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ORIGINAL PAPER

Sugar fermentation by *Fusarium oxysporum* to produce ethanol

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Abstract As a first step in the research on ethanol production from lignocellulose residues, sugar fermentation by *Fusarium oxysporum* in oxygen-limited conditions is studied in this work. As a substrate, solutions of arabinose, glucose, xylose and glucose/xylose mixtures are employed. The main kinetic and yield parameters of the process are determined according to a time-dependent model. The microorganism growth is characterized by the maximum specific growth rate and biomass productivity, the substrate consumption is studied through the specific consumption rate and biomass yield, and the product formation via the specific production rate and product yields. In conclusion, *F. oxysporum* can convert glucose and xylose into ethanol with product yields of 0.38 and 0.25, respectively; when using a glucose/xylose mixture as carbon source, the sugars are utilized sequentially and a maximum value of 0.28 g/g ethanol yield is determined from a 50% glucose/50% xylose mixture. Although fermentation performance by *F. oxysporum* is somewhat lower than that of other fermenting microorganisms, its ability for simultaneous lignocellulose-residue saccharification and fermentation is considered as a potential advantage.

Keywords Ethanol · Fermentation · *Fusarium oxysporum* · Glucose · Xylose

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Abbreviations

b	biomass productivity (g biomass/l h)
E	ethanol concentration (g/l)
E_T	theoretical ethanol production (g/l)
q_E	specific ethanol production rate (g ethanol/g biomass h)
q_E^D	specific ethanol production rate by differential method (g ethanol/g biomass h)
q_s	specific substrate consumption rate (g substrate/g biomass h)
q_s^D	specific substrate consumption rate by differential method (g substrate/g biomass h)
s	substrate concentration (g/l)
s_0	initial substrate concentration (g/l)
t	time (h)
x	biomass concentration (g/l)
x_0	initial biomass concentration (g/l)
Xy	xylitol concentration (g/l)
$Y_{E/s}$	instantaneous ethanol yield (g ethanol/g substrate)
$Y_{E/s}^G$	overall ethanol yield (g ethanol/g substrate)
$Y_{E/x}$	instantaneous ethanol productivity (g ethanol/g biomass)
$Y_{x/s}$	instantaneous biomass yield (g biomass/g substrate)
$Y_{x/s}^G$	overall biomass yield (g biomass/g substrate)
$Y_{Xy/s}$	instantaneous xylitol yield (g xylitol/g substrate)
$Y_{Xy/s}^G$	overall xylitol yield (g xylitol/g substrate)

Greek symbols

μ	specific growth rate (h^{-1})
μ_m	maximum specific growth rate (h^{-1})

Introduction

Lignocellulose materials constitute a major part of available biomass in nature. Their main components are cellulose (25–53%), hemicellulose (20–35%), lignin (10–25%) and extractives (Knauf and Moniruzzaman 2004). Cellulose, a highly stable glucose polymer, is the most abundant organic compound on Earth as the main component of plant cellular walls. Hemicellulose is also a sugar polymer whose types and distribution depend on the particular lignocellulose material. Xylan, composed primarily by xylose, is the most common polymer found in the hemicellulose fraction of many materials (Pavarina and Durrant 2002; Sapre et al. 2005). Other sugars like arabinose, mannose or galactose may also be present in this fraction.

The production of ethanol from lignocellulose materials has deserved a great deal of interest in the last years. Conventional methods, applied for bioconversion of cellulose and hemicellulose to ethanol, involve acid or enzyme hydrolysis of the biopolymers to soluble sugars followed by fermentation to ethanol (Martín et al. 2002; Panagiotou et al. 2005a) although some bottlenecks need still to be overcome to achieve widespread commercialization (Knauf and Moniruzzaman 2004; Mosier et al. 2005). For example, enzymatic hydrolysis of the cellulose fraction offers the potential for higher glucose yields and milder process conditions; however, the cost of cellulose enzymes represents a major barrier for the commercialization of a cellulose-to-ethanol process (Panagiotou et al. 2005b). On the other hand, successful industrial production of ethanol from lignocellulose-containing raw materials depends on the quantitative conversion of carbon sources. Several industrial microorganisms can readily utilize hexose sugars, but efficient pentose utilizing microorganisms are less common (Panagiotou et al. 2005c).

An alternative approach includes a direct process in which one or more microorganisms carry on simultaneous production of cellulases, hydrolysis and fermentation in the same bioreactor (Panagiotou et al. 2005b). Among the microorganisms capable of a direct conversion of cellulose to ethanol, a promising one is *Fusarium oxysporum*. Besides cellulases, xylanases produced by *F. oxysporum* have been also characterized; moreover it produces enough β-glucosidase activity to prevent cellobiose inhibition during hydrolysis (Christakopoulos et al. 1999; Panagiotou et al. 2005b). The production of cellulolytic and xylanolytic enzymes by *F. oxysporum* grown on different lignocellulose substrates like corn stover (Panagiotou et al. 2003), rice chaff (Tao et al. 1998), sugar beet pulp (Cheilas et al. 2000), sugarcanne bagasse and sawdust (Pavarina and Durrant 2002) has been reported. In the same way, the capability of this microorganism to transform both hexoses and pentoses into ethanol has been established (Panagiotou et al. 2005a, c, d). However, byproducts like xylitol and acetic acid may also been formed in the process, although operation under oxygen-limited conditions (Panagiotou et al. 2004; 2005a) may reduce byproduct formation.

The objective of this work is to study the fermentation by *F. oxysporum* of the most abundant sugars found in lignocellulose residues. Glucose, xylose and arabinose as well as different glucose/xylose mixtures have been analyzed as fermentation substrates. Kinetic and yield process parameters have been determined to describe cellular growth, substrate uptake and product formation by *F. oxysporum*.

Materials and methods

Microorganism and culture conditions

Fermentations were performed by *Fusarium oxysporum* VTT-D-80134. The microorganism was stored between 5 and 10°C in 100 ml-test tubes on a sterilized solid culture medium with a composition (in g/l): yeast extract 3; malt extract 3; peptone 5; xylose 10; agar–agar 20. Before the start of each experiment the microorganism was inoculated under sterile conditions into glass test tubes containing the same solid culture medium. These tubes were then kept in an incubator at 30°C for 60 h in order to obtain cells at the same growth stage for every experiment.

The fermentation medium (Lindegren et al. 1958) contained (in g/l) $MgSO_4$, 1; KH_2PO_4, 2; $(NH_4)_2SO_4$, 3; peptone, 3.6; yeast extract, 4; and xylose, arabinose, glucose or different xylose/glucose mixtures, 25. Fermentations were started by adding, as an inoculum, 20 ml mycelia and spore suspension of *F. oxysporum* from a 60 h-old culture grown on the above mentioned solid culture medium.

The culture medium was sterilized using a pre-filter of glasswool and cellulose nitrate filters with 0.2 μm pore size. All the experiments were made in a 2-l batch reactor without external air supply. The volume of culture medium was 0.5 l. The stirring speed was 500 rpm, and the stirring rod was 4 cm long and 0.8 cm in diameter. The cultures may be considered as oxygen-limited, taking into account the oxygen introduced through the stirring vortex. Under these conditions, the overall volumetric mass-transfer coefficient at the

beginning of each experiment was 2.9 h^{-1}. The temperature was kept at 30°C and the pH was maintained at 4.5 by additions of either NaOH or HCl when necessary. The cultures were sampled at fixed intervals and the concentrations of biomass, sugars and products were determined.

Analytical methods

The sugar solution was analyzed for the concentration of reducing sugars (Miller 1959) and glucose by enzymatic methods (Trinder 1969; Bergmeyer et al. 1974). Cell concentration was determined by filtering culture samples and dry weight calculation. Concerning bioproduct formation, the concentrations of ethanol and xylitol were quantified based on enzymatic methods (Beutler and Michal 1977; Beutler and Becker 1977). According to these methods, ethanol is oxidized to acetaldehyde (in the presence of the enzyme alcohol dehydrogenase) by NAD; acetaldehyde is further and quantitatively oxidized to acetic acid in the presence of aldehyde dehydrogenase, the concentration of ethanol being calculated from absorbance measures at 340 nm. Xylitol is oxidized by NAD to D-xylulose in the presence of the enzyme polyiol dehydrogenase with the formation of NADH, which is in turn removed in a subsequent reaction by reducing iodonitrotetrazolium chloride to formazan in the presence of diaphorase; the concentration of xylitol is measured through the absorbance of formazan at 492 nm.

Results and discussion

Figure 1 shows the fermentation results for concentrations of biomass, substrate, ethanol and xylitol throughout the experiments. Figures 1a–e correspond to the experiments in which glucose, xylose or a mixture glucose/xylose were used as a substrate. These fermentations lasted for a period of time decreasing as the proportion of glucose was higher. Ethanol concentrations produced were always much greater than xylitol concentrations and, in general, the higher proportion of glucose in the substrate the higher ethanol concentration obtained. Regarding xylitol concentrations, a decrease from maxima values was detected (Fig. 1a–c), virtually disappearing for fermentation time above approx. 100 h. This may be attributable to the ability of *F. oxysporum* to utilize xylitol as a carbon source, as reported by Suihko (1983). Figure 1f illustrates the fermentation experiment made with arabinose as the sole carbon source; as can be seen, little biomass growth was detected but there was neither substrate consumption nor product formation. A similar result is reported by Panagiotou et al. 2005c when studying the fermentation by *F. oxysporum*; these authors found that only a very small fraction (14%) of the available arabinose was transformed into ethanol under anaerobic conditions after 160 h, suggesting difficulties regarding the arabinose transport into the cell.

To describe the behavior of *F. oxysporum* kinetic and yield parameters have been determined. Cellular growth was monitored by maximum specific growth rate. Specific substrate consumption rates and global biomass yields were used to describe substrate consumption. Finally, the product formation was studied by means of specific product formation rates and product yields.

Cellular growth

Growth curves were obtained by plotting $\ln(x/x_o)$ versus time as shown in Fig. 2. After inoculation, there was scarcely no lag phase and the cultures entered the exponential growth phase (solid lines in Fig. 2) almost immediately. The maximum specific growth rate, μ_m, for each culture was evaluated by least-squares adjustment as the slope of the linear interval in growth curves. The values of μ_m are listed out in Table 1. In general, increasing the proportion of glucose in the substrate mixture leads to higher values for μ_m. A similar behavior is found when other ethanol-producing microorganisms grow on xylose/glucose mixtures, (Sánchez et al. 2002). In accordance to our results, Panagiotou et al. 2005c using *F. oxysporum* on glucose reported higher values of μ_m than those obtained when xylose was used as a substrate. Regarding growth on arabinose, a value of 0.080 h^{-1} for μ_m was found, even if no substrate uptake or product formation was detected. This may be attributed to cell growth based on the culture medium composition. In fact, higher μ_m values than this one have been reported in other microorganism cultures with no carbon source (Sánchez et al. 1999); for example, using the same culture medium, Sánchez et al. 2002 calculated maxima specific growth rates of 0.33, 0.19 and 0.26 h^{-1} in cultures of *Pichia stipitis*, *Candida shehatae* and *Pachysolen tannophilus*, respectively, in the absence of carbon source.

Next to the exponential phase the cultures continued to grow but at slower rates; in fact, growth can be described along this phase by a linear relationship with time:

$$x = c + b \cdot t \quad (1)$$

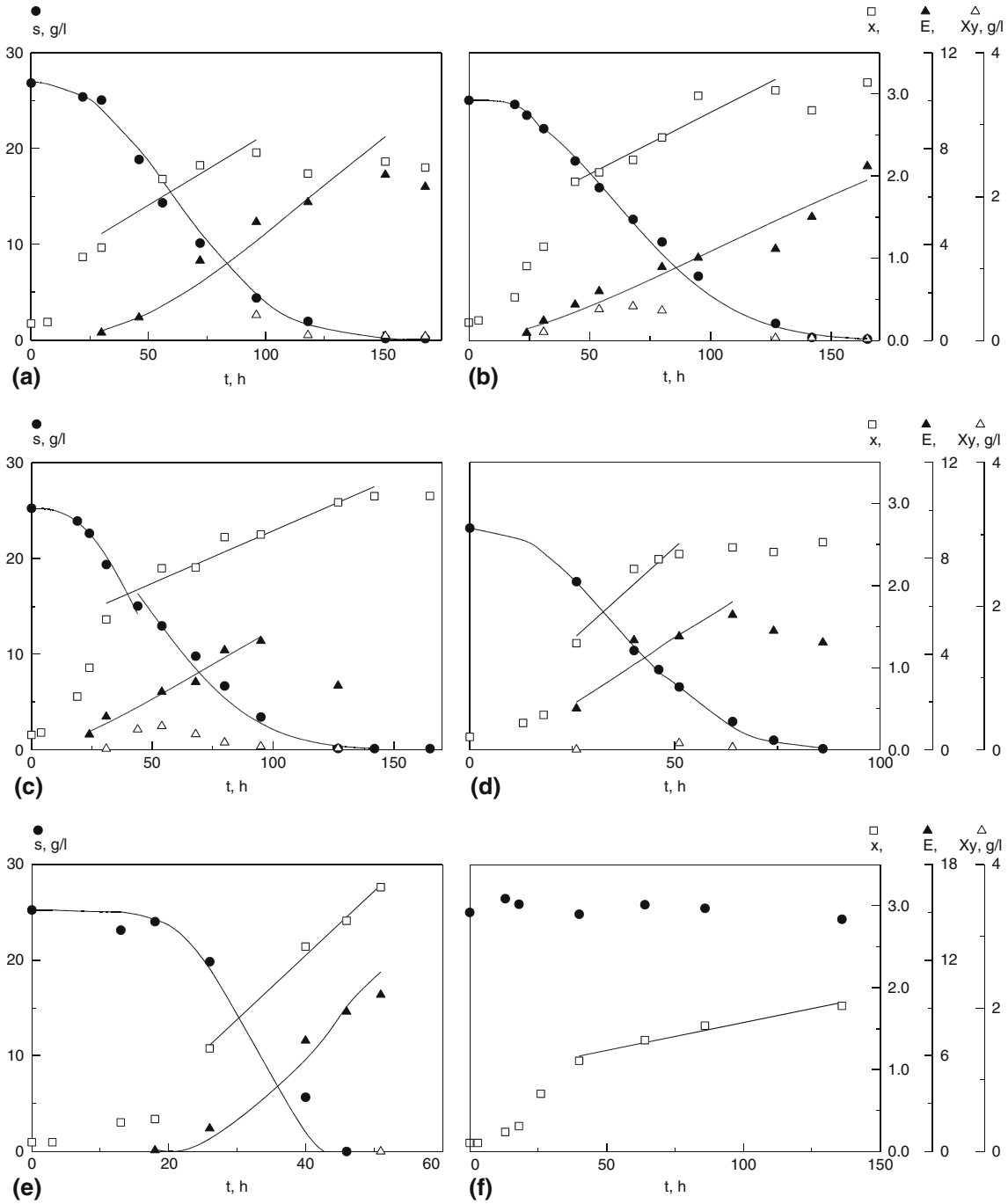

Fig. 1 Concentrations of biomass (□), total substrate (•), ethanol (▲) and xylitol (Δ) as a function of time. (**a**) Xylose, 25 g/l; (**b**) xylose, 24 g/l, glucose, 1 g/l; (**c**) xylose, 20 g/l, glucose, 5 g/l; (**d**) xylose, 12.5 g/l, glucose, 12.5 g/l; (**e**) glucose, 25 g/l; (**f**) arabinose, 25 g/l

From the representations of the experimental results of *x*–*t* the duration of this growth period can be determined (lines in Fig. 1) and the values for biomass productivity, *b*, can be calculated by least-squares adjusts. These values are set out together with those of μ_m in Table 1.

Finally, the cultures came to a stationary period in which almost no cellular growth was detected.

Substrate consumption

The experimental results concerning substrate consumption are represented in Fig. 1. As can be seen, glucose is rapidly consumed by *F. oxysporum*. As the proportion of xylose in the culture medium was increased, the microorganism needed a longer period of time for the total utilization of sugars, glucose being the first to be assimilated. This suggests that glucose

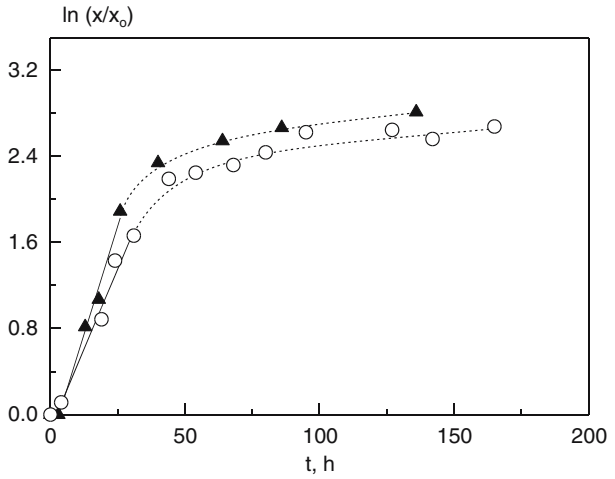

Fig. 2 Growth curves for the experiments with 24 g/l xylose and 1 g/l glucose (○) and 25 g/l arabinose (▲)

Table 1 Maximum specific growth rates (μ_m) and biomass productivities (b)

Initial substrate concentration (g/l)	μ_m (h^{-1})	$b{\cdot}10^2$ (g/l h)
Xylose, 25	0.075	1.7
Xylose, 24; glucose, 1	0.059	1.5
Xylose, 20; glucose, 5	0.075	1.3
Xylose, 12.5; glucose, 12.5	0.11	4.5
Glucose, 25	0.10	7.8
Arabinose, 25	0.080	0.68

blocks the production of the enzymes responsible for the metabolism of xylose. This blocking disappears at low concentrations of glucose (Lee 1992). Finally, when arabinose was used as carbon source, no consumption was detected.

To describe substrate consumption both differential and integral methods for analysis of kinetic data were used. In the first case, experimental data of substrate concentration, s (g/l), were fitted to an empirical equation, so that the specific substrate consumption rate could be evaluated. Among the different equations assayed that allowing the best reproducibility of experimental data was as follows:

$$s = s_0 \alpha^{-t^\beta} \tag{2}$$

where s_0 stands for the initial substrate concentration and α and β are two adjustment parameters. Application of linearized form of Eq. (2) to the experimental data and further adjustment by least-squares resulted in a straight line for the experiments with just one substrate or two straight lines when mixtures of glucose and xylose were used. As an example, Fig. 3 shows that two stretches, corresponding to the consumption of glucose first and, that exhausted, xylose, can be discerned. From these plots, parameters α and β for each culture can be determined and the evaluation of substrate concentration at any culture time can be predicted. These predicted substrate values are represented by means of lines in Fig. 1, together with the experimental discrete results (symbols).

From Eq. (2), the derivative $d(s_0–s)/dt$ can be calculated analytically and, replacing in the definition of specific substrate consumption rate:

$$q_s = \frac{1}{x}\frac{d(s_0 - s)}{dt} \tag{3}$$

the following equation can be derived:

$$q_s^D = \frac{s_0 \beta (\ln \alpha)\left(t^{\beta-1}\right)\left(\alpha^{-t^\beta}\right)}{x} \tag{4}$$

where x represents the biomass concentration for the time considered.

The values of q_s^D are listed in Table 2 at two different culture times.

The specific substrate consumption rates have been also evaluated using the integral method. With that purpose, the global biomass yield $Y_{x/s}^G$ (g biomass/g substrate) has been firstly calculated from plots of net biomass formed, $x–x_0$ (g/l) versus net substrate consumed, $s_0–s$ (g/l) similar to that shown in Fig. 4. Again, the experiments in which a mixture of glucose/xylose was used present two stretches corresponding to the consumption of each sugar and two biomass yields were determined (Table 2), the one due to glucose-consumption being always greater than the one derived from xylose uptake.

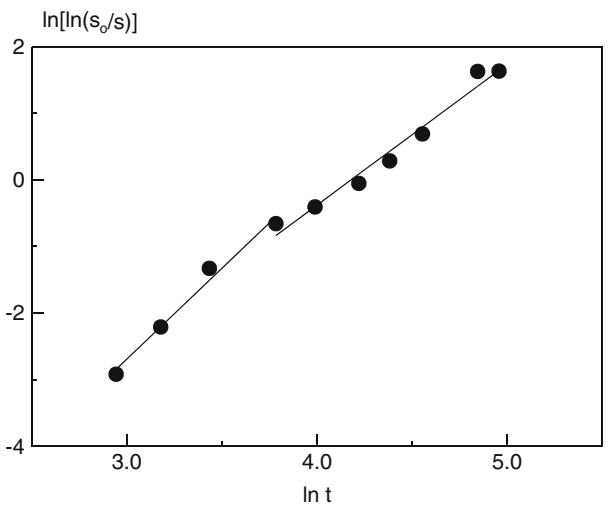

Fig. 3 Application of linearized form of Eq. 2 for the experiment with 20 g/l xylose and 5 g/l glucose

Table 2 Global biomass yields ($Y^{G}_{x/s}$) and specific substrate consumption rates calculated by differential (q^{D}_{s}) and integral (q_s) methods

Initial substrate concentration (g/l)	$Y^{G}_{x/s}$ (g/g)	t (h)	q^{D}_{s} (g/g h)	q_s (g/g h)
Xylose, 25	0.079	30	0.18	0.19
		50	0.20	0.13
Xylose, 24; glucose, 1	0.25[a]	25[b]	0.24	0.23
	0.074	100	0.063	0.073
Xylose, 20; glucose, 5	0.23[a]	25[b]	0.29	0.32
	0.074	100	0.042	0.065
Xylose, 12.5; glucose, 12.5	0.16[a]	30	0.30	0.18
	0.034	50	0.18	0.54
Glucose, 25	0.11	25[b]	0.91	0.92
		40	0.27	0.30

[a] Values corresponding to glucose consumption

[b] Exponential growth time

By combination of the definitions of biomass yield $Y_{x/s} = \mathrm{d}(x - x_0)/\mathrm{d}(s_0 - s)$, specific growth rate $\mu = (1/x)(\mathrm{d}x/\mathrm{d}t)$ and specific substrate consumption rate (Eq. 3), it can be deduced that:

$$q_s = \frac{\mu}{Y^{G}_{x/s}} \tag{5}$$

This equation allows the specific substrate consumption rate q_s (integral method) to be calculated.

For a comparison of the values of q^{D}_{s} and q_s, Table 2 shows the specific substrate consumption rates at various times obtained by both methods. In general, the values calculated by the two procedures were similar.

Product formation

Ethanol is the main product obtained in the fermentation by *F. oxysporum* under the operation conditions assayed. Little concentrations of xylitol are also detected. A similar result is found by Panagiotou et al. 2005a, who report that no xylitol is produced under oxygen-limited conditions from a xylose/glucose mixture and that a small fraction of xylose is converted mainly to xylitol under anaerobic conditions.

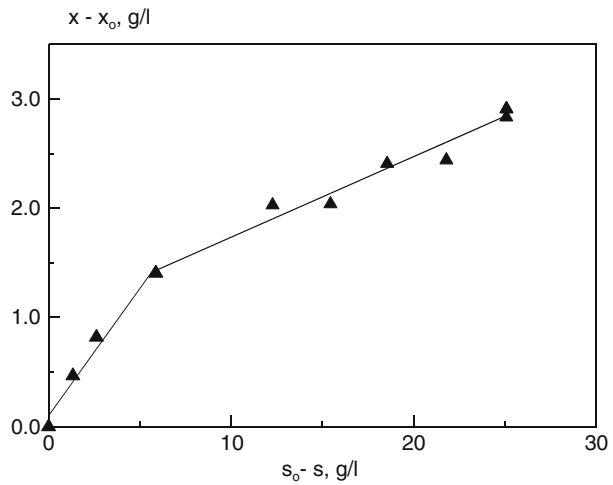

Fig. 4 Biomass production versus substrate consumption for the experiment with 20 g/l xylose and 5 g/l glucose

For the calculation of the specific ethanol-production rate, differential (q^{D}_{E}) and integral (q_E) methods were also used.

In the application of the differential method, certain equations were tested for acceptable reproduction of the ethanol concentration data produced in the longest time intervals. Among those assayed, the best reproduction of the experimental variations proved to be:

$$\frac{E_T}{E_T - E} = A^{t^B} \tag{6}$$

where E_T represents the maximum concentration attainable if the conversion of substrate into ethanol were theoretic, and A and B are two adjustment parameters. For the determination of these parameters, Eq. (6) can be linearized in the form

$$\ln\left(\ln\frac{E_T}{E_T - E}\right) = \ln(\ln A) + B \ln t \tag{7}$$

and by least-squares fits of the first member against ln t, the values of these parameters were calculated. This enables the time-course concentration of ethanol to be evaluated, as shown in the ethanol lines appearing in Fig. 1, together with the experimental results (symbols).

Afterwards, from Eq. (6) the derivative $\mathrm{d}E/\mathrm{d}t$ can be determined, enabling the determination of specific rate of ethanol formation, q^{D}_{E}, similarly to q^{D}_{s}. For example, Fig. 5 provides the representations of Eq. (7) for the fermentations of xylose and glucose.

The specific rates of ethanol formation for the various times of culture were almost constant, so a mean value for each culture, $\overline{q}^{D}_{E}$, could be calculated, Table 3.

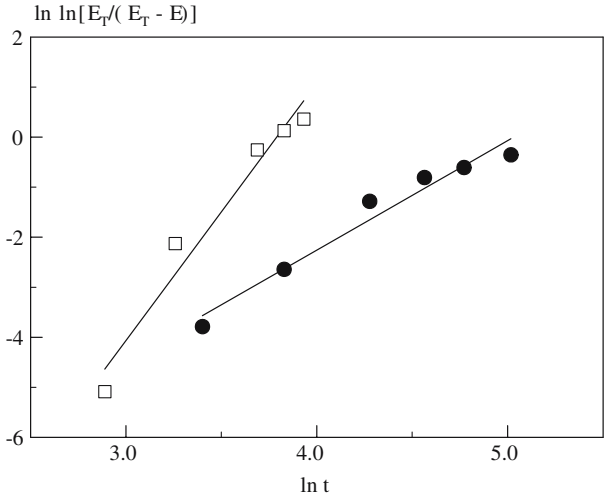

Fig. 5 Linearized form of ethanol production model (Eq. 7) for the experiments with 25 g/l xylose (•) and 25 g/l glucose (□)

On the other hand, since at the beginning of the experiment the ethanol concentration is 0, the instantaneous ethanol productivity is defined by:

$$Y_{E/x} = \frac{\mathrm{d}E}{\mathrm{d}x} = \frac{q_{\mathrm{E}}}{\mu} \tag{8}$$

If the specific ethanol-production rate is assumed to be constant, then, according to the definition of biomass productivity b (Eq. 1) and Eq. (8), it can be deduced that, during the linear growth phase:

$$\frac{\mathrm{d}E}{x\mathrm{d}x} = \frac{\mathrm{d}E}{\mathrm{d}(x^2/2)} = \frac{q_{\mathrm{E}}}{b} \tag{9}$$

so that, a representation of E versus $x^2/2$ confined to the linear growth phase should give a straight line, from the slope of which q_{E} can be determined. As an example Fig. 6 shows these representations for two experiments.

By this procedure, the q_{E} values (integral method) were calculated, as shown in Table 3 for the same times in which $\overline{q}_{\mathrm{E}}^{\mathrm{D}}$ was determined. An acceptable agreement was achieved in the values of the parameter calculated by the two procedures proposed.

Product yields

The yields of ethanol and xylitol were calculated in the same way as for biomass yields. Firstly, to check whether the yields of these products were constant, their concentrations throughout experiment time were plotted versus the quantity of sugars used up, always obtaining graphs similar to that shown in Fig. 7. From the slopes of these straight lines, the overall yields in ethanol ($Y^{\mathrm{G}}_{E/s}$) and xylitol ($Y^{\mathrm{G}}_{\mathrm{Xy}/s}$) were calculated, all of which are set out in Table 3.

As can be seen, the overall ethanol yield calculated when glucose was the only carbon source is greater than those obtained from any xylose/glucose mixture or from xylose alone. The values agree with those reported by Panagiotou et al. 2005d and by Panagiotou and Christakopoulos 2004, who found ethanol yields of 0.32 and 0.20 g ethanol/g substrate in the oxygen-limited fermentation of glucose and xylose, respectively, by *F. oxysporum*. Our results (0.38 and 0.25 g/g, respectively) are slightly higher although the initial substrate concentration in the above mentioned works was 40 g/l instead of 25 g/l used in this work.

Regarding ethanol yields from substrate mixtures, no clear relationship may be established between glucose proportion and ethanol yield values, the highest one being obtained at the highest glucose proportion assayed (50% xylose/50% glucose); at the same substrate proportion Panagiotou et al. 2005a report an ethanol yield of 0.25 g/g, comparing with our 0.28 g/g result.

Comparing with other fermenting microorganisms, *F. oxysporum* shows in general a little lower fermentation performance in terms of ethanol yields. For example, the fermentation of sugars in the same operational conditions by *Pichia stipitis, Candida shehatae* and *Pachysolen tannophilus* led to $Y^{\mathrm{G}}_{E/s}$ values of 0.37, 0.41 and 0.42 g ethanol/g xylose, and 0.42, 0.38 and 0.41 g ethanol/g glucose, respectively (Sánchez et al. 2002). When a sugar mixture of 5 g/l glucose and 20 g/l xylose was used as carbon source, the overall ethanol yields determined were 0.39, 0.35 and 0.34 g ethanol/g substrate, respectively. These three microorganisms are known as pentose-fermenting yeasts

Table 3 Overall ethanol ($Y^{\mathrm{G}}_{E/s}$) and xylitol ($Y^{\mathrm{G}}_{\mathrm{Xy}/s}$) yields and specific ethanol production rates by differential ($\overline{q}_{\mathrm{E}}^{\mathrm{D}}$) and integral ($q_{\mathrm{E}}$) methods

Initial substrate concentration (g/l)	$Y^{\mathrm{G}}_{E/s}$ (g/g)	$Y^{\mathrm{G}}_{\mathrm{Xy}/s}$ (g/g)	$\overline{q}_{\mathrm{E}}^{\mathrm{D}}$ (g/g h)	q_{E} (g/g h)
Xylose, 25	0.25	0.015	0.033	0.038
Xylose, 24; glucose, 1	0.22	0.041	0.02	0.011
Xylose, 20; glucose, 5	0.21	0.051	0.029	0.018
Xylose, 12.5; glucose, 12.5	0.28	0.0059	0.055	0.072
Glucose, 25	0.38	–	0.14	0.15

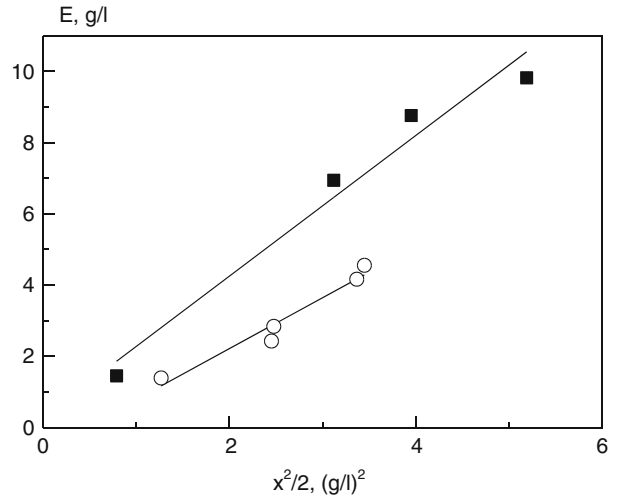

Fig. 6 Application of Eq. 9 for experiments with 25 g/l glucose (■), and 20 g/l xylose and 5 g/l glucose (○)

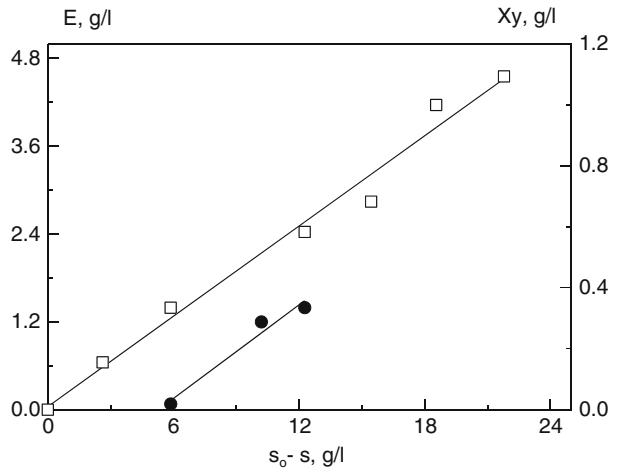

Fig. 7 Ethanol (□) and xylitol (•) concentrations against substrate consumption for the experiment with 20 g/l xylose and 5 g/l glucose

with relatively high performance levels, but they are not able to produce hydrolysis and fermentation simultaneously.

Conclusions

In this work the fermentation of the main sugars found in lignocellulose residues by *F. oxysporum* is studied by means of kinetic and yield parameters derived from a time-dependent model. *F. oxysporum* can convert glucose and xylose into ethanol but not arabinose. In the case of sugar mixtures, a sequential uptake of glucose and, that exhausted, xylose, has been evidenced. Kinetic and yield parameters determined from pure glucose are higher than those obtained from glucose/xylose mixtures or xylose alone. Further studies on its behavior in natural media, e.g., lignocellulose hydrolysates, and an economic assessment of the whole process are needed.

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