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AN INVESTIGATION OF ETHANOL INHIBITION AND OTHER LIMITATIONS OCCURRING DURING THE FERMENTATION OF CONCENTRATED WHEY PERMEATE BY KLUYVEROMYCES FRAGILIS.

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ABSTRACT

Based on the well-known fact that Kluyveromyces fragilis strains show sub-optimal performance when grown in concentrated whey permeate, previously optimized medium was investigated for possible limitations appearing at high concentrations. Shaken flask cultures showed that no additional vitamin or mineral sources were required when the optimized amount of yeast extract was added to the concentrated permeate. Several aspects of the ethanol inhibition of the growth of K. fragilis NRRL 665 were investigated in continuous culture. The maximum ethanol concentration tolerated by this yeast, i.e. 45 g/l, was much lower than commonly reported for other strains. Ethanol and biomass production were also influenced by the increased ethanol concentration of the medium. At 31 g/l of alcohol product yield was reduced to 0.23 g/g whereas biomass yield was 0.05 g/g. Some evidence suggested that residence time and residual lactose concentration played a significant role in modulating the toxic effect of ethanol.

INTRODUCTION

Over the last few years, whey disposal through alcoholic fermentation gained a renewed interest. After the necessary screening step (Burgess and Kelly, 1979; Laham-Guillaume et al, 1979; Vienne and von Stockar, 1983), various developments of the fermentation process were reported. Continuous culture with cell recycling (Cheryan and Mehaia, 1983; Janssens et al, 1984) and cell immobilization (Marwaha and Kennedy, 1984) were proposed. Some authors attempted to define appropriate growth condition in order to achieve higher metabolic activity of their cultivated yeast. Optimization of temperature, pH and medium composition were thus reported by Burgess and Kelly (1979), Castillo et al (1982), Chen and Zall (1982) and Vienne and von Stockar (1983 and 1985). Under optimized growth conditions specific growth rate, lactose consumption rate and ethanol yield of K. fragilis NRRL 665 were markedly reduced when the lactose concentration of the medium was increased from 5% to 15% (Vienne and von Stockar, 1985). This observation spurred further investigations of the factors influencing the growth of this yeast in a concentrated whey permeate.

MATERIALS AND METHODS

Strain and culture conditions

Kluyveromyces fragilis NRRL 665 was maintained on YM agar slants. Spray dried whey permeate was purchased from Nestec (La Tour-de-Peilz, Switzerland). The stoichiometric limitation study was performed in 250 ml Erlenmeyer flasks containing 100 ml of medium with a 10% inoculum. The whey permeate powder was reconstituted at 90 g/l of lactose and the optimized 8.25 g/l yeast extract was added (Vienne and von Stockar, 1983). This composition constituted the basal medium to which various supplements were added as listed in Figure 1. The ethanol inhibition experiment was carried out in continuous culture using a 2 litre KLF 2000 fermenter (Bioengineering AG, Wald, Switzerland). The fermentation volume of 1350 ml was maintained by an overflow device. Whey permeate powder was reconstituted at 46 g/l with the addition of 3.75 g/l yeast extract. As a result of previous optimization studies, all experiments were performed at 38 °C and pH 4.0 (Vienne and von Stockar, 1983 and 1985).

Analytical procedures

Cell densities (expressed as dry weight) were determined by centrifuging 20 ml of culture (15 min at 10'000 g and 4 °C) and drying at 105 °C overnight. Lactose and ethanol were assayed by means of enzymatic and gas chromatographic methods respectively as previously described (Vienne and von Stockar, 1983).

RESULTS AND DISCUSSION

Possibility of a stoichiometric limitation of concentrated permeate

The observation that a doubling of the concentration of the optimized permeate medium resulted in a decrease in the biomass yield of K. fragilis from 0.071 g/g to 0.042 g/g led to the hypothesis of a stoichiometric limitation occurring in the concentrated medium (Vienne and von Stockar, 1985). Figure 1 shows that neither specific growth rate nor biomass yield of K. fragilis could be significantly improved by the addition of various mineral elements, vitamins or combination of both. It appears however that a slightly higher biomass yield was sustained by the addition of some amino acids. It is nevertheless not clear whether this effect resulted from the addition of building elements of proteins allowing a greater efficiency of the biosynthetic process, as stated by Jones et al (1969) or could be accounted for by supplementary growth on these nutrients.

If the possibility of a stoichiometric limitation was no longer valid, Figure 1 indicates that the decrease of the metabolic activity of the yeast in a concentrated medium might be explained by the toxic effect of ethanol. Addition of 20 g/l ethanol indeed resulted in the reduction of the specific growth rate and of the biomass yield by 42% and 58%, respectively, compared to the values obtained in the control medium.

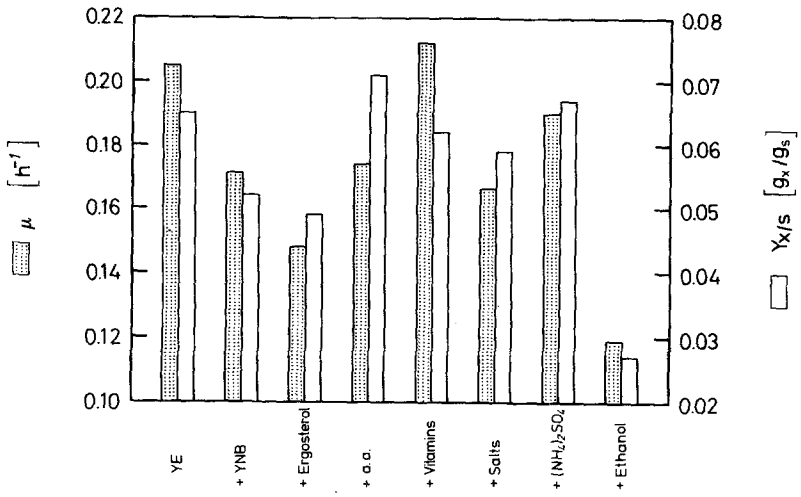

Figure 1: Maximum specific growth rate and biomass yield of K. fragilis NRRL 665 grown in batch cultures on concentrated whey permeate with various supplements:
YE: Basal medium (BM) (lactose: 90 g/l; yeast extract: 8.15 g/l);
YNB: BM + yeast nitrogen base (19 g/l); Ergosterol: BM + ergosterol (20 mg/l); a.a: BM + alanine, cystine, tryptophane, tyrosine, threonine (50 mg/l); Vitamins: biotin,folic acid (0.01 mg/l),inositol (10 mg/l) nicotinic acid, vit. B_2, B_6, thiamine (1.6 mg/l); Salts: K_2HPO_4(5 g/l), $MgSO_4$ (2.4 g/l), NaCl (0,6 g/l), $CaCl_2$ (0.06 g/l) $(NH_4)_2SO_4$: 10 g/l; Ethanol: 20 g/l.

Ethanol inhibition of the growth of K. fragilis

The observation made on Figure 1 called for further investigation of the effect of ethanol on the growth of K. fragilis. This experiment was performed in continuous culture by adding various amounts of ethanol to the medium (0, 10, 21.5 g/l). Steady-state parameters were measured at different dilution rates and were plotted in Figure 2. Some of the underlying raw data will be presented in more detail elsewhere (Vienne and von Stockar, 1985).

This Lineweaver-Burk plot shows that a non-competitive inhibition model could describe the effect of ethanol on the growth of K. fragilis. Although it was not possible to propose a quantitative model based on the limited data available, it permitted an evaluation of the maximum specific growth rate as a function of inhibitor concentration. This function, normalized by the maximum specific growth rate at zero inhibitor added to the medium, was compared to similar data reported by other researchers.

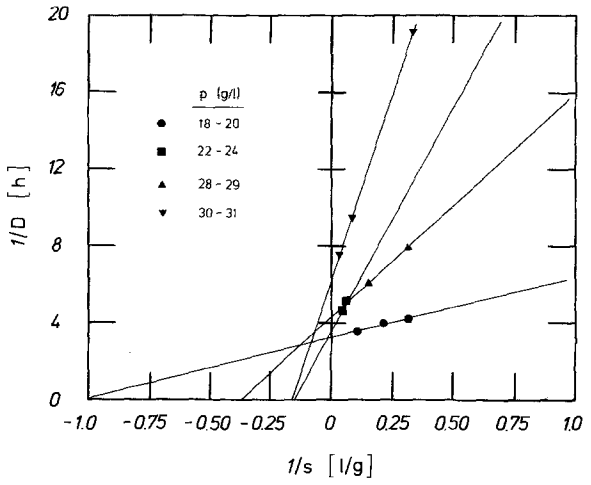

Figure 2 : Lineweaver-Burk plot of the continuous culture of K. fragilis NRRL 665 on permeate supplemented with 3.75 g/l yeast extract and with various amounts of ethanol added to the medium. S_0:46 g/l. Parameter: steady-state ethanol concentration.

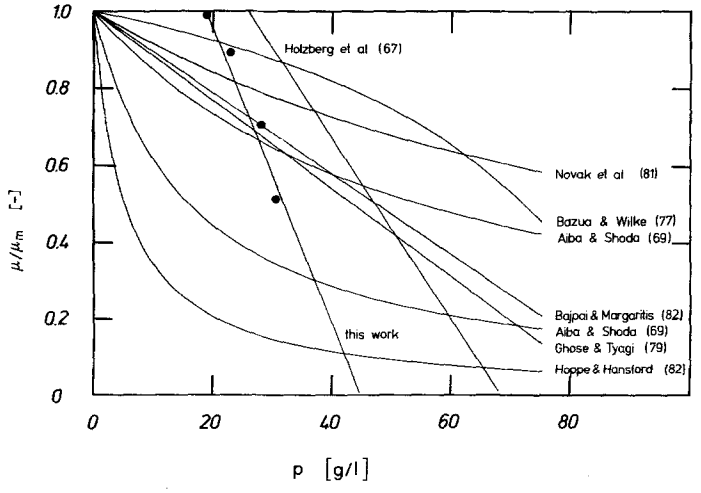

Figure 3 : Comparison of various functions obtained from the literature representing the effect of ethanol on the specific growth rate of yeasts.

The large scatter among literature data observed in Figure 3 certainly results from the different experimental procedures, medium and yeast strains used. It nevertheless can be concluded that under these experimental conditions, K. fragilis NRRL 665 is more affected by ethanol inhibition than other yeast strains. From the models the maximum concentration of ethanol tolerated by some yeast (ie. the concentration which completely supresses growth) can be determined (Table 1). The results show that the value of 45 g/l for K. fragilis NRRL 665 is less than half the average figure reported for the other strains.

STRAIN	ETHANOL [g/l]	REFERENCE
S. ceverisiae	69	Holzberg et al, 1967
S. cerevisiae ATCC 4126	94	Bazua and Wilke, 1977
S. cerevisiae NRRL 132	87	Ghose and Tyagi, 1979
S. ceverisiae NCYC 479	120	Brown et al, 1981
K. marxianus	95	Bajpai and Margaritis, 1982
K. fragilis NRRL 665	45	This work

Table 1: Maximum ethanol concentration tolerated by various yeast strains

Figure 4 shows the ethanol yield ($Y_{P/S}$) and the biomass yield ($Y_{X/S}$) for some of these experiments as a function of both the dilution rate and the steady state concentration of ethanol in the fermentor. The ethanol yield $Y_{P/S}$ dropped by 53% to 0.23 g/g when the ethanol concentration increased from the normal 18-20 g/l to 31 g/l. This dilution rate seems to have a progressively stronger influence on $Y_{P/S}$ as the ethanol concentration increases.

Analysis of the biomass yield $Y_{X/S}$ is even more complicated, in that it seems to depend also on substrate concentration. Comparing values of $Y_{X/S}$ for the same residual lactose concentration on figure 4, it appears that $Y_{X/S}$ is markedly depressed by an increase of alcohol concentration. Comparing points, however, which represent the influence of an increase of ethanol concentration at similar dilution rates, it seems that the inhibitory effect can be compensated for, and a high $Y_{X/S}$ restored by a higher residual lactose concentration, the net effect again

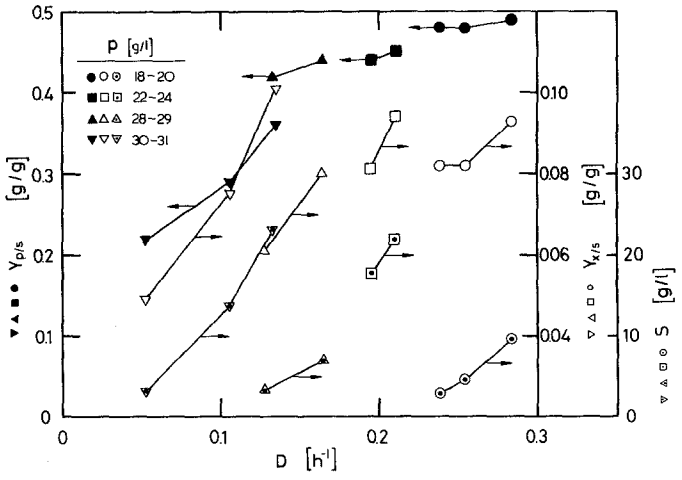

Figure 4: Effect of ethanol on biomass and product yield in continuous culture of K. fragilis NRRL 665 in non-concentrated permeate with 3.75 g/l yeast extract. S^o = 46 g/l.

Solid symbols : product yield
Open symbols : biomass yield
Dotted symbols : residual substrate

being a progressively stronger influence of the dilution rate at higher ethanol concentration. This influence of dilution rate on both $Y_{X/S}$ and $Y_{P/S}$ might reflect the effect of variing times of exposure to the toxic agent.

As shown by Panchal and Stewart (1980) for S. uvarum, high osmotic pressures in the medium can also account for a reduction in ethanol yield and viability by bringing about a higher internal level of ethanol, which is still the ultimate effector. This observation could be used to explain both the unsatisfactory performance of K. fragilis in concentrated permeate and its lower ethanol tolerance, since the toxic effect of the alcohol is expected to be amplified by the high osmotic pressure of whey.

CONCLUSIONS

The observed depression of the performance of K. fragilis in concentrated whey permeates (Vienne and von Stockar, 1985) cannot be attributed to limitations of the medium provided the correct amount of yeast extract is added. The most probable cause is the adverse effect of ethanol which appears to depend on the time the cells are exposed to it, and might also be amplified by the high osmotic pressure of the medium. With respect to the design of a technical fermentation process, these findings might preclude the use of highly concentrated whey permeate in conventional continuous culture.

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