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Excretion of proline by *Saccharomyces cerevisiae* during fermentation of arginine-supplemented high gravity wheat mash

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SUMMARY

The rate of ethanolic fermentation of high gravity wheat mashes by *Saccharomyces cerevisiae* was increased by nitrogen sources such as ammonium sulfate or arginine. This stimulation was mediated through increased proliferation of cells. Large quantities of proline, however, were excreted by the yeast into the medium when arginine was added as a nutrient supplement. The amount of proline excreted was proportional to the concentration of arginine supplied. Nitrogen sources such as ammonium sulfate or lysine enhanced the production of proline from arginine and its excretion into the medium. Results show that the stimulation of very high gravity fermentation by arginine is not merely through provision of a source of nitrogen but also because it serves as a precursor for the production of proline, a compound which may play a significant role in alleviating the effects of osmotic stress.

INTRODUCTION

The yeast *Saccharomyces cerevisiae* can produce and tolerate relatively high concentrations of ethanol [3,22]. Nutritional and environmental factors, in addition to the genetic makeup of this yeast, greatly influence the concentration of ethanol that can be made [4,5,19]. By controlling these factors it is possible to achieve an ethanol yield in excess of 20% (v/v). The time required to produce such high concentrations, however, may vary with the nature of the substrate, the temperature of fermentation and the nutritional supplements provided. During sake fermentation, which is carried out at about 20 °C, an ethanol yield of 20% (v/v) is quite normal, although 4 or more weeks may be required for the fermentation to complete [10,13,14]. We showed that by fermenting wheat mashes at 20 °C an alcohol yield of 21.5% (v/v) can be obtained within 5 days [21].

In spite of its low assimilable nitrogen content, wheat mash proved to be an excellent medium for the production of ethanol. We have suggested that efficient utilization of wheat mash for ethanol production was related to its free amino acid composition [19,20]. The dissolved solids in wheat mash consist mainly of dextrins which will be saccharified into glucose but small amounts of soluble proteins, free amino nitrogen (FAN) and minerals are also present. The main source of usable nitrogen in wheat mash is the FAN (amino acids). The growth and the extent of fermentation are limited by the availability of this assimilable nitrogen. Interestingly, the proportion of various amino acids in wheat mash was found to be conducive to very efficient utilization of FAN for growth. For example, wheat mash contains low amounts of lysine and relatively large amounts of arginine and asparagine. This is beneficial for cell growth and a rapid rate of fermentation [20].

We have also demonstrated previously that if the growth medium was deficient in assimilable nitrogen (or present in growth-limiting concentrations), lysine present naturally in the medium (as in wheat mash) or added as a nutrient supplement would become inhibitory to yeast growth and retard fermentation [20]. Lysine-induced inhibition, however, was relieved by other exogenously supplied nitrogen sources. Among the nitrogen compounds tested arginine was most effective in relieving this inhibition.

Arginine from the medium is readily taken up by the yeast and hydrolyzed to urea and ornithine through the action of arginase (for reviews on nitrogen metabolism in *S. cerevisiae* see references [6] and [9]). Urea is an excellent source of nitrogen and is second only to arginine in relieving lysine-induced inhibition of yeast growth [20]. Ornithine, on the other hand, is poorly utilized by *S. cerevisiae* as a nitrogen source [6]. It is transaminated to glutamic semialdehyde which is then converted spontaneously to Δ^1-pyrroline-5-carboxylic acid (P5C). Depending on growth conditions, P5C may be reduced to form proline. Where proline can be used as a source of nitrogen it is converted to glutamic acid with P5C serving as an intermediate [1].

It is a common observation that proline is the major free amino acid in juices and fermented alcoholic beverages such as wine [11] and beer [8]. In this communication we present evidence to suggest that at least part of the proline in fermented products may be actively synthesized by the yeast,

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with the rest being present as a result of the inability of the yeast under anaerobic conditions to catabolize the proline present in raw materials [12].

MATERIALS AND METHODS

Materials

Active dry yeast, High-T (high temperature α-amylase) and Allcoholase II (glucoamylase) were supplied by Alltech Biotechnology Center (Nicholasville, KY, USA). The active dry yeast was stored under nitrogen at 4 °C. Amino acids were purchased from Sigma Chemical Company (St Louis, MO, USA). Phenyl isothiocyanate reagent, and Pico·Tag eluents 1 and 2 for free amino acid analysis were purchased from Waters Chromatographic Division (Milford, MA, USA). Amino acid standards were obtained from Pierce (Rockford, IL, USA). All other chemicals were obtained through local suppliers and were of reagent grade. Red hard spring wheat obtained locally was used throughout the study.

Grinding and mashing of wheat

One part of ground wheat was dispersed in 3 parts by weight of water at 60 °C containing 10 μM calcium chloride. To each liter 1.25 ml of high-temperature α-amylase was added. After 5 min, the temperature of the starch slurry was raised to 95–97 °C and held at that temperature for 1 h with continuous stirring. The volume lost because of evaporation was made up by adding sterile distilled water, and the temperature lowered to 80 °C. The gelatinized starch was liquefied further by adding another 1.25 ml of α-amylase per liter mash and incubated for 30 min. This liquefaction step hydrolyzed all of the starch to soluble dextrins. The water-soluble portion of the mashes thus prepared contained 20–22 g of dissolved solids per 100 ml. This mash could be saccharified without further treatment and then fermented, or most of the particulate matter from the mash could be removed by straining through a stainless-steel food strainer (20 mesh) followed by saccharification and fermentation.

Fermentation

In most experiments 500-g samples of the wheat mash were transferred to jacketed sterile Celstir fermentors (Wheaton Instruments, Millville, NJ, USA) which either contained 10 ml sterile distilled water or 10 ml sterile solutions of the supplements used. The fermentors were connected to a water bath circulator maintained at 30 °C. Dextrins in these mashes were saccharified to fermentable sugars by adding 1 ml of Allcoholase II (glucoamylase) to each fermentor. After 30 min, the fermentors were cooled to 20 °C and inoculated with active dry yeast to give an initial cell number of approximately 3×10^7 cells per g of mash. Preparation of the inoculum has been described previously [19].

Measurement of total dissolved solids

Samples withdrawn from the fermentors at various times were centrifuged at 10 300 × *g* for 15 min and the specific gravities of clear supernatant liquids were determined at 20 °C with a digital density meter (DMA-45, Anton Paar KG, Graz, Austria). With the aid of appropriate tables, the results were converted to grams of dissolved solids (expressed as grams of sucrose) per 100 ml.

Cell number and cell viability

Total cell counts and viable cell counts were determined by the direct microscopic method described previously [19].

Analysis of free amino acids by high performance liquid chromatography (HPLC)

The free amino acids in the mashes and fermentation liquids were measured by the HPLC method developed for the determination of free amino acids in physiological samples (Waters Chromatographic Division). Clear supernatant liquids obtained by centrifugation of samples at 10 300 × *g* for 15 min were derivatized by the Pico·Tag procedure and analyzed by injecting into a Pico·Tag free amino acid column (3.9 mm × 30 cm) maintained at 46 °C. Derivatized amino acids were separated by eluting with a proprietary gradient solvent system delivered at the rate of 1 ml min^{-1}, and were detected and quantified by measuring absorbance at 254 nm with a UV detector (model 410, Waters Chromatographic Division). The results were processed with the Maxima 510 computer program developed for the analysis of chromatographic data.

RESULTS

Rate of fermentation

All of the sugars in an unsupplemented wheat mash which initially contained 21–22 g of dissolved solids per 100 ml were fermented within 120 h (Fig. 1A). This is in agreement with results reported earlier [19]. Ammonium sulfate added to the mash at a concentration of 10 mM reduced the fermentation time to 72 h. Arginine supplementation (10 mM) of the mash stimulated the fermentation to a greater extent and allowed it to be completed within 48 h. There was no further improvement in the rate of fermentation or reduction in the time required for the complete utilization of sugars when varied amounts of arginine along with 10 mM ammonium sulfate were added to the wheat mash. As expected, the rate of cell proliferation and maximum cell number attained reflected the rate of fermentation (Fig. 1B).

Arginine alleviates lysine-induced inhibition of fermentation

Although wheat mash is deficient in assimilable nitrogen, all of the sugars in the mash can be fermented by *S. cerevisiae* at 20 °C. It was reported earlier that a mash containing 20 g dissolved solids per 100 ml (mainly fermentable sugars) could be fermented in 5 days at 20 °C [19–21]. On supplementing the mash with an assimilable source of nitrogen, the duration of fermentation was reduced to 2 days or less. We also showed that under nitrogen-deficient conditions, the amino acid lysine either naturally present in the mash or added exogenously as a source of nitrogen became inhibitory to yeast growth. This resulted in a reduced

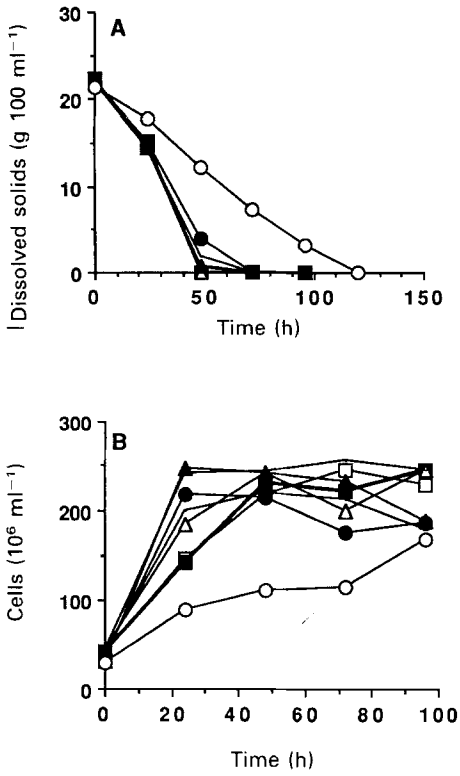

Fig. 1. Rate of depletion of dissolved solids (A) and the increase in cell numbers (B) during fermentation of high gravity wheat mashes by *S. cerevisiae*. The mashes were: unsupplemented (control) (○); supplemented with 10 mM arginine (▽), or with 10 mM ammonium sulfate (●). All other mashes contained 10 mM ammonium sulfate along with arginine at the following concentrations: line without symbol, 2 mM; ▲, 4 mM; △, 6 mM; ■, 8 mM; □, 10 mM.

rate of fermentation and an incomplete utilization of fermentable sugars. As shown in Fig. 2A, the fermentation was 'stuck' when lysine was added to the mash at a concentration of 10 mM and only 57% of the dissolved solids were used by the yeast by 120 h. The lysine-induced inhibition was prevented by adding various nitrogen sources to the mash at the beginning of fermentation. The rate of fermentation increased with increasing concentrations of arginine (the most effective nitrogen source in relieving lysine-induced inhibition [20]), even though the mash initially contained 10 mmol lysine L^{-1} (Fig. 2A). All of the fermentable sugars in the mash were consumed within 48 h when the added arginine concentration in the medium was 6 mM or greater. Even at a concentration of 2 mM arginine stimulated fermentation, although in this case 96 h were required for the complete utilization of sugars.

It is clear that arginine relieved the lysine-induced inhibition of fermentation by increasing cell proliferation and by maintaining the viability of yeast cells at high levels (Fig. 2B,C). When lysine was the only supplement, a maximum of 9.0×10^7 cells per ml were produced (a three-fold increase), but the viability of the yeast population dropped from 80% to less than 20%. In the control (unsupplemented mash) there was a four-fold increase in

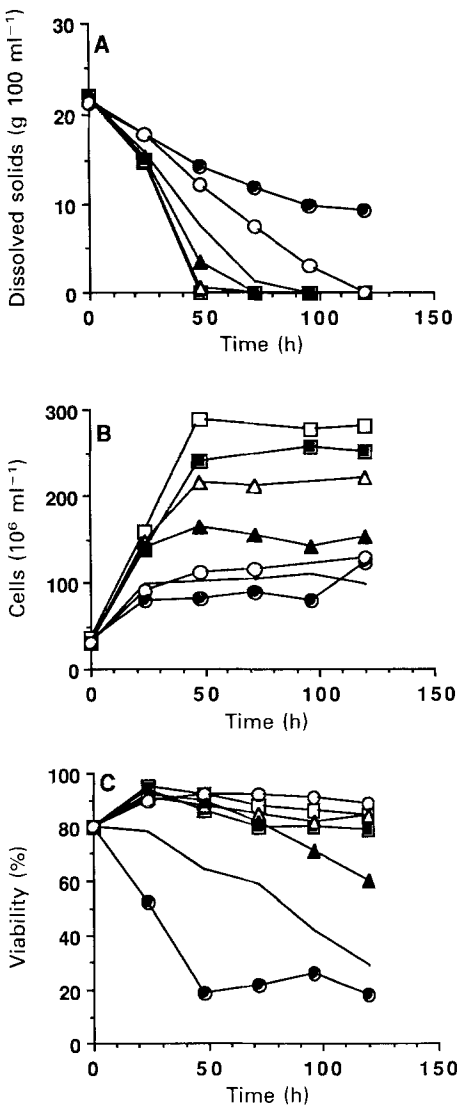

Fig. 2. Rate of depletion of dissolved solids (A), increases in cell number (B), and changes in cell viability (C) during the fermentation of wheat mashes supplemented with lysine and arginine. The mashes were: unsupplemented (control) (○), or supplemented with 10 mM lyine (●). All other mashes contained 10 mM lysine along with arginine at the following concentrations: line without symbol, 2 mM; ▲, 4 mM; △, 6 mM; ■, 8 mM; □, 10 mM.

cell number and viability of the yeast cell population remained over 90% throughout fermentation. Both percentage viability and maximum cell number increased as the concentration of arginine in the lysine-supplemented mash was raised from 0 to 10 mM. At an arginine concentration of 10 mM the maximum cell number in the lysine-supplemented mash was 2.9×10^8 ml^{-1}, a 9.6-fold increase.

Excretion of proline into the medium

The wheat mash used in this work contained only 35 nmol proline L^{-1}, most of which was taken up within the first 24 h and used by the yeast for growth (Table 1). Addition of lysine to the mash at a concentration of 10 mM not only seemed to inhibit the uptake of proline but also increased its concentration in the mash. Supplementing the mash with

TABLE 1

Excretion of proline by *S. cerevisiae* during the early stages of fermentation of high gravity wheat mash supplemented with 10 mM lysine or 10 mM lysine plus 10 mM arginine

Time (h)	nmol proline per ml supernatant liquid		
	Control	lys	(lys + arg)
0	35	35	35
3.0	41	96	139
6.0	49	70	147
12.0	26	47	194
24.0	5	73	3790

arginine which is known to reverse lysine-induced inhibition of yeast growth [20], further increased the concentration of proline in the mash and this increase became quite pronounced after 12 h of fermentation. By 24 h the concentration of proline in the medium had increased to 3790 nmol ml^{-1}.

Proline excretion into the medium by the yeast appears to be dependent on exogenously supplied arginine. Readily assimilable nitrogen sources such as aspartic acid, asparagine, glutamic acid, or leucine did not cause excretion of proline into the medium (data not shown). When no supplement was added or when the only supplement was ammonium sulfate, no proline was detected in the medium after 24 h of fermentation (Table 2). Although ammonium sulfate did not promote excretion of proline into the medium, it appeared to have a synergistic effect on proline production in the presence of added arginine. For example, when arginine (10 mM) was the only supplement, the proline concentration reached a maximum value of 5653 nmol ml^{-1}. When ammonium sulfate (10 mM) was also added the yield of proline increased to 8732 nmol ml^{-1} (Table 2). A similar synergistic effect was observed when lysine and arginine were added to the mash.

Although arginine was available from the beginning of the fermentation there was no significant amount of proline produced during the first 12 h of fermentation (Table 1). Even at a high initial arginine concentration of 10 mM, over 70% was taken up in 24 h by the yeast. By 48 h there was no detectable amount of arginine present in the medium (Table 3). This suggested that there was some delay either in the conversion of absorbed arginine to proline or in its excretion into the medium. For example in the medium which originally contained 10 mmol arginine L^{-1}, proline at 24 h accounted for only half of the arginine consumed. Its concentration gradually increased and by 48 h the amount of proline in the medium was equivalent to 95% of the arginine added at the beginning of fermentation.

When the yield of proline was plotted against initial arginine concentration in the medium a linear correlation ($r^2 = 0.993$) was observed if the medium also contained 10 mmol lysine L^{-1} (Fig. 3A). When lysine was replaced with ammonium sulfate (10 mM) as the supplement the relationship was not linear (Fig. 3B), although the amount of proline excreted still increased with increasing concentrations of arginine in the medium.

TABLE 2

Excretion of proline by *S. cerevisiae* during fermentation of wheat mash supplemented with ammonium sulfate (10 mM) and increasing amounts of arginine. Proline concentration was determined at 48 h

Addition to wheat mash		nmol proline per ml supernatant
Arginine (mM)	$(NH_4)_2SO_4$ (mM)	
0	10	0
2	10	568
4	10	2126
6	10	4672
8	10	7546
10	10	8732
10	0	5653

DISCUSSION

The amino acid arginine can serve as a source of nitrogen for *S. cerevisiae* and it has been shown that in a simple, defined medium arginine can promote growth at rates comparable to those by ammonium sulfate [23]. We have previously shown that of the nitrogen sources tested, arginine was the most effective in relieving lysine-induced inhibition

TABLE 3

Effect of arginine addition on excretion of proline and uptake of arginine by *S. cerevisiae* during fermentation of lysine-supplemented (10 mM) wheat mash

Arginine concentration (mM)	Time (h)	nmol per ml sample of supernatant	
		Arginine	Proline
2	24	282	1269
2	48	0	1441
2	96	0	1467
4	24	271	2380
4	48	0	3723
4	96	0	3031
6	24	449	2672
6	48	0	4983
6	96	0	4177
8	24	1396	3392
8	48	0	7440
8	96	0	7098
10	24	2862	3569
10	48	0	9492
10	96	0	8246

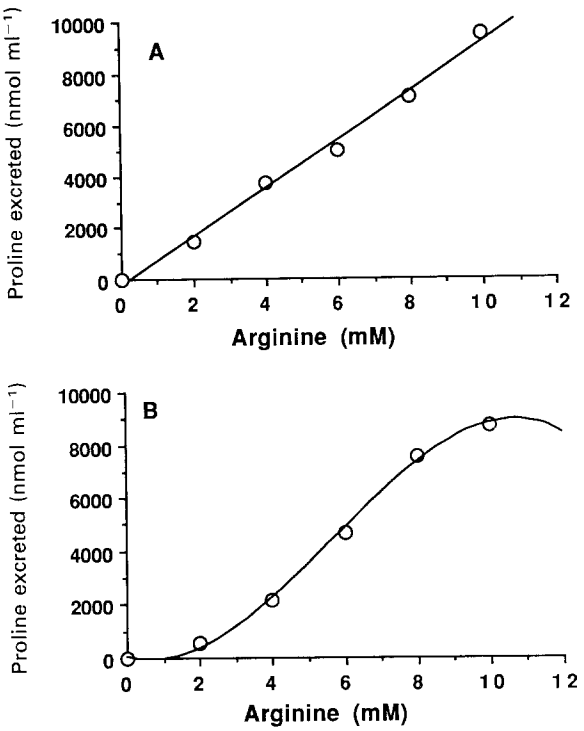

Fig. 3. Relationship between the amount of proline excreted by *S. cerevisiae* and the concentration of arginine in the mash supplemented with 10 mmol lysine (A) or with 10 mmol ammonium sulfate (B) per liter of mash.

of yeast growth [20]. Indirect evidence suggested that it was urea derived from arginine that prevented or alleviated the lysine-induced inhibition. Apart from being a source of nitrogen, added arginine appeared to play a significant role in promoting fermentation of wheat mashes containing high concentrations of sugars. For example, very high gravity wheat mashes containing 30–35% sugar (w/v) could be fermented within 4 days at 20 °C if they were supplemented with 20 mmol arginine L^{-1}. Other nitrogen sources such as ammonium sulfate, yeast extract or other amino acids, although added to give a nitrogen concentration equal to or higher than that provided by 20 mM arginine, failed to accelerate the fermentation to the same extent (data not shown). This suggested that in promoting very high gravity fermentation, arginine may have a role(s) other than serving merely as a source of nitrogen. Arginine could serve as a precursor for several compounds such as citrulline, ornithine, glutamic acid and proline [9]. Previous workers showed that basic amino acids are taken up by the yeast and that carbon from these compounds is retained within the cells against large concentration gradients [24]. Although the pathway of formation of proline from arginine in *S. cerevisiae* is well known, there exists some uncertainty as to the fate of the proline thus derived. Brandriss and Magasanik [1] did not observe excretion of proline into the medium by wild-type *S. cerevisiae* growing with arginine or ornithine as sole nitrogen source. In contrast to this, the results reported here have clearly shown that proline is produced by the yeast and is excreted into the medium. This excretion is dependent on the presence of arginine in the medium.

A decrease in the intracellular arginine concentration is correlated with a derepression of arginase, which occurs only in the absence of nitrogen-containing compounds [2]. However, our results show that the conversion of a given amount of arginine to proline and its excretion into the medium was greater in the presence of other nitrogen compounds such as ammonium sulfate or lysine than in their absence. This indirectly suggests that arginase was not repressed or that under the high gravity fermentation conditions employed in this study, the activity of the enzyme was increased. Since intracellular arginine is compartmentalized in vacuoles and the cytosol [9], its mobilization from these locations may be under the control of different mechanisms.

Fermentation of high gravity wheat mash was stimulated by exogenously supplied arginine and this stimulation may be partly related to the production and excretion of proline into the medium. Preliminary studies with a defined medium containing high concentrations of sugars showed that additions of proline increased the rate of uptake and fermentation of sugars (manuscript in preparation, to be presented at the SIM meeting at Toronto, Canada in August 1993). Although proline under repressed and anaerobic conditions cannot serve as a source of nitrogen [12], it may be involved in protecting yeast cells from osmotic stress during fermentation of these high gravity wheat mashes.

Proline has been shown to have diverse roles in biological systems. Apart from being an important nutrient, it appears to alleviate effects of osmotic stress in plants [16,17], and in bacteria under low water activity [7], and it serves as a cryoprotectant under certain conditions [15]. It is interesting to note that honey always contains a certain amount of proline which appears to be made mostly by the bees [18], and it may have an unrecognized biological role(s) in protecting bees from severe osmotic stress caused by the low water activity. Research into the effects of exogenously added proline on yeast metabolism during very high gravity fermentation is currently in progress.

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