as nitrogen source as function of initial pH.

asparaginase production, glucose consumption, and change in pH for these fermentations. The already discussed proline data were included for comparison. Equivalent and somewhat higher specific cell growth rates of 0.35 and 0.34 h^{-1} were respectively observed for the ammonium and the urea medium, compared with the proline medium (0.31 h^{-1}). These results are in agreement with the literature, since according to Magasanik and Kaiser *(1)*, the differences in growth rate supported by very different nitrogen sources are often small. A different pattern of response, though, was observed for enzyme production regarding the nitrogen substrates.

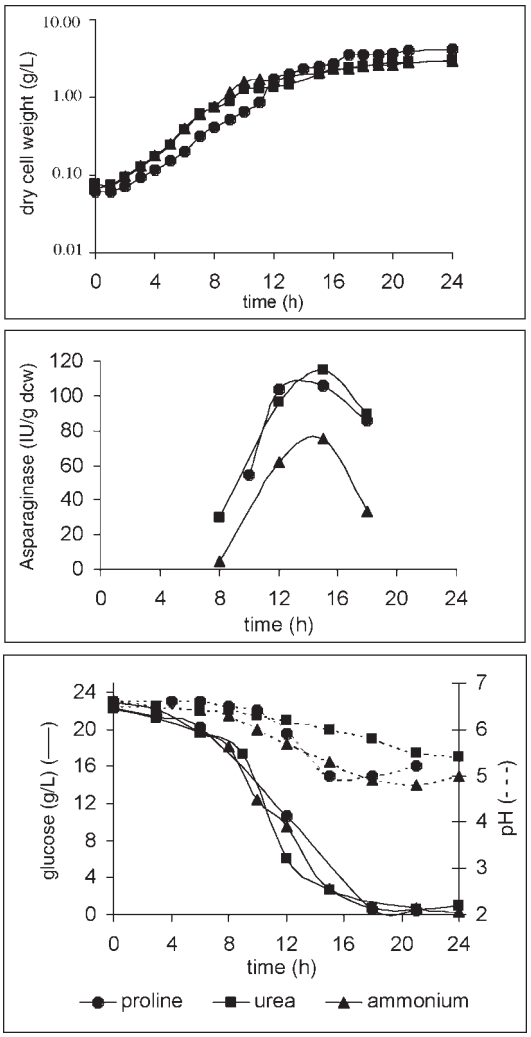

Fig. 3. Time course of *S. cerevisiae ure2dal80* mutant strain culture in pH 6.5 buffered medium containing proline, urea, or ammonium sulfate as nitrogen source.

Equivalent peak enzyme levels of 115 IU/g of DCW (265 IU/L) and 106 IU/g of DCW (265 IU/L) were observed, within 15 h, in the nonrepressive nitrogen sources urea and proline, respectively, and a lower enzyme level of 75 IU/g of DCW (157 IU/L) was measured in the ammonium medium. Therefore, the effect of the nitrogen sources on the general cell metabolism, as evaluated by cell growth, and on the regulation of *ASP3* expression were quite dissociated. This result was expected since in this *S. cerevisiae ure2dal80* mutant strain, *ASP3* expression in the ammonium culture would be repressed by the GATA factor Nil2p *(5)*. According to the data presented in Fig. 3, in all fermentations glucose depletion occurred within 16 h and a similar pattern for glucose consumption was observed.

At peak asparaginase concentration, the urea medium showed a higher pH value (6.0) in comparison with the proline (5.0) and ammonium (5.3) media.

All in all, the study of the effect of nitrogen nutrition and pH of the medium on the kinetics of cell growth, substrate consumption, and periplasmic asparaginase II accumulation using a nitrogen partially derepressed *S. cerevisiae ure2dal80* mutant strain showed that the conditions studied did not significantly affect cell growth. Enzyme production, however, was affected by both nitrogen source and pH of the medium: the use of buffer protected the periplasmic enzyme from inactivation, and the nitrogen nonrepressive urea or proline substrates allowed 50% higher enzyme levels in comparison to the repressive source ammonium. Since urea, a low-cost source, showed to be as derepressive as proline in the asparaginase II fermentations, this source is a valuable option to study nitrogen regulation in a range of other nitrogen-repressed enzymes and metabolites of industrial interest. Moreover, the use of urea is also advantageous, because its metabolism did not result in a sharp pH decline during the fermentation, preventing the inactivation of external enzymes.

Acknowledgments

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