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BATCH FERMENTATION KINETICS OF ETHANOL PRODUCTION BY *ZYMOMONAS MOBILIS* ON CELLULOSE HYDROLYSATE

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SUMMARY

Growth and ethanol production by three strains (MSN77, thermotolerant, SBE15, osmotolerant and wild type ZM4) of the bacterium *Zymomonas mobilis* were tested in a rich medium containing the hexose fraction from a cellulose hydrolysate (Aspen wood). The variations of yield and kinetic parameters with fermentation time revealed an inhibition of growth by the ethanol produced. This inhibition may result from the increase in medium osmolality due to ethanol formation from glucose.

INTRODUCTION

In the ethanol fermentation, the greatest barrier to economic feasibility of a process is the cost of the carbon source (around 70% of the production costs). Therefore, a great interest has been devoted to cheap carbon sources from renewable agricultural resources such as inulin (Allais *et al.*, 1987; Favela-Torres *et al.*, 1986), molasses (Park and Baratti, 1991a and b, 1992, 1993) or starch (Fein *et al.*, 1984; Favela-Torres and Baratti, 1988). In addition to these glycosidic substrates, lignocellulosic materials from forestry, agriculture and wastes represent excellent alternative feedstocks for the production of bulk chemicals such as ethanol by fermentation (Lynd *et al.*, 1991).

Lignocellulose is a complex polymer composed of cellulose, hemicellulose and lignin (Jeffries, 1988). Cellulose and hemicellulose must be depolymerized into monomeric sugars before fermentation. Cellulose hydrolysis requires drastic conditions including a thermal pre-treatment and the presence of strong acids or of several enzymes (Jeffries, 1988). Three enzymes are used for cellulose depolymerization: endoglucanases, exoglucanases and β-glucosidases. At each step of this hydrolysis process the reaction products act as competitive inhibitors reducing the reaction rate and the final hydrolysis yield. To reduce this inhibition it is possible to run simultaneously the saccharification and fermentation steps (Eklund and Zacchi, 1995).

However, most of the enzymes utilised for cellulose hydrolysis showed maximal activity around 50°C, a temperature greatly above the optimal fermentation temperature of yeasts used for ethanol fermentation. Therefore, we decided to check the possibility of using the bacterium *Zymomonas mobilis*, a highly efficient ethanol producer (Lee *et al.*, 1979; Rogers *et al.*, 1979 and 1982; Swings and De Ley, 1977) for two main reasons: a) the wild type strains are more thermotolerant than yeasts; b) thermotolerant mutants are available in our laboratory.

Several reports have appeared recently using recombinant *Escherichia coli* (Ingram and Doran, 1995; Padukone *et al.* 1995) or *Klebsiella oxytoca* (Wood and Ingram, 1992; Brooks and Ingram, 1995) containing genes for ethanol production from *Z. mobilis*. To our knowledge, there is only one report on the direct fermentation of a cellulose hydrolysate by *Z. mobilis* which demonstrated inhibition of the bacterial growth by several chemicals of the hydrolysate (Fein *et al.*, 1984).

In this work a cellulose hydrolysate from Aspen wood, containing mainly hexoses, was used as a feedstock for ethanol fermentation. Three bacterial strains of *Z. mobilis* were tested on a rich medium and the fermentation kinetics of the best strain were analysed on a mineral medium.

MATERIALS AND METHODS

Bacterial strains. Three laboratory strains of *Z. mobilis* were used: the wild strain ZM4 (ATCC 31821) and two mutants SBE15 and MSN77 isolated from ZM4 after chemical mutagenesis. Strain SBE15 is an osmotolerant mutant which grows faster than

ZM4 on media containing added KCl or on molasses (Park and Baratti, 1991a and b; 1992; 1993). Strain MSN77 is a thermotolerant mutant isolated after chemical mutagenesis of ZM4 and plating at 40°C (Rangheard and Baratti, unpublished results). This strain grows faster than the wild type on a glucose rich medium at 40°C.

Cellulose hydrolysate. Cellulose hydrolysate from Aspen wood (*Populus tremuloïdes*) was obtained from Institut Français du Pétrole (Rueil-Malmaison, France). This hydrolysate was prepared by enzymatic cellulose hydrolysis after pre-treatment by steam explosion under acidic conditions (205 °C at a pressure of 16/17 bars and during 2.5 min.) and elimination of the pentose fraction. The sugar composition of this hydrolysate was (per kg): glucose 427g; cellobiose 16g and xylose 13g. Density was 1.22. Glucose was the only carbon source utilised by *Z. mobilis*. The hydrolysate was first sterilised at 110°C during 30 min. and centrifuged to eliminate any precipitate (6,000 rpm, 25°C, 5 min.). The pH was ajusted to 5.0 with 5N KOH before a second sterilisation in same conditions as above.

Culture media. Two culture media were used. Rich medium contained (in gram per liter): $(NH_4)_2SO_4$ 1; $MgSO_4.7H_2O$ 0.5; KH_2PO_4 2 and yeast extract 10. The mineral medium has the same composition as rich medium with the exception that yeast extract was removed and calcium pantothenate 0.005 g/l was supplied. The pH was ajusted to 5.0 with 5N KOH before sterilisation. Both media were supplemented with the required amount of cellulose hydrolysate to obtain a final glucose concentration of 60 g/l. For erlenmeyer cultures, the potassium phosphate concentration was increased up to 10 g/l.

Culture conditions. Cultures were carried in a 2 l glass fermentor (Biolafitte, France) containing 1.6 l of culture (including inoculum) without aeration. Agitation was set at 180 rpm, temperature was maintained at 40°C and pH was regulated at 5.0 by addition of 5N KOH. Inocula (6% in volume), were from an over-night culture on the same medium. These later cultures were incubated at 40°C without pH regulation or shaking.

Analytical methods. Samples from the fermentation broth were centrifuged (7,000 rpm, 5 min., 4°C) and supernatants were utilised for ethanol and glucose determinations. The pellets were washed twice with distilled water and used for dry cell weight determination after incubation at 105°C until constant weight (usually 48 h). Ethanol was determined by gas chromatography (Park and Baratti, 1991a). Glucose concentration was determined by enzymatic method using the glucose oxidase. Osmolality data were collected using an osmometer (Vogel 6300) standardized with a 300 milliosmol kg^{-1} NaCl solution (9.463 g NaCl/1000 g distilled water) (Park and Baratti, 1991a). Kinetics and yield parameters describing the batch fermentation were determined using a polynomial fit as described by Aït-Abdelkader and Baratti (1993).

RESULTS AND DISCUSSION

Strains comparaison

The fermentation of cellulose hydrolysate by the three *Z. mobilis* strains ZM4, SBE15 and MSN77 was tested on rich medium. This hydrolysate contained mainly glucose as fermentable sugar with low amounts of cellobiose and xylose, both of them being not utilised by *Z. mobilis*. A temperature of 40°C was selected as a good compromise between the maximum activity of cellulase (around 50°C) and the usual growth temperature of the *Z. mobilis* strains (30°C).

The selected strains of *Z. mobilis* were cultivated in fermentors, without aeration, on the rich medium containing the cellulose hydrolysate as carbon cource. The three strains were able to grow and to produce ethanol in the conditions tested (Table I). The final biomass concentration was in the range 1 to 1.3 g/L which is similar to that obtained on a rich glucose medium (without cellulose hydrolysate).

Table I : Fermentation parameters of *Z. mobilis* strains ZM4, SBE15 and MSN77 grown at 40°C on a cellulose hydrolysate rich medium.

Parameters	ZM4	SBE15	MSN77
Initial glucose concentration, S (g/L)	56	56.5	57.7
Glucose conversion, C (%)	100	100	100
Fermentation time, t (h)	15	12	12
Time interval for calculations, (h)*	7-12	5-9.5	4-8
Growth rate, μ (h^{-1})	0.34	0.43	0.41
Specific glucose uptake rate, q_S (g/g.h)	10.3	11.3	10.9
Specific ethanol productivity, q_p (g/g.h)	5.8	6.1	6.0
Biomass yield, $Y_{x/s}$ (g/g)	0.019	0.018	0.020
Ethanol yield, $Y_{p/s}$ (g/g)	0.49	0.48	0.48
Volumetric ethanol productivity, Q_p (g/L.h)	1.8	2.3	2.3

* for estimation of q_S and q_p

All the glucose was consumed and ethanol reached a final concentration of 27-29 g/L.
The specific growth rate (calculated from plots of log of biomass concentration versus time) was significantly lower for strain ZM4 compared to SBE15 and MSN77. As a result, the fermentation time was shorter for SBE15 (12h) and MSN77 (12h) compared to ZM4 (15h). In contrast, the mutant strain MSN77 showed good growth as expected for a thermotolerant mutant. More surprising was the observed good growth of strain SBE15. This mutant showed higher performances for ethanol fermentations than the wild type strain on substrates such as molasses (Park and Baratti, 1991a), sugar based substrates (Park and Baratti, 1991b) and media with added KCl (Park and

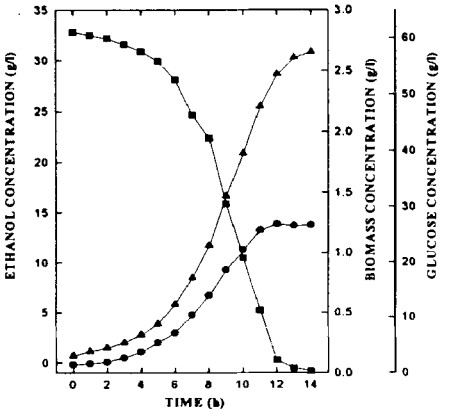

Figure 1. Batch fermentation of a cellulose hydrolysate in mineral medium by *Z. mobilis* strain MSN 77. Biomass (circles), ethanol (triangles) and glucose (squares) concentrations.

Baratti, 1993) as expected from its phenotype of osmotolerance. However, these properties were found at 30°C at the usual growth temperature for *Z. mobilis* strains. Therefore, it was concluded that the osmotic pressure is an important parameter in fermentation with cellulose hydrolysate as carbon source. High osmotic pressure may be due to the addition of chemicals during the pre-treatment and the depolymerisation steps.
The biomass yields were similar for the three strains and in the same range as those observed on a glucose rich medium. The ethanol yields (calculated on the glucose consumed) were quite high (more than 94% of theoretical) and similar for the three strains.
The kinetics of ethanol production were also compared using the specific ethanol productivity (q_p) and the specific glucose uptake rate (q_s). This lafer parameter is mostly the reflect of ethanol production since more than 96% of glucose is transformed into ethanol. The two parameters were estimated using a polynomial fitting of the experimental results according to a recently published method (Aït-Abdelkader and Baratti, 1993). Both parameters (q_p and q_s) were very similar for the three strains. Therefore, the cellulose hydrolysate demonstrated no inhibition of ethanol production in the conditions used.
In vue of the preceding results, the thermotolerant mutant strain MSN77 was selected for further work on cellulose hydrolysate.

Medium comparison

Next, a suitable medium for large scale industrial ethanol production was defined. It is well known (Swings and De Ley, 1977) that the most important effect of yeast extract in rich medium is to supply calcium pantothenate, a necessary growth factor for the bacterium. Calcium pantothenate addition has previously been successful in preliminary work done on the same substrate with a different strain (Park and Baratti, 1993).
Thus, strain MSN77 was cultivated at 40°C on a mineral medium supplemented with cellulose hydrolysate to get a final glucose concentration of 60g/l. The variations of biomass, ethanol and glucose concentrations with time are presented in

Table II : Fermentation parameters of *Z. mobilis* strain MSN77 at 40°C on a cellulose hydrolysate mineral and rich media

Parameters	Rich Medium	Mineral Medium
Intial glucose concentration, S (g/L)	57.7	61.1
Glucose conversion, C (%)	100	100
Fermentation time, t (h)	12	13
Time interval for calculations (h)*	4-8	6-10
Growth rate, μ (h^{-1})	0.41	0.33
Specific glucose uptake rate, q_s (g/g.h)	10.9	12.0
Specific ethanol productivity, q_p (g/g.h)	6.0	6.4
Biomass yield, $Y_{x/s}$ (g/g)	0.020	0.019
Ethanol yield, $Y_{p/s}$ (g/g)	0.48	0.50
Volumetric ethanol productivity, Q_p (g/L.h)	2.3	2.3
Initial osmolality (mOsmol.Kg^{-1})	614	552

* for estimation of q_s and q_p

Fig. 1. All the glucose was consumed after 14 hours of fermentation with the parallel production of 1.3 g/L of biomass and 30.5 g/L of ethanol. These results are very similar to those obtained with the rich medium.

The quantitative data on the rich and mineral media are shown in Table II. As expected, cell growth was significantly slower on the mineral medium as reflected by the drop in specific growth rate. In contrast, ethanol production was at least identical or even slightly higher on the mineral medium in terms of yield ($Y_{p/s}$) and specific ethanol productivity (q_p). On the whole, the fermentation time was only slightly affected with an increase from 12 to 13 hours and the volumetric ethanol productivity was similar to that on rich medium.

These results agreed with those reported by Belaich and Senez (1965), Galani *et al.* (1985) Fein *et al.* (1984) and Toran-Diaz *et al.* (1983) on the use of calcium pantothenate instead of yeast extract.

Fermentation kinetics

In the third step of this work, a detailed analysis of fermentation kinetics was undertaken to get informations on growth and ethanol production on the mineral medium. Therefore, the crude data presented in Fig. 1 were further analysed using a recently described method (Aït-Abdelkader and Baratti, 1993). This method involved the calculation of «intantaneous» parameters and the study of their variations with fermentation time. According to this methodology the specific growth rate μ, specific glucose uptake rate q_S, specific ethanol productivity q_p, biomass and ethanol yields $Y_{x/s}$ and $Y_{p/s}$ were calculated and plotted against fermentation time as shown in Fig. 2 and 3.

The growth rate showed a typical pattern of

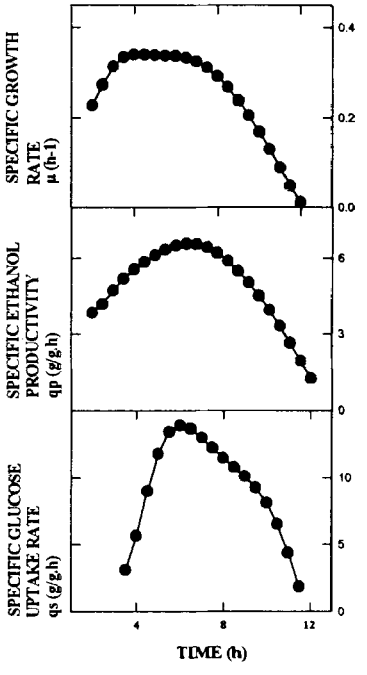

Figure 2. Variations of kinetics "intantaneous" parameters with fermentation time

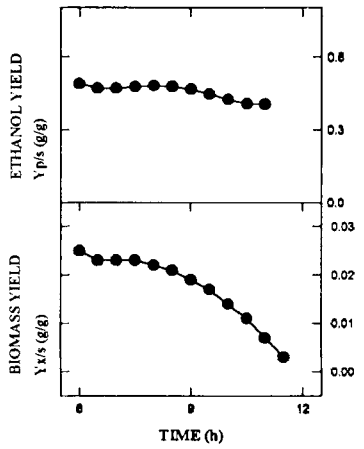

Figure 3. Variations of yield "intantaneous" parameters with fermentation time.

product-inhibited growth. The curve can be separated into two main parts: one from 3 to 7 h with a constant value around 0.33 h^{-1} and a second one from 7 to 12 h with a strong drop to almost 0. It should be noted that the maximum value observed here (Fig. 2) is identical to that (Table II) determined by a plot of log of biomass versus time. Therefore, ethanol produced strongly inhibited cell growth in our conditions.

This conclusion was fully confirmed by examining the variations of the biomass yield with time (Fig. 3). The intantaneous biomass yield ($Y_{x/s}$) was almost constant until 7 hours of fermentation and then decreased sharply in parallel to the drop of specific growth rate (μ). Thus, growth inhibition could be detected on both kinetics and yield parameters. In contrast, the ethanol yield $Y_{p/s}$ (Fig. 3) was almost constant during the whole fermentation. The kinetic parameters q_S and q_p (Fig. 2) were maximal in the middle of the fermentation and then decreased slowly. These

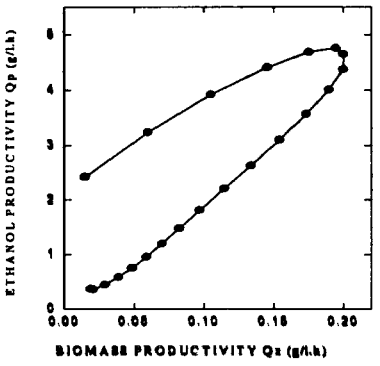

Figure 4. Variations of ethanol productivity with biomass productivity.

variations are quite similar to the theoretical one demonstrating little or no effect of ethanol inhibition on the kinetics of ethanol production.
On the whole, we concluded that growth was inhibited during the fermentation but not ethanol production. This result is in good agreement with previous reports indicating a higher sensitivity of cell growth than ethanol production towards ethanol inhibition during fermentation (Fein *et al.*, 1984). In addition, this general trends of all *Z. mobilis* fermentations was most probably enhanced here due to the high temperature used. It is well known that ethanol inhibition increased with the temperature of fermentation (Laudrin-Seiller *et al.*, 1984).
A clear demonstration of the above conclusion is given by examining the variation of the ethanol production rate (volumetric productivity, Q_p) versus biomass production rate (volumetric biomass productivity, Q_X) as plotted in Fig. 4. For a product kinetics of type I according to Gaden's classification, i.e. growth associated, Q_p should be proportional to Q_X during the whole fermentation curve. In our case, this assumption was only valid during the first part of the growth curve, i.e. "exponential growth phase". During the second part of fermentation, the ethanol productivity was higher than expected from proportionality. At the end of fermentation (Q_X near zero) non growing cells are still actively converting glucose into ethanol at a volumetric rate about half of the maximal value. This phenomenon is well known in *Z. mobilis* as «uncoupled growth» and it has been previously reported in earlier studies as an effect of either temperature (Forrest, 1967; Swings and De Ley, 1977) or substrate concentration (Park and Baratti, 1991b).

Effect of medium osmolality

The above described growth inhibition in the second part of the fermentation was attributed to a product, i.e. ethanol inhibition. This effect may be due to a direct action on cells, for instance on cell membranes by alteration of their fluidity, or to an indirect action, for instance by increasing the medium osmotic pressure. All the solutes in cellulose hydrolysate contributed to get an important osmotic pressure in the fermentation broth, i.e. glucose and other sugars, mineral ions... In such non ideal solutions, the valuable thermodynamic variable is the osmolality expressed in osmol: the mass of solute which when dissolved in 1 kg of solvent exert the same osmotic pressure as one mole of an ideal substance dissolved in the same conditions (Jones and greenfield, 1986).
To check a possible effect of osmotic pressure on the fermentation the variation of medium osmolality was measured as a function of time. The initial osmolality was 552 mosmol/kg and it increased in parallel to ethanol production as shown in Fig. 5. This increase was mainly due to the breakdown of one mole of glucose into two moles of ethanol increasing the total solute concentration in broth.

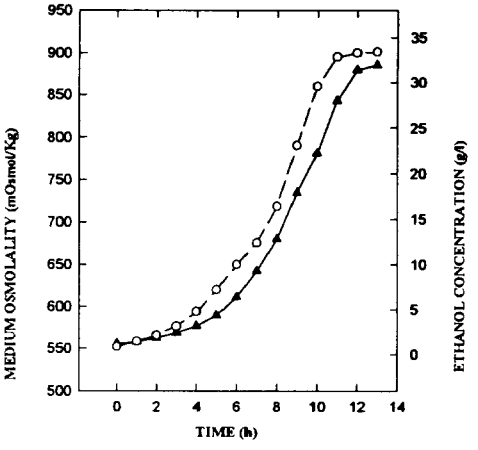

Figure 5. Variations of ethanol concentration and medium osmolality with fermentation time.

As a result, medium osmolality reached 900 mosmol/kg at the end of fermentation. During this period, bacterial cells must adapt their metabolism to this drastic change of their environment, for instance by adjusting the osmotic pressure inside the cell. In this situation, some of the nutrients will most probably be derived from growth to the synthesis of intracellular compounds to maintain the internal osmotic pressure. It resulted a decrease of the specific growth rate (μ) and biomass yield ($Y_{X/S}$) as observed experimentally.
Therefore, ethanol inhibition may well go through

the non specific way of an increase in medium osmolality instead of a specific action of ethanol on the cell. However, based on these data, it cannot be determined whether one or both mechanism were active. A very similar conclusion has been drawn with cells grown on media containing molasses (Park and Baratti, 1991a). Therefore, the growth kinetics on industrial media such as cellulose hydrolysate or molasses are quite complex and difficult to analyse. Moreover, the utilisation of intantaneous parameters revealed to be an useful tool for this analysis.

CONCLUSION

During this work the thermotolerant mutant MSN77 and the osmotolerant mutant SBE15 of *Z. mobilis* were found to have higher fermentation performances than the wild type strain ZM4 when grown at 40°C on a cellulose hydrolysate rich medium. Strain MSN77 was also able to convert efficiently glucose into ethanol when cultivated on a mineral medium with the same cellulose hydrolysate as above. This medium may be very useful on an industrial scale. By studying the variations of instantaneous parameters with fermentation time it was shown that ethanol produced inhibited growth during the second half of the fermentation. This inhibition could well result from an increase in medium osmolality due to the formation of ethanol from glucose.

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NOMENCLATURE

S = glucose concentration (g/L)
C = conversion of glucose (%)
t = fermentation time (h)
q_S = specific glucose uptake rate (g/g.h)
q_p = specific ethanol productivity (g/g.h)
Q_p = volumetric ethanol productivity (g/L.h)
Q_x = volumetric biomass productivity (g/L.h)
$Y_{x/s}$ = biomass yield (g/g)
$Y_{p/s}$ = ethanol yield (g/g)
μ = specific growth rate (h^{-1})

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