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Nutrient-Enhanced Production of Remarkably High Concentrations of Ethanol by *Saccharomyces bayanus* through Soy Flour Supplementation

CRISTINA A. VIEGAS, ISABEL SÁ-CORREIA,* AND JÚLIO M. NOVAIS

Laboratório de Engenharia Bioquímica, Instituto Superior Técnico, 1096 Lisbon Codex, Portugal

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The supplementation of a simple medium with soy flour led to an increase in the specific growth rate and viable cell concentration of *Saccharomyces bayanus* during fermentation. Increasing the amount of soy flour led to an increase in the maximum number of viable yeast cells and the percentage of glucose fermented. It was possible in 64 h to reach 12.8% (wt/vol) ethanol by adding 4% soy flour (wt/vol) to a simple medium with 300 g of glucose per liter. The aqueous extract from soy flour was nearly as effective as whole-soy flour, whereas the lipidic fraction had no positive effect.

The addition of soy flour, an abundant and inexpensive source of proteins (38%) and lipids (21%), has been shown to lead to a significant increase in batch and continuous alcoholic fermentation productivity by both *Saccharomyces cerevisiae* (2) and *Zymomonas mobilis* (9).

Large amounts of fermentable sugars are often incompletely used during fermentation, but improvements as a result of broth supplementation with lipids, proteins, or vitamins have been reported (1, 3, 5–7). Most conventional processes yield about 8 to 10% (wt/vol) ethanol, after which no fermentation of any remaining sugar is achieved. However, it is well known that in traditional saké production, concentrations of ethanol as high as 16% (wt/vol) can be obtained. It has also been shown that if oryzenin (a rice protein), albumin, or koji mold micelia are added to a usual fermentation medium, the final ethanol concentration can also reach similar levels (5). Significant improvements of alcoholic fermentation as a result of the addition of unsaturated fatty acids and sterols or the lipid-protein complex have also been reported (1, 3, 6, 7), their effect being attributed to the enhancement of resistance of yeast cells to ethanol. This higher tolerance has been related to a more favorable membrane composition in terms of unsaturated fatty acids and sterols (14, 15). In fact, ethanol toxicity and tolerance of yeast cells have been pointed out as the limiting factor of final ethanol concentration and fermentation rate (N. van Uden, *in* G. T. Tsao, ed., *Annual Reports on Fermentation Processes*, vol. 8, in press), and the effect of the action of additives has been attributed to the enhancement of resistance of yeast cells to ethanol (3, 6, 7). However, it has been suggested that nutritional deficiencies rather than ethanol toxicity are responsible for the premature stoppages of fermentations (1). Improvements by nutritional enrichments have been recognized in the wine and beer industries (1, 11).

We previously studied the mechanism of action of soy flour supplementation (16). The possibility that an increase in the tolerance of yeast cells to ethanol could be responsible for fermentation improvement was rejected because inhibition of the glucose consumption rate by ethanol was identical for cells grown in supplemented and unsupplemented media. The positive action of soy flour was attributed to the satisfaction of nutritional deficiencies: its addition to a stopped fermentation caused its rapid resumption.

To thoroughly examine the role and the potentialities of soy flour and to recognize the missing nutrients, batch alcoholic fermentations were carried out with an industrial strain of *Saccharomyces bayanus* distributed by the Institut Oenologique de Champagne, Éparnay, Montréal, France, and prepared by LALVIN, Canada, in the form of an active dry granulated material. The simple fermentation medium was composed (in grams per liter) of yeast extract (Difco Laboratories), 5; $(NH_4)_2SO_4$, 5; KH_2PO_4, 5; $MgSO_4 \cdot 7H_2O$, 1; and glucose, 300 or 400; soy flour was added at concentrations of 0, 2, 4, or 6% (wt/vol). The effect of whole-soy flour was compared with the effect of its water-soluble fraction, which was obtained by washing soy flour with tap water at ambient temperature, and with its lipidic fraction, which was obtained by extraction with ethyl ether followed by solvent evaporation. The aqueous and the lipidic fractions were added to the fermentation medium at levels corresponding to the extracts of 6% (wt/vol) soy flour. All media were sterilized at 120°C for 20 min. Batch alcoholic fermentations were carried out in conical flasks closed with a rubber bung perforated by a needle; the flasks were incubated in a shaker at 30°C. Inoculation was done by rehydration of the active dry yeast in the fermentation medium (0.1 g/liter). For sampling during fermentation, the bungs were taken off. The glucose concentration was determined by a glucose analyzer (YSI model 27; Yellow Spring Instrument Co.). Ethanol was analyzed by gas chromatography. Viable cells were counted by plating 0.1 ml of the fermentation medium, after adequate dilution, on the surface of petri dishes with a medium composed of 2% glucose, 1% peptone, 0.5% yeast extract, and 2% agar.

In an unsupplemented medium, a concentration of 300 g of glucose per liter was incompletely fermented by *S. bayanus* (Fig. 1), and the final concentration of ethanol did not exceed 8% (wt/vol), as is common for traditional processes. However, the addition of 2 or 4% (wt/vol) soy flour led to a significant increase in the amount of glucose fermented and to remarkably high final concentrations of ethanol, reaching 11.6 and 12.8% (wt/vol), respectively (Fig. 1). Such unusual concentrations of ethanol were obtained after 64 h of fermentation, with an identical maximum productivity (2 g/liter

* Corresponding author.

per h), and only a slight loss in the ethanol yield (respectively, 0.48, 0.47, and 0.44).

The presence of soy flour led to an increase in the rate and extent of growth (Fig. 2). After 18 to 24 h of fermentation for both supplemented and unsupplemented media, growth stopped, but the maximum viable yeast concentration increased with soy flour concentration. Afterwards, death occurred, and the death rate increased with the concentration of ethanol accumulated (Fig. 2). After the cessation of growth, glucose fermentation still continued, but more slowly, and stopped when the concentration of viable cells attained an insignificant level. The addition of 4% soy flour did not allow the total fermentation of a higher concentration of glucose (345 g/liter); however, this could perhaps be possible if a higher concentration of soy flour were used.

A medium with 400 g of glucose per liter and 6% (wt/vol) soy flour was fermented in an attempt to produce a concentration of ethanol as high as the 16% (wt/vol) attained in saké fermentations. After 66 h the fermentation stopped. By that time only 316 g of glucose per liter was consumed, and 12.2% (wt/vol) ethanol was produced, with a low yield (0.39), compared with the value obtained with the lower glucose concentration. An initial concentration of glucose as high as 400 g/liter strongly inhibits fermentation (8). Inhibition by sugar and ethanol appears to be synergistic (8), and for such

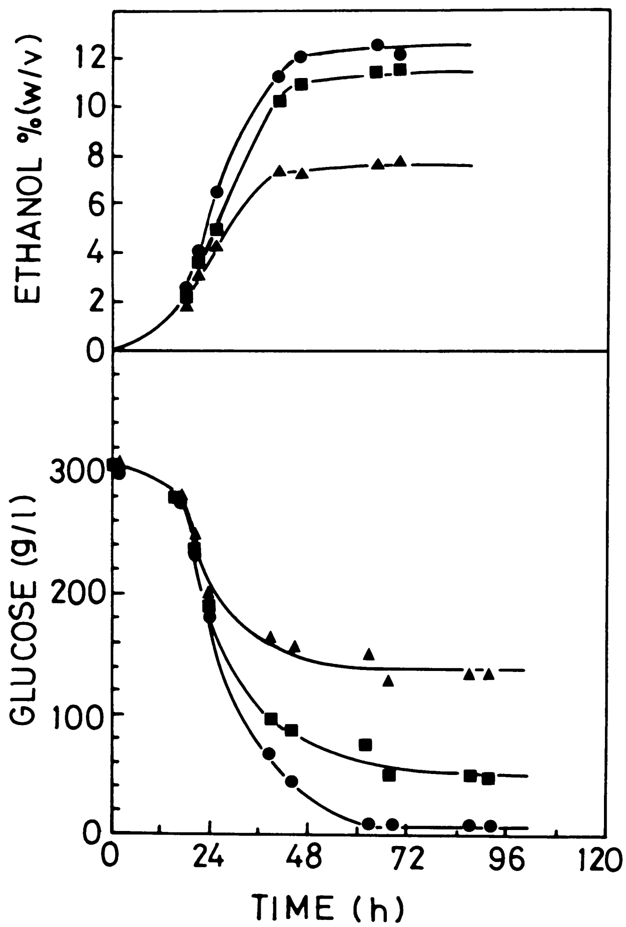

FIG. 1. Profiles for the fermentation of 300 g of glucose per liter in a simple medium (▲) or a medium supplemented with 2% (■) or 4% (●) soy flour.

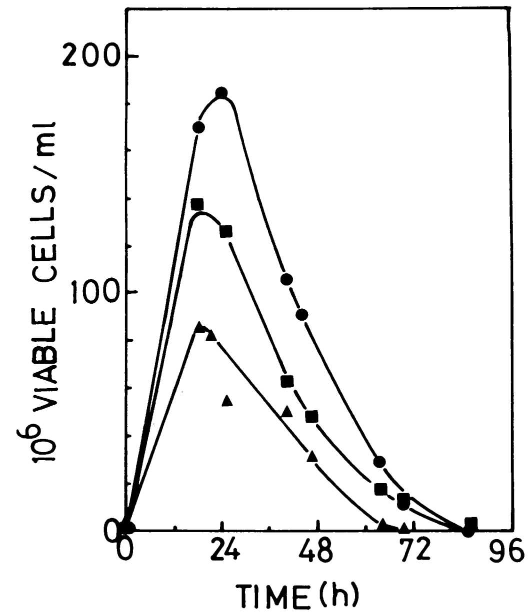

FIG. 2. Concentration of the viable cells during the fermentation of 300 g of glucose per liter in a simple medium (▲) or supplemented with 2% (■) or 4% (●) soy flour.

a high glucose concentration, even with the addition of 6% (wt/vol) soy flour, it was impossible to increase the amount of sugar fermented (Fig. 1 and 3). However, it is our feeling that through optimization of the initial glucose and soy flour concentrations it would be possible to increase the final ethanol concentration to >13% (wt/vol). It is well known for beer production that to get a rapid and full fermentation it is necessary to extend the growth phase, because nongrowing yeast ferments slowly, whereas rapidly growing yeast ferments rapidly (10). Soy flour, acting to overcome nutritional deficiencies, allowed more growth.

The addition of the water-soluble fraction of soy flour led to an ethanol productivity similar to that possible by using the whole-soy flour. However, when the whole-soy flour was added, the amount of fermented glucose was slightly higher, but the ethanol yield appeared to be slightly lower (0.42 and 0.38, respectively; Fig. 3). This could be partially due to ethanol adsorption by soy flour particles, which at the concentration used (6% [wt/vol]) presented a significant surface area. The effect of the addition of the water-soluble fraction indicates that proteins and ions (e.g., Zn^{2+} for beer fermentations [12]) could be the missing nutrients. Recent results confirmed this hypothesis.

No positive effect on fermentation pattern by the addition of the lipidic fraction (Fig. 3) could be found.

Present results call attention to a judicious addition of nutrients in fuel ethanol production now that in the wine and beer industry this procedure is already recognized (1, 11). Premature stoppage of alcoholic fermentations cannot be attributed only to ethanol toxicity and tolerance of yeast cells. In fact, brewers' yeasts are able to produce high concentrations of ethanol, previously associated only with winery yeasts, when nitrogenous and lipid nutritional deficiences are satisfied (3). Some of us (F. C. Rosa, I. Sá-Correia, and J. M. Novais, unpublished results) also observed that a low-ethanol-tolerant strain of *Kluyveromyces fragilis* fermented Jerusalem artichoke juice (a rich medium containing proteins and lipids [4]) to a concentration of 11.2% (wt/vol),

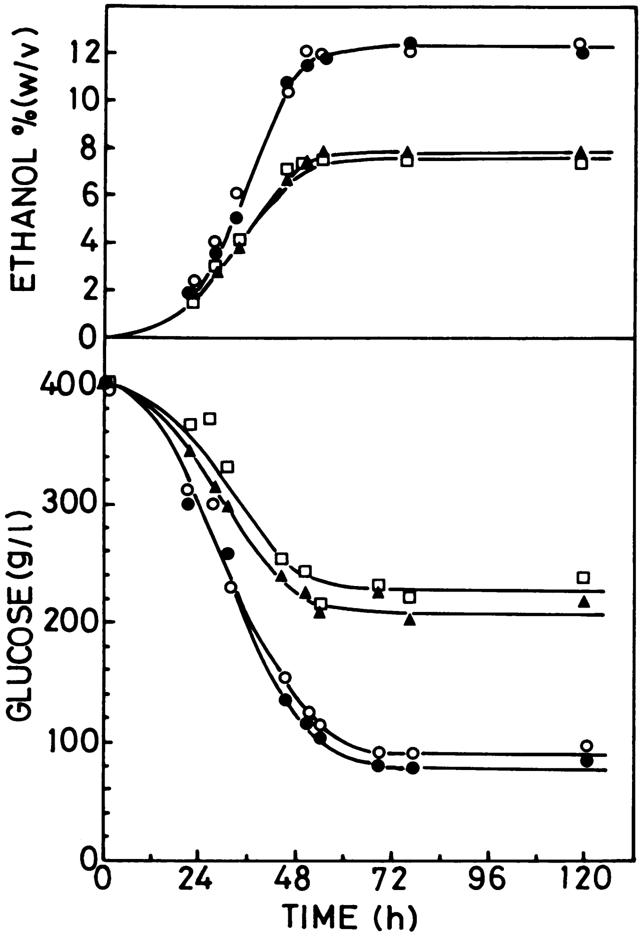

FIG. 3. Profiles for the fermentation of 400 g of glucose per liter in a simple medium (▲) or in a medium supplemented with (●) 6% soy flour, (○) the water-soluble fraction equivalent to 6% soy flour, (□) the lipidic fraction equivalent to 6% soy flour.

although the maximum concentration of ethanol which allowed growth in a simple medium was only 6.4% (wt/vol) (13).

In conclusion, these results show that soy flour is a good yeast nutrient supplement. Moreover, the water-soluble fraction leads to identical results and allows the use of a clean, fermented medium, easy to centrifugate and free from soy flour particles.

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