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Effect of Sugar Transport Inactivation in *Saccharomyces cerevisiae* on Sluggish and Stuck Enological Fermentations

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Sluggish and stuck (i.e., very delayed or incomplete) fermentations have been often observed in wine making. Some of them appeared to be associated with insufficient levels of yeast nutrients such as assimilable nitrogen. In these conditions, sugar transport catabolite inactivation, which is triggered by the protein synthesis arrest, may account in part for the inhibition of fermentation. Moreover, this mechanism of inhibition may explain the failure of added ammoniacal nitrogen to nitrogen-limited musts to restore or elevate rate of fermentation after the early yeast growth phase.

The hexose transport system of *Saccharomyces cerevisiae* consists of at least two components with two different affinity constants (high and low affinity) (5, 6). The high-affinity component is subject to catabolite repression and thus is not detectable in cells grown in a high concentration of glucose (7). Moreover, when protein synthesis is inhibited, the hexose transport systems are irreversibly inactivated by a catabolite-inactivation process requiring the utilization of a fermentable substrate (8). This inactivation is responsible for the drastic decrease in fermentation observed in ammonium-starved yeasts (13).

In the enology field, the quantity of assimilable nitrogen plays an important role in the duration and progress of the alcoholic fermentation (W. A. Agenbach, Proc. S. Afr. Soc. Enol. Vitic., p. 66–88, 1977). Recently, the growth-limiting assimilable nitrogen concentrations for *S. cerevisiae* during alcoholic fermentation of grape musts were quantified (2, 4). These studies reveal that ammoniacal nitrogen is totally degraded by yeasts and seems to compensate for some unidentified inhibitory effects appearing during the fermentation progress (for example, the toxic effect of produced ethanol or other unidentified effects).

Moreover, it is well known that the more grape must is nitrogen limited, the more residual sugar concentration remains at the end of the fermentation. To avoid such technological problems, the addition of ammonia during fermentation to nitrogen-limited musts was performed successfully (10, 20) but appears to be efficient only during the early yeast growth phase (M. C. Bezenger, thesis, Université des Sciences et Techniques du Languedoc, Montpellier, France, 1987).

All of these results led us to investigate the possible effect of the glucose transport inactivation on the important decrease of the production rate which appears in the enological alcoholic fermentation progress concurrently with inhibition by produced ethanol (19) or secondary metabolites (12). Such a hypothesis was made a few years ago but was not established (14).

The purpose of the present paper is to evaluate the influence of this physiological mechanism on the production rate by yeast during alcoholic fermentation of a synthetic medium simulating a standard grape must (22, 23) with different ammoniacal nitrogen contents as a unique nitrogen source.

MATERIALS AND METHODS

Yeast strain. The enological strain of *S. cerevisiae* used in this study was isolated during a continuous vinification of white wine in the south of France and was conserved in our laboratory collection (strain V8-6, Institut National de la Recherche Agronomique, Montpellier, France).

Culture conditions. Batch cultures were carried out under anaerobiosis at 28°C in 300- or 1,200-ml fermentors with bubbling CO_2 outlet, containing 250 and 1,000 ml of medium, respectively. Cultures were run with permanent magnetic stirring.

Growth media. The growth medium, previously described (22, 23), was a synthetic medium simulating a standard grape must except for nitrogen source. This medium contained the following components: 30 mM citric acid/citrate buffer (pH 3.5); nitrogen source, NH_4Cl concentrations as indicated; carbon source, 37 mM L-malic acid, 1.1 M glucose; vitamins, 6.3 μM calcium pantothenate, 111 μM *myo*-inositol, 16 μM nicotinic acid, 1.2 μM pyridoxine hydrochloride, 0.74 μM thiamine hydrochloride, 41 nM biotin; trace elements, 8 μM H_3BO_3, 1 μM $CuSO_4 \cdot 5H_2O$, 0.6 μM KI, 12 μM $MnSO_4 \cdot H_2O$, 0.9 μM $NaMoO_4 \cdot 2H_2O$, 6.5 μM $Fe_2(SO_4)_3$, 14 nM $ZnSO_4 \cdot 7H_2O$, 0.5 μM $CoCl_2 \cdot 6H_2O$, 0.5 μM $NiSO_4 \cdot 6H_2O$; mineral salts, 11 mM KH_2PO_4, 2 mM $MgSO_4 \cdot 7H_2O$, 3.5 mM $CaCl_2 \cdot 2H_2O$; anaerobic growth factors, 2.16 g of Tween 80 $liter^{-1}$ (2 ml $liter^{-1}$), 66 μM sodium oleate, 151 μM ergosterol.

Monitoring of fermentation. The amount of CO_2 released was determined by automatic measurement of fermentor weight loss. The validity of this technique and the description of the automatic monitoring of fermentation were described elsewhere (3).

Biomass. The number of cells and the cellular volume were determined by using a Coulter Channelyser 256 linked to an electronic particle counter (model ZBI; Coulter-Counter Coultronics). It was possible to define two distinct cellular populations: the first one with a high mean cellular volume corresponding to mother cells, and the second one with a lower mean cellular volume corresponding to daughter cells. The cellular dry weight was obtained by filtering 5 ml of the culture medium through membrane filters (pore size, 1.2 μm; Millipore Corp.), rinsed with the same amount of distilled water, and desiccated at 108°C until constant weight.

Reducing sugar determination. The reducing sugar concentration in the medium was determined by using dinitrosali-

cylic reagent (18). The fermentation progress was calculated by determining the disappearance of sugars in the culture medium with the following equation: $FP = 1 - (S/S_0)$, where FP is fermentation progress, S is the sugar concentration at time t, and S_0 is the initial sugar concentration.

Ammonia determination. Ammonia concentration in the culture medium was enzymatically determined (1). In the same way, we had verified the accuracy of the method by monitoring the pH of the medium during the fermentation; we observed an acidification of the medium proportional to the disappearance of ammonia as previously stated (9, 21).

Intracellular ammonia concentration was estimated by hydrolyzing, for 24 h at 105°C in 6 M hydrochloric acid, cell biomass corresponding to 10 ml of culture medium. After centrifugation, the supernatant was neutralized and the ammonia concentration was enzymatically determined (1). The mean cellular volume used for the intracellular concentration estimation was the total population mean cellular volume given by the electronic particle counter.

Total cellular proteins. Cell proteins were extracted as described by Jayamaran et al. (11) and determined by the method of Lowry et al. (17).

Activity of the glucose transport systems. The glucose transport activity was studied by using 200 mM D-[U-^{14}C]xylose as described by Serrano and Delafuente (24) with the modifications described by Busturia and Lagunas (8). The use of this concentration of xylose allowed us to measure the activities of the two glucose transport systems. In these conditions, 45 and 55% of the labeled xylose was transported by the systems with high and low affinity, respectively.

The maximum velocities obtained by various authors for glucose uptake were 3.0 and 24.0 mmol g of proteins^{-1} h^{-1} for high- and low-affinity systems, respectively, while in our conditions, we obtained a maximum velocity for xylose uptake of 9.0 mmol g of proteins^{-1} h^{-1}. Standard deviation in our xylose uptake rate determination represents 10 to 12% of the given values.

RESULTS

Appearance of stuck and sluggish fermentations by limiting assimilable nitrogen. The analysis of sugar consumption (Fig. 1A) and biomass production (Fig. 1B) kinetics shows that very low concentrations of assimilable nitrogen in the medium lead to stuck or sluggish fermentations (i.e., incomplete fermentation or extremely slow sugar utilization at the end of fermentation), as stated previously (10).

At the same time, total consumption of ammoniacal nitrogen occurs after about 20 h of fermentation whatever initial nitrogen concentration was used (Table 1). The use of fermentation progress instead of fermentation time allows a better knowledge of the total nitrogen consumption time.

Physiological study of the cells. The physiological behavior of yeast cells during such fermentations may be divided into three phases which are dependent on extracellular ammoniacal nitrogen concentration and fermentation progress (data shown in Fig. 2 summarize situations resulting from the two extreme extracellular ammoniacal nitrogen concentrations used).

(i) Phase A. In phase A, ammoniacal nitrogen intracellular concentration increases parallel with ammoniacal nitrogen disappearance from the medium (Fig. 2). It appears that lowering extracellular initial nitrogen concentration increases this phenomenon of nitrogen intracellular concentration, which is particularly clear for a fermentation progress of 0.2 (Fig. 2A). This increase seems to have no effect on the biomass protein content.

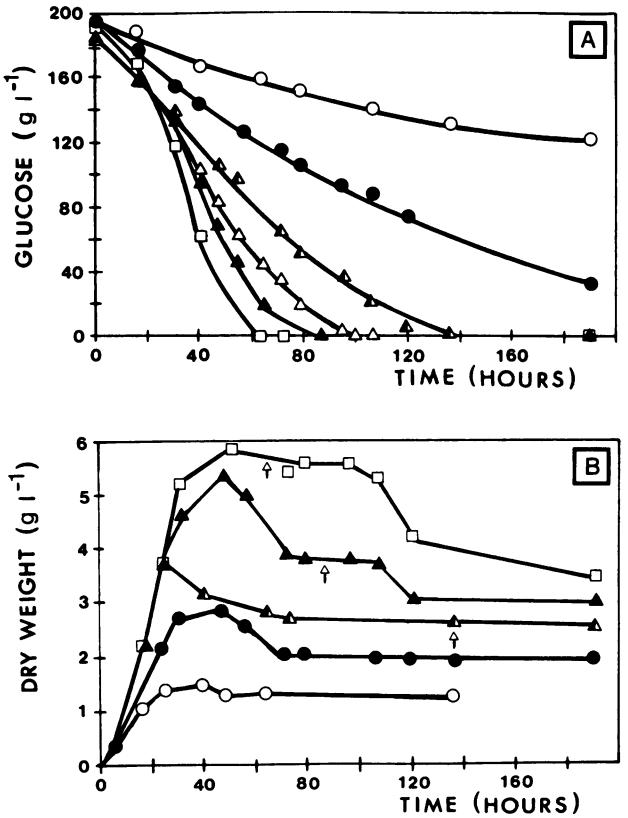

FIG. 1. Disappearance of glucose (A) and biomass production (B) at different ammoniacal nitrogen concentrations (□, 27.9 mM; ▲, 17.9 mM; △, 10.7 mM; ◭, 6.7 mM; ●, 4.5 mM; ○, 1.7 mM). ↑. Complete exhausting of glucose from the medium.

(ii) Phase B. In phase B, extracellular concentration of nitrogen is nil and biomass protein content increases (Fig. 2). During this phase, we observed that an increase of nitrogen limitation in the medium resulted in an increase of mean cellular volumes of the two classes of cells (Fig. 3). On the basis of the initial nitrogen content of the medium, we defined two types of fermentation behavior: biomass protein

TABLE 1. Effect of initial ammoniacal nitrogen concentration on the fermentation time required for obtaining its complete depletion from the medium

Initial ammoniacal nitrogen concn		Fermentation time for ammonium depletion		
mg liter^{-1}	mM	Time (h)	Fermentation progress	Equivalent degraded sugar concn (g liter^{-1})
23.5	1.7	18–20	≈0.10	≈20
37	2.6	18–20	≈0.10	≈20
63	4.5	18–20	≈0.14	≈28
75	5.4	18–20	≈0.14	≈28
94	6.7	20	0.16	32
150	10.7	21	0.17	34
250	17.9	23	0.26	52
300	21.4	25	0.28	56
390	27.9	31	0.37	74

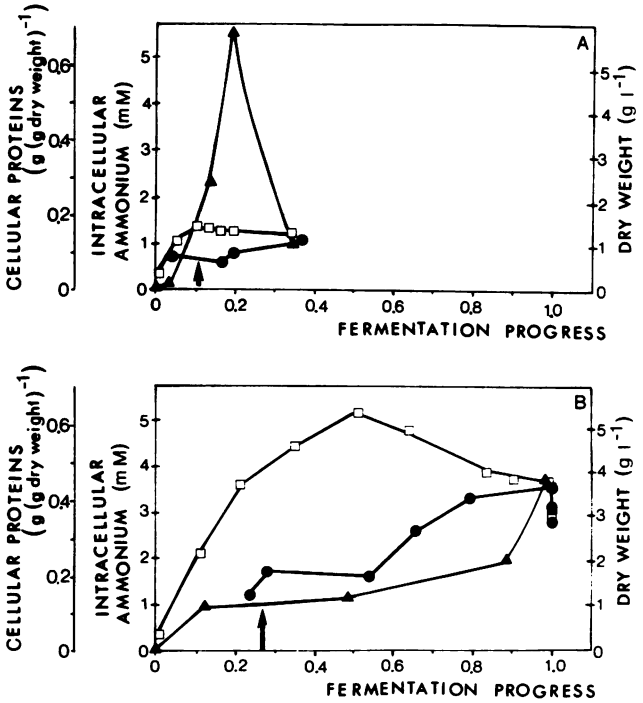

FIG. 2. Intracellular ammoniacal nitrogen concentration (▲), biomass (□), and biomass protein content (●) versus fermentation progress at an initial ammoniacal nitrogen concentration of 1.7 (A) or 17.9 (B) mM.

content increases with a strong decrease of ammoniacal nitrogen intracellular contents (Fig. 2A), leading to stuck or sluggish fermentations, or biomass protein content increases with constant ammoniacal nitrogen intracellular content (Fig. 2B), leading to normal and complete fermentations.

(iii) Phase C. Lastly, a specific phase exists for complete fermentations (initial concentration of ammoniacal nitrogen of 10.7 mM or above), where mean cellular volume decreases (Fig. 3) parallel the lost of cell viability. During this last phase (phase C), protein and ammoniacal contents decrease strongly. These decreases may result from intracellular ammonium assimilation and protein turnover in order to support continued protein synthesis.

These data indicate that after ammoniacal nitrogen depletion of the medium, the cells tend to maintain a sufficient rate of protein synthesis (increase of biomass protein content) in two different ways: by using intracellular reserves of ammoniacal nitrogen without decrease of the biomass, leading to incomplete fermentations, and by decreasing biomass in order to maintain a constant intracellular nitrogen concentration, leading to normal fermentations.

Thus, the only difference between these two yeast populations seems to be the availability of an intracellular ammoniacal nitrogen pool and, therefore, their ability to synthesize proteins.

Relationship between protein synthesis arrest and glucose transport inactivation. We investigated the ammoniacal nitrogen limitation effect on the glucose transport inactivation and the possible role of this phenomenon on the appearance of sluggish and stuck fermentations.

First, we monitored the time course of the CO_2-specific

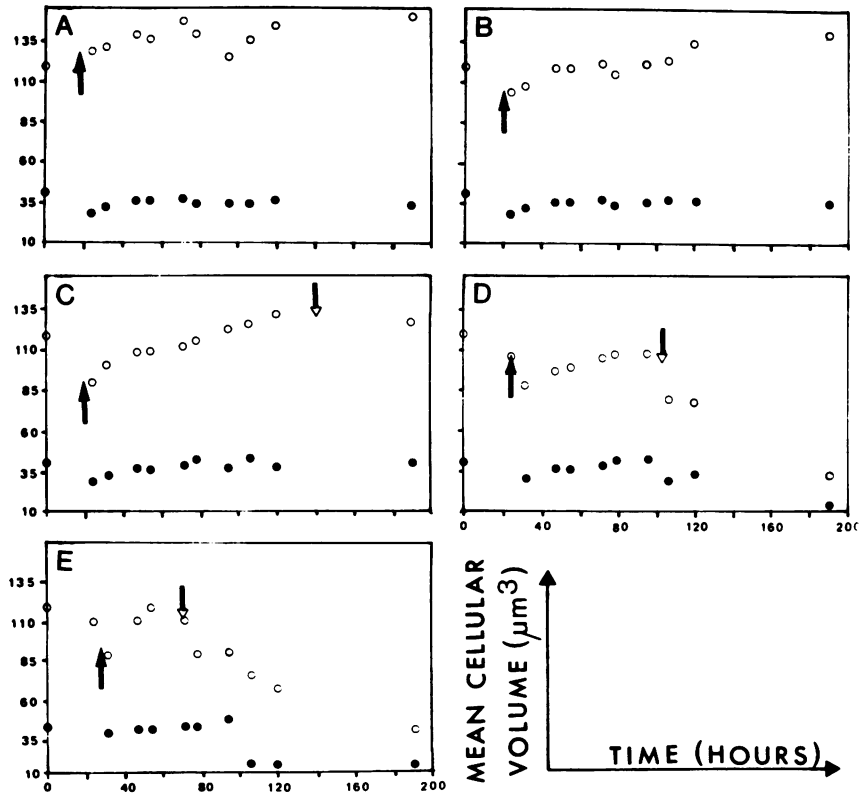

FIG. 3. Mean cellular volumes of mother (○) and daughter (●) cells at different initial ammoniacal nitrogen concentrations: 2.6 mM (A), 4.5 mM (B), 6.7 mM (C), 10.7 mM (D), and 21.4 mM (E). ↓. Complete exhausting of ammoniacal nitrogen from the medium; ↓, complete exhausting of glucose from the medium.

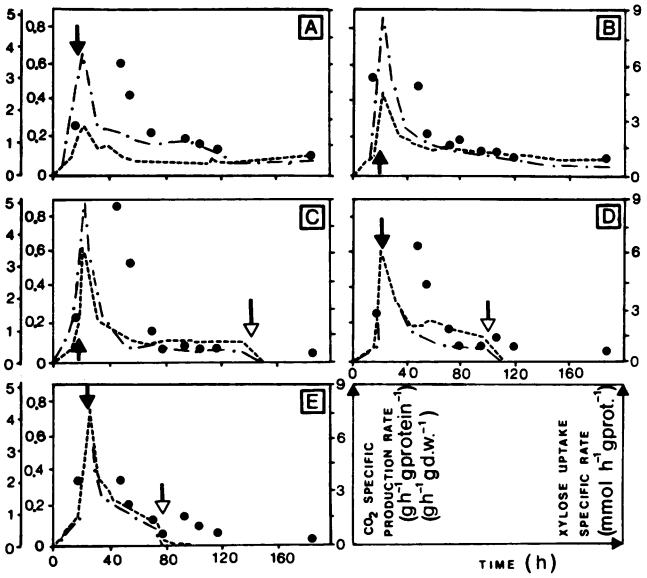

FIG. 4. CO_2-specific production rate (· – · , related to proteins [prot.]; – – – –, related to dry weight [d.w.]) and specific xylose uptake (●) at different initial ammoniacal nitrogen concentrations: 2.6 mM (A), 4.5 mM (B), 6.7 mM (C), 10.7 mM (D), and 21.4 mM (E). ↑, Complete exhausting of ammoniacal nitrogen from the medium; ↓, complete exhausting of glucose from the medium.

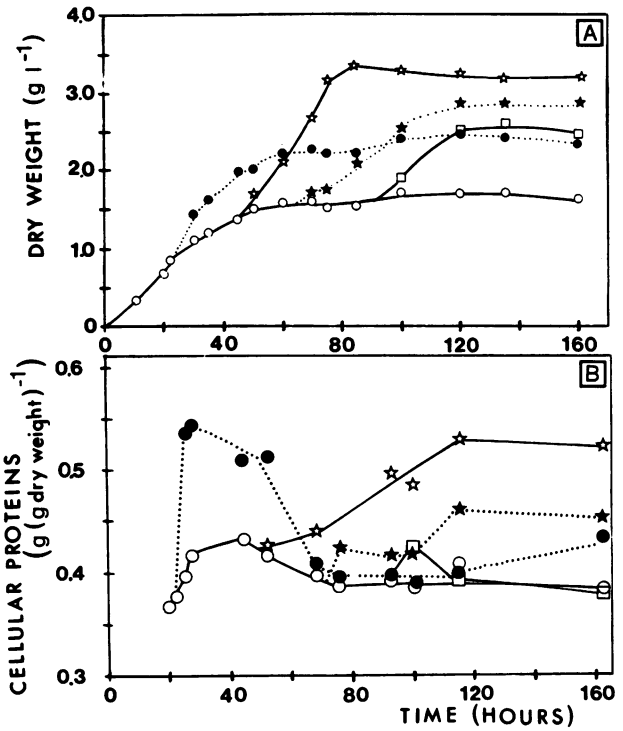

FIG. 5. Effect of ammoniacal nitrogen addition (3 mM) on biomass (A) and biomass protein content (B) during fermentation of a nitrogen-limited medium containing 5.8 mM initial ammoniacal nitrogen. ○, Without addition; ●, addition at 23 h; ☆, addition at 45 h; ★, addition at 69 h; □, addition at 93 h.

production rate (Fig. 4); this specific rate increased as long as ammoniacal nitrogen was available in the medium (phase A), reached a maximum when ammonium concentration in the medium became nil, and then decreased (phase B). Then, we tried to correlate this decrease in the CO_2-specific production rate with the inactivation of glucose transport systems (Fig. 4); inactivation occurred and seemed to be almost complete at 50 h after ammonium depletion from the medium, but the CO_2-specific production rate decreased before inactivation.

It is difficult to measure xylose uptake rate exactly at the peak of CO_2 evolution, which is very brief. Nevertheless, cells harvested during exponential growth on synthetic medium with a high concentration of NH_4Cl as the sole nitrogen source (50 mM) showed a xylose uptake rate of ≈9 mmol g of proteins^{-1} h^{-1}. This value should be approximately the same at the peak of CO_2 evolution.

Besides, at the end of fermentation we found that values of hexose uptake rate (i.e., ≈0.5 mmol h^{-1} liter^{-1} measured for xylose; therefore, ≈0.2 to 3.5 mmol h^{-1} liter^{-1} calculated for glucose, depending on the transport system used) are similar to the CO_2 production values measured at the same time (≈4 mmol h^{-1} liter^{-1}). These data are compatible with a limiting role of sugar uptake in the fermentation rate when glucose transport inactivation has taken place. This result could be of significance in the case of ammonium-limited fermentations where inactivation is complete for fermentation progress values around 0.2.

Moreover, Fig. 4 shows that the complete exhausting of sugar from the medium led to an arrest of inactivation of the sugar transport systems and to a partial de novo synthesis of the sugar transport systems. Exhausting of fermentable sugar from the medium or enhancement of protein synthesis by the addition of ammonium must have the same effect on this de novo synthesis of the sugar transport systems. So, we have tested the possible role of this process after ammonium addition to ammonium-limited medium at this physiological stage.

Relationship between ammoniacal nitrogen addition and glucose transport system activities. The addition of ammoniacal nitrogen to ammonium-limited medium during fermentation reveals different effects on biomass production (Fig. 5A) and especially on CO_2-specific production rates (Fig. 6), depending on the yeast growth stage, as already stated by Bezenger et al. (2). The efficiency of these additions seems to be all the greater if they are made before the end of cellular growth.

As a matter of fact, addition of ammoniacal nitrogen before the maximum cell protein content was reached led to an important increase in the protein synthesis rate (Fig. 5B). On the other hand, later additions of ammoniacal nitrogen led only to limited or even nonexistent protein synthesis. Moreover, the pattern of dry weight increase (Fig. 5A) followed essentially the pattern of cellular protein increase upon subsequent addition of ammoniacal nitrogen. These data indicate that cell protein synthesis ability in response to ammoniacal nitrogen addition depends on the physiological stage of the culture.

Figure 6 shows that a de novo synthesis of glucose transport systems upon addition of ammoniacal nitrogen takes place but is limited by a nonefficient protein synthesis. These results agree with the irreversible character of the glucose transport inactivation (8). This inactivation can be removed only by a high protein synthesis rate, which cannot be obtained in enological conditions after depletion of assimilable nitrogen from the medium.

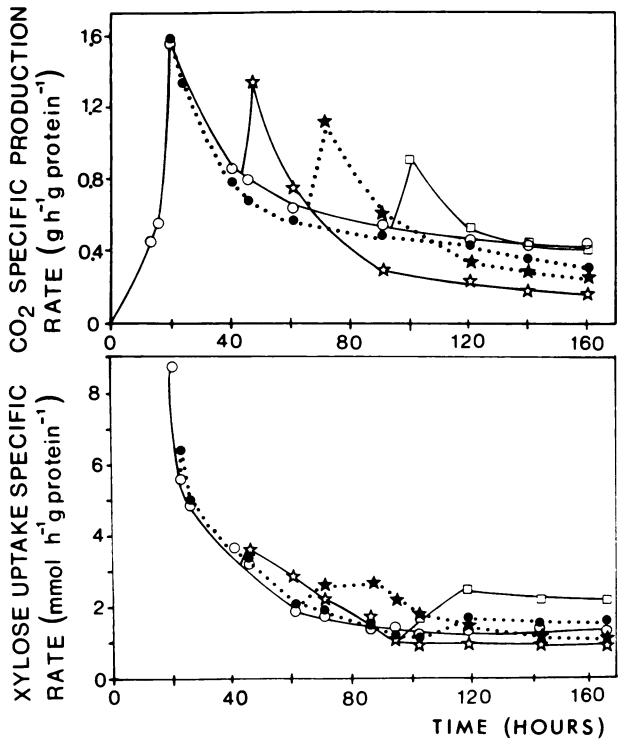

FIG. 6. Effect of ammoniacal nitrogen addition (3 mM) on CO_2-specific production rate and specific xylose uptake during fermentation of a nitrogen-limited medium containing 5.8 mM initial ammoniacal nitrogen. ○, Without addition; ●, addition at 23 h; ☆, addition at 45 h; ★, addition at 69 h; □, addition at 93 h.

DISCUSSION

In enological conditions, i.e., during fermentation of grape musts, the yeast growth arrest generally occurs when the medium still contains high sugar concentrations. At this stage of fermentation, the level of activity of alcoholic fermentation enzymes seems to be normal (15); nevertheless, it appears that there is a rapid inhibition of fermentative metabolism, sometimes leading to stuck fermentations.

This inhibition of fermentative metabolism has been attributed to several mechanisms: intracellular accumulation of ethanol (19), toxicity of some yeast fermentation by-products such as fatty acids (12), sugar transport inefficiency (14), or a conjoined intervention of all or some of these mechanisms (16, 24, 25).

The present study on ammoniacal nitrogen-limited stuck fermentations shows that irreversible glucose transport inactivation may account in part for the inhibition of CO_2 production. In these conditions, glucose transport may appear to be a limiting factor of fermentation when inactivation has taken place. Moreover, the strong irreversibility of this inactivation process seems to explain the poor efficiency of additions of ammoniacal nitrogen to nitrogen-limited musts after cellular growth arrest.

It is noteworthy that all these experiments have been made on synthetic medium with ammoniacal nitrogen as the sole nitrogen source, which is far from the actual must assimilable nitrogen content. Nevertheless, the extrapolation of such results to the fermentative abilities of some musts may shed light on the relationship between assimilable nitrogen and sugar content in the enological field.

Further investigations will consist of studies of the effect of free alpha amino nitrogen limitations on the catabolite inactivation of sugar transport systems during enological fermentations.

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