

Full Length Research Paper

Kinetic behavior of *Candida tropicalis* during xylitol production using semi-synthetic and hydrolysate based media

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The effects of the xylose concentration and the air supply on the production of xylitol from D-xylose by the yeast *Candida tropicalis* (AY 2007) using semi-synthetic and hydrolysate based media were investigated in four fermentation batch runs. The xylitol formation was favored under oxygen-limited conditions (2nd experiment, air flow rate 0.3 vvm) with a maximum xylitol production (36.0gm/l) after 59h as compared with that obtained in the 1st experiment (30.99 g/L) after 66 h under relatively less oxygen limited condition (air flow rate 1.5 vvm). Increasing the initial xylose concentration from 45.5 g/L in the 2nd experiment to 96.9 g/L in the 3rd experiment led to a great decrease in the xylitol accumulation rate (Qp) from 0.506 to 0.134 g/L, xylitol yield coefficient (Yp/s) from 0.704 to 0.180 g/g and a mild increase in the xylose consumption rate (Qs) from 0.70 to 0.75 g/L/h. The semi-synthetic medium (1st and 2nd experiments) had a maximum xylitol yield coefficient (Yp/s) of 0.701 and 0.704 g/g, respectively which was less than that obtained in the hydrolysate experiment (0.783 g/g). This is probably due to the low hydrolysate initial xylose content (29.8 g/L).

Key words: Xylitol, *Candida tropicalis*, xylose, pH, aeration, kinetics.

INTRODUCTION

Commercial demand for bulk sugar substitutes that are suitable for diabetics and are non cariogenic is of great interest. One of the most promising sweeteners is xylitol when used alone or formulated with other sugars (Fran, 2003; Jannesson et al., 2002). On the industrial scale, xylitol is produced by the catalytic hydrogenation of D-xylose in hemicellulosic hydrolysates (Winkelhausen and Kuzmanova, 1998). With this method, it is difficult to obtain a high yield (50 to 60% based on the converted xylan) because considerable amounts of byproducts are formed, making the downstream processing for the purification of xylitol expensive (Parajo et al., 1998). The biotechnical production of xylitol is an alternative and potentially a more economical process since many yeasts and mycelial fungi contain a xylose reductase enzyme

(EC1.1.1.21) that catalyses the reduction of xylose to xylitol has been patented in Japan using *Candida tropicalis* (Kim et al., 1997). Few data have been determined in bioreactors and the references show many different and conflicting results. Therefore, it is important to determine the factors that influence the process of xylose bioconversion to xylitol under controlled conditions for the development of an efficient technology for large-scale xylitol production.

Many species of yeast contain NADP⁺ - dependant xylose reductase that catalyses the reduction of D-xylose to xylitol as the first step in D-xylose metabolism (Sanchez et al., 1997). The xylitol is oxidized to xylulose by NAD-dependent xylitol dehydrogenase, which is further phosphorylated and channeled into the pentose phosphate metabolic pathway (Winkelhausen and Kuzmanova, 1998). Under anaerobic conditions or at very low oxygen transfer rates, the electron transport system of the yeast is unable to oxidize NADH

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completely, leading to an accumulation of intracellular NADH, and paralysis of the subsequent metabolic reactions (the Custer effect). This consequently stimulates the accumulation of xylitol in the culture medium (Silva et al., 1996). *C. tropicalis* has a potential to be industrially important because of its high xylose uptake rate, xylitol production capacity and alkane and fatty acid degradation in its peroxisomes (Granstrom et al., 2002).

In the present work, we reported the kinetic results of xylitol production by *C. tropicalis* (AY 2007) which has been grown on semi-synthetic medium under different microaerobic conditions, and also on bagasse hydrolysate under batch cultivation conditions in order to assess the feasibility of natural substrate utilization for xylitol production.

MATERIALS AND METHODS

C. tropicalis, which has been previously isolated and identified by the same author (Elbaz et al., 2011), was used in the fermentation experiments. The yeast was grown and sub cultured on a modified Wickerham's agar medium composed of (g/L): malt extract, 3.0; yeast extract, 3.0; peptone, 5.0; agar, 20 and xylose, 10.0 in place of glucose (pH was adjusted at 5.5 ± 0.1) and incubated for 24 h at 30°C.

Preparation of inoculum

The inoculum was prepared by transferring a loop-full of a four-day old yeast culture, which was grown on a modified Wickerham's agar slant into 25 ml of pre culture medium composed of (g/L): D- xylose, 25; $(\text{NH}_4)_2 \text{SO}_4$, 2.0; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.1 and rice bran, 15 (pH was adjusted at 6.0 ± 0.2) in a 250 ml Erlenmeyer flask plugged with a cotton-cloth sandwich and incubated in an orbital shaking incubator at 100 rpm for 24 h at 30°C. The resultant growth was centrifuged, washed with sterile saline, re-centrifuged and then resuspended in a sterile growth medium to attain the desired inoculum concentration. 10% was taken as the inoculum volume for the fermentation medium, which has the same composition of the precultured medium except the xylose concentration (measured after addition to the autoclaved medium) which was 45.5 g/L for the first and the second experiments and 96.9 g/L for the third experiment and finally 29.87 g/L for the hydrolysate experiment.

Preparation of sugar cane bagasse hydrolysate

Sugar cane bagasse hydrolysate was prepared according to Silva et al. (1997). The treated hydrolysate was autoclaved at 100°C for 20 min and then supplemented with 15 g/L of rice bran (El-Baz et al., 2011).

Fermentation condition

Different fermentation runs were carried out in a 7.5 L bioreactor with 3.0 L working volume (New Brunswick scientific model-310) equipped with instruments and controllers for agitation temperature, pH and fitted with a reflux cooler in the gas exhaust to minimize evaporation. Silicon oil was used to control foaming. Cultures were run at 30°C under different micro aerobic conditions (500 rpm, 1.5 vvm for the first experiment and 200 rpm, 0.3 vvm for the second, third and fourth experiments).

Kinetic analysis

Rates of cellular growth, xylose uptake, glucose uptake and xylitol production were determined from the slope of the measured concentration vs. time. Specific rates were calculated as the ratio of the aforementioned rates to the corresponding biomass concentration. Values of the yields for growth and xylitol formation with respect to xylose were calculated from the slopes of biomass vs. D-xylose and xylitol vs. D-xylose plots, respectively. The value of xylitol formation with respect to biomass was calculated from the slope of xylitol vs. biomass.

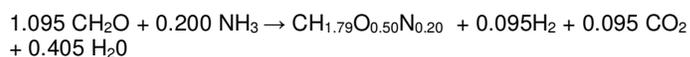
Analytical methods

The xylose and xylitol concentrations were measured by the methods of Trinder (1975) and Sanchez (1998), respectively and glucose was determined by Trinder enzymatic kit (Trinder, 1969). Cell concentration was determined by means of a calibration curve (dry weight \times optical density at 600 nm).

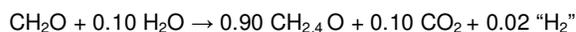
Carbon material balance

The microaerobic behavior of *C. tropicalis* was studied through simple carbon material balances, using the batch experimental data of xylitol, D-xylose and biomass concentration (Sampaio et al., 2005). The metabolic scheme for this isolate is based on the observation that no ethanol was formed during the bioconversion. It consists of the following equations with stoichiometric coefficients expressed as C-mol:

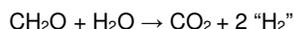
(a) Biomass growth:



(b) Xylitol formation from D-xylose:



(c) Catabolic reaction:



Where, "H₂" stands for reducing equivalents in the form of NADH or any other equivalent form of metabolic reducer.

RESULTS AND DISCUSSION

The fermentation behavior of the *C. tropicalis* in semi synthetic medium was verified under two different air flow rates (0.3 and 1.5 vvm) using two different initial xylose concentrations (45.4 and 96.9 g/L). Hemicellulose hydrolysate based medium (29.86 g/L of xylose) was also evaluated using air flow rate of 0.3 vvm. The fermentation parameters, namely xylitol yield coefficient (Y_p/s), xylitol volumetric productivity (Q_p), xylose volumetric consumption (Q_s), cell mass yield coefficient (Y_x/s) and xylitol yield coefficient with respect to cell mass (Y_p/x) were calculated by linear regression of the values attained in the fermentation assays (Figure 2A to E).

Table 1. Kinetic parameters of xylose batch fermentation into xylitol by *Candida tropicalis*

Experiment number	Kinetic parameter											
	S ₀ (g/l)	Air flow (vvm)	Fermentation time (h)	Max. xylitol (g/L)	Q _s (g/L/h)	Q _p (g/L/h)	Q _g (g/L/h)	Y _{x/s} (g/g)	Y _{p/s} (g/g)	η (%)	Y _{p/x} (g/g)	Max q _P g/L/h/g
1 st Experiment.	45.5	1.5	71	30.99 after 66 hr	0.565	0.428		0.045	0.701	77	14.69	1.334
2 nd Experiment.	45.5	0.3	65	36.0 after 59 hr	0.7	0.506		0.031	0.704	77.3	5-Oct	0.98
3 rd Experiment.	96.9	0.3	78	14.05 after 60 hr	0.75	0.134		0.032	0.18	19.7	6.2	0.376
4 th Experiment.	29.87	0.3	94	22.56 after 82 hr	0.292	0.239	0.405	0.06	0.783	86	12.41	0.377

Effect of the initial xylose concentration

Increasing the xylose concentration from 45.5g/L in the 1st and the 2nd experiments to 96.6 g/L in the 3rd experiment led to a great decrease in the xylitol accumulation rate Q_p (from 0.428 and 0.506 g/L/h to 0.134), xylitol yield coefficient Y_{p/s} (from 0.701 and 0.704 g/g to 0.180) and a mild increase in the xylose consumption rate Q_s (from 0.565 and 0.700 g/L/h to 0.750) (Table 1).

This can be explained by the substrate inhibition phenomenon and was probably related to the switch of xylose metabolism to the formation of by-products such as ethanol and citric acid (Silva et al., 1998). The maximum specific xylitol productivity (q_p) was also greatly reduced from 1.334 and 0.98 g/g/h to 0.376 (Table 1). This means that the lowest substrate inhibiting value is given for a D-xylose concentration of 45.5 g/L at which the conversion of D-xylose into xylitol progresses at maximum speed. Similar results were also reported by Silva and Afscher (Silva and Afschar, 1994) on *C. tropicalis* DSM 7524.

In the 1st experiment, the growth was associated with xylitol accumulation from the start to the end of fermentation process. Biomass was nearly unchanged during the first 17 h (lag phase)

with low increment in the xylitol production (Figure 1A). Log phase was extended to the 16th hour of growth with no notable stationary growth phase. 83.2% of the consumed D-xylose was utilized for xylitol production, 4.66% for growth and 12.13% for catabolic reaction (Table 2). In the 2nd experiment, the growth appeared to be dissociated from xylitol accumulation and the cells grew exponentially at the first 20h without releasing the desired product (Figure 1B). After about 30 h of growth, the physiological state of the cells was responsible for the good D-xylose consumption and xylitol accumulation, whereas biomass showed the typical slow growth of the late exponential or decelerated growth phase (Figure 1B).

Only 5.63% of the D-xylose (2.4 g/L) was consumed and utilized for biomass production and 2.21% (0.95g/L) being addressed to energy production through the catabolic reactions (Table 2). As a consequence, 92.16% of the consumed D-xylose was addressed to xylitol production, leading to the highest values of the kinetic parameters of xylitol formation (Q_p=0.506g/L/h and Y_{p/s}=0.704 g/g) (Table 1). The decrease in the xylitol concentration in the 2nd experiment after 60 h was referred to its re-assimilation

(Figure 1B) and this was confirmed by Winkelhausen and Kuzmanova (1998) who stated that it is necessary to maintain the xylose concentration above a certain limit during the cultivation of *Debaromyces hansenii* unless it re-assimilates the xylitol, even if it has insufficient oxygen. Also, in the 3rd experiment, the growth was dissociated from xylitol accumulation from the start to the end of fermentation process. Biomass was greatly enhanced during the first 20 h without comparable increment in the xylitol production (Figure 1C). More than 27.45% of the consumed D-xylose was utilized for xylitol production, 3.9% for growth and 68.63% for catabolic reaction (Table 2), thus, leading to the worst production kinetics of Q_p = 0.134 g/L/h and Y_{p/s} = 0.18 g/g (Table 1).

Effect of air supply

In the xylose metabolism of *C. tropicalis*, xylose was taken by a specific transferase and reduced to xylitol by xylose reductase (XR) with NADPH followed by conversion to xylulose by xylitol dehydrogenase (XDH) with NAD⁺. In this work, independent of the employed growth conditions,

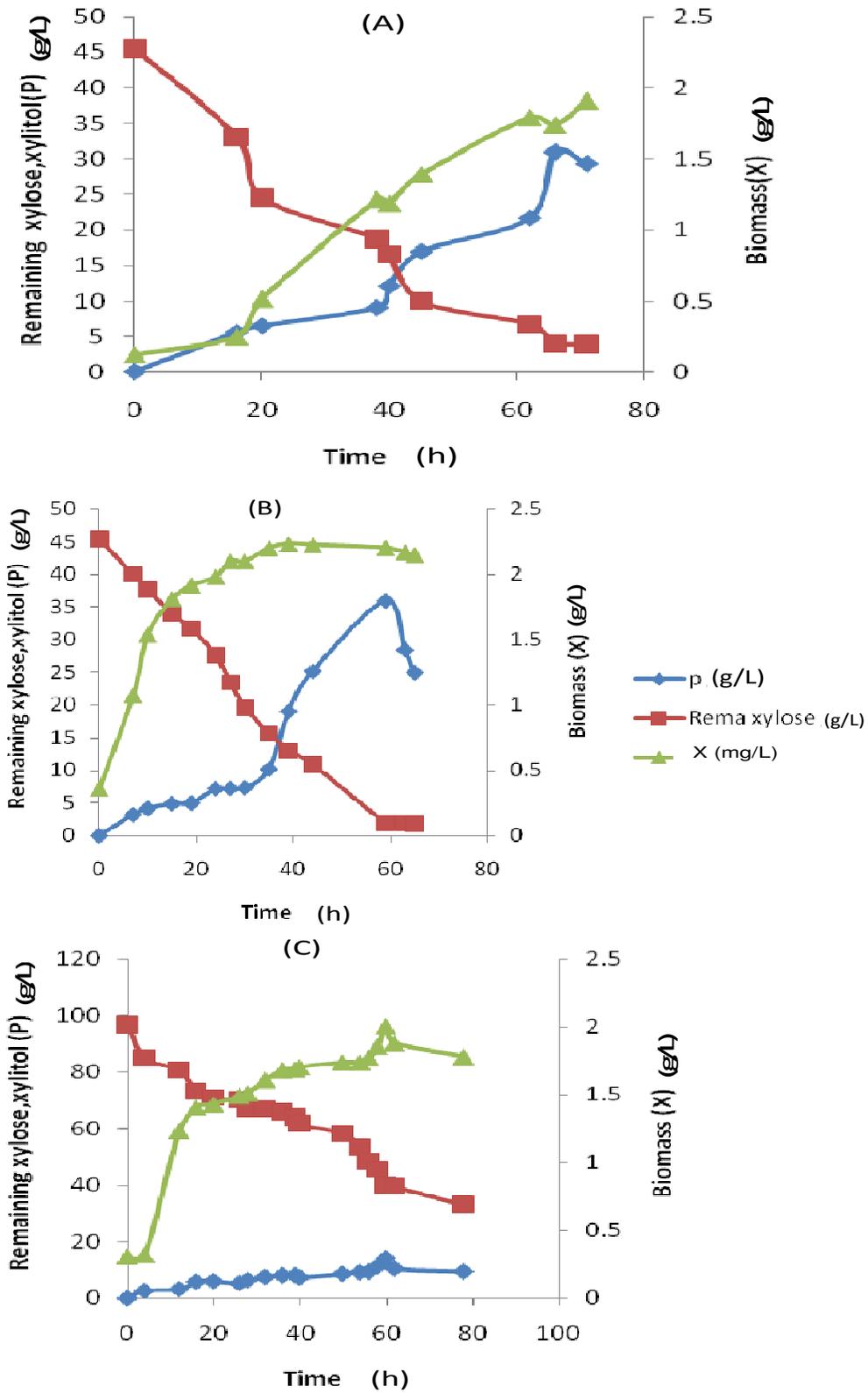


Figure 1. Xylose consumption, xylitol and biomass production during the time course of *Candida tropicalis* cultivations performed on the four fermentation experiments (A; 1s , B; 2nd , C; 3rd and D;4th).

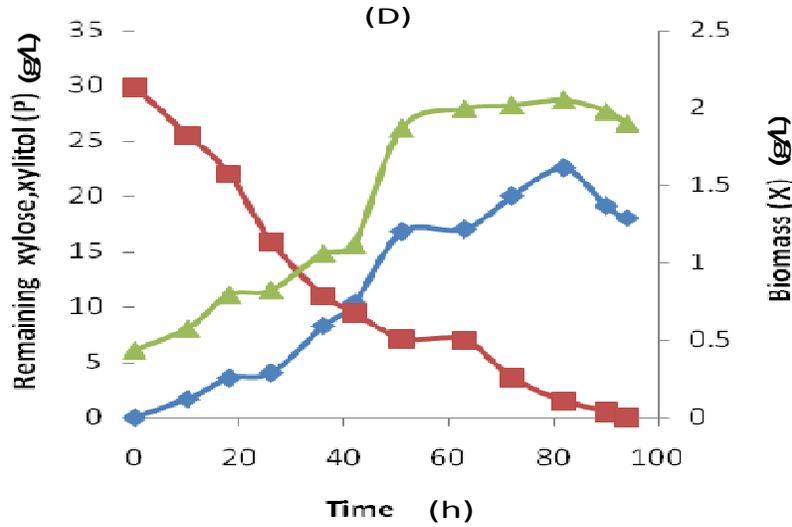


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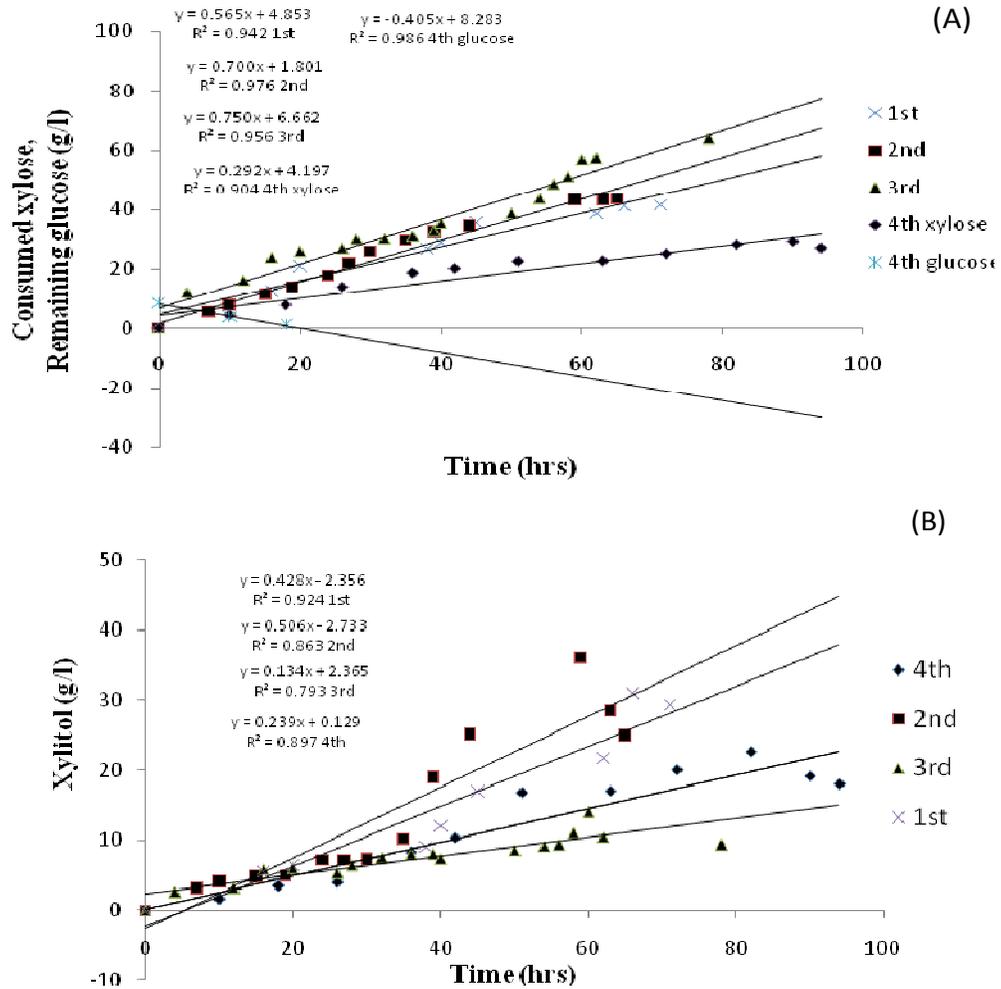


Figure 2. Fermentation kinetic parameters of xylitol production under different growth conditions (1st, 2nd, 3rd and 4th experiments). (A) Xylose and glucose volumetric consumption (B) Xylitol volumetric productivity (C) Xylitol yield coefficient (D) Specific xylitol formation with respect to cell mass (E) Biomass yield coefficient.

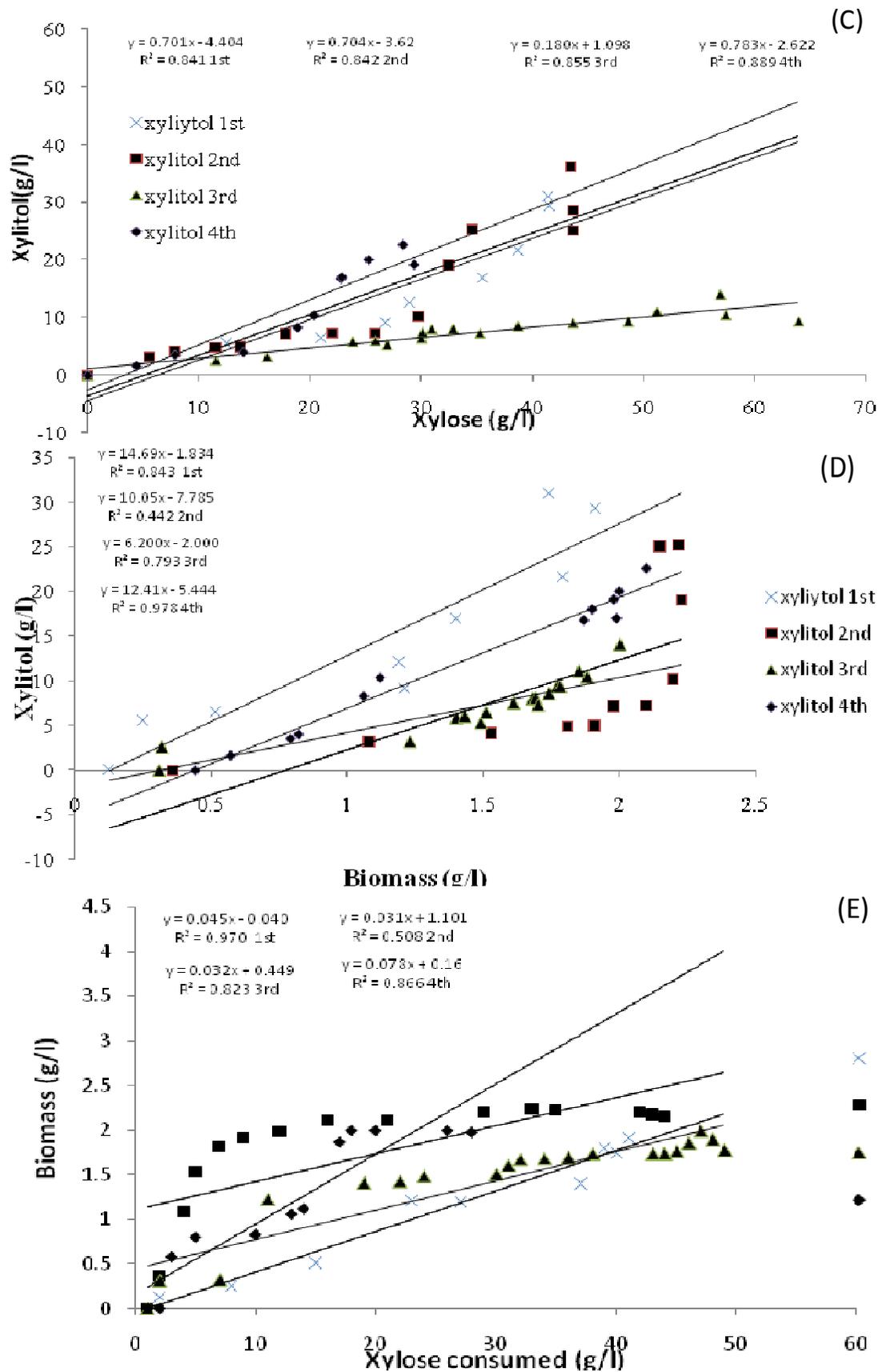


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Table 2. Fractions of xylose consumed by different metabolic activities during different fermentation batches of *Candida tropicalis*.

Experiment number	Time for maximum xylitol production (h)	Total consumed Xylose (g/L)	Xylose converted to Xylitol (g/L)	Xylose converted to biomass (g/L)	Xylose directed to catabolic reactions (g/L)
1 st Experiment.	66	41.38	34.43 (83.2%)	1.93 (4.66%)	5.02 (12.13%)
2 nd Experiment.	59	43.4	40 (92.16%)	2.446 (5.63%)	0.954 (2.21%)
3 rd Experiment.	60	56.85	15.61 (27.45%)	2.22 (3.9%)	39.02 (68.63%)
4 th Experiment.	82	28.37	24.960 (88%)	2.269 (8%)	1.134 (4%)

xylitol was accumulated in the broth medium of the four batch runs after approximately 20 h of the cultivation (Figure 1A to D). This showed that the fermentation conditions used were suitable for enhancing the first step of xylose metabolism and assuming the mechanism described by Taylor et al. (1990) is operative. Depending on the oxygen availability, xylose uptake can preferentially be addressed towards xylitol production, biomass growth or respiration. To obtain a high xylitol yield, the xylose flux to xylulose has to be controlled by an oxygen supply sufficient for regeneration of NADPH and cell maintenance. Low oxygen levels also favor xylitol production because they decrease the NAD⁺/NADH ratio, which favors the xylitol dehydrogenase-catalyzed reaction to xylitol accumulation by changing the equilibrium constant. Full aeration is detrimental to xylitol production as proved in previous studies (Faria et al., 2002; Martinez et al., 2000; Silva and Afschar, 1994).

In the present investigation, microaerobic conditions were fulfilled by applying two micro aeration rates (0.3 and 1.5 vvm) as selected from previous references (Aranda et al., 2000; Belloy et al., 2000), while keeping constant the initial xylose concentration (45.5 g/L) for the 1st experiment and 2nd experiment with increasing xylose concentration up to 96.5 in the 3rd experiment. By decreasing the air flow from 1.5 vvm in the 1st experiment into 0.3 vvm in the 2nd experiment, the volumetric xylitol production (Q_p) and volumetric xylose consumption (Q_s) were increased by 18 and 24%, respectively while the xylitol yield coefficient Y_{p/s} was not changed (Table 1). We can believe that the aeration level of 0.3 vvm ensured the best micro aerobic conditions for xylitol formation, under which the cell utilized most xylose for this activity (92.16%), while the catabolic reaction was practically greatly decreased to be 2.21% (Table 2). In the 1st experiment, an aeration rate of 1.5 vvm may lead to NADH being oxidized to NAD⁺. A high NAD⁺/NADH ratio led to the oxidation of xylitol to xylulose, which was further metabolized to cell materials (4.66%); thus less xylitol and more cells were accumulated (Guo et al., 2006).

Hydrolysate experiment

Fermentation of hemicellulose hydrolysate is normally complex and critical, as the hydrolysate contains a

mixture of sugars and several types of compounds formed during hydrolysis, some of which are toxic to the organism (Parajo et al., 1998). When the hydrolysate is concentrated to increase the sugar content, the concentration of non volatile compounds are also increased and the medium becomes more toxic (Mussatto and Roberto, 2003). Therefore, the hydro-lysate was concentrated only to get 29.87g/L of xylose to minimize the toxic effect of certain hydrolysate containing substances.

The xylose consumption rate (Q_s) was enhanced (from 0.398 to 0.710 g/L/h) just after the complete depletion of glucose (Figure 2A), which could be attributed partially to the glucose inhibitory effect on the xylose reductase enzyme (Silva et al., 1998). Maximum Q_p (0.714 g/L/h), was achieved after 51 h of fermentation (Figure 2B) with a xylose consumption ratio of 76%, after which the yeast entered the stationary phase indicating that the flow of the remaining xylose (24%) was almost directed only to the xylitol production. After 82 h, the maximum xylitol production was achieved, the xylose was nearly depleted (Figure 1D) and it appears that the yeast started up taking little amounts of xylitol as explained thus. A percentage of 88% of the xylose was directed to xylitol with the least xylose percentage (4%) directed to catabolic reactions (Table 2). After approximately 90 h of growth, a very low xylose consumption rate (Q_s = 0.112 g/L/h) was accompanied with a rapid increase in xylitol utilization rate (Q_p = -0.43 g/L/h) (Figure 2B), to get the energy needed for yeast maintenance, indicating that the fermentation should be interrupted as has been mentioned by Roels (1983). Sampaio et al. (2007) also stated that on starvation conditions, such a carbon source, xylitol was preferentially consumed by the catabolic reaction likely to produce the energy necessary for the yeast sustenance.

The semi-synthetic medium (1st and 2nd experiments) has a xylitol yield coefficient (Y_{p/s}) values of 0.704 and 0.701 g/g (Figure 2C). Correspondingly, they are less than that obtained in the hydrolysate experiment (0.783 g/g) and this could be attributed to its low starting xylose content. These results are harmonized with that obtained by Mussatto and Roberto (2003) and Nollet et al. (1993) who worked with *Candida parapsilosis*, and found that the highest xylitol yield coefficient was 0.74 g/g. Generally, in this work there was a continuous decrease in the pH values of the growth medium till the end of the

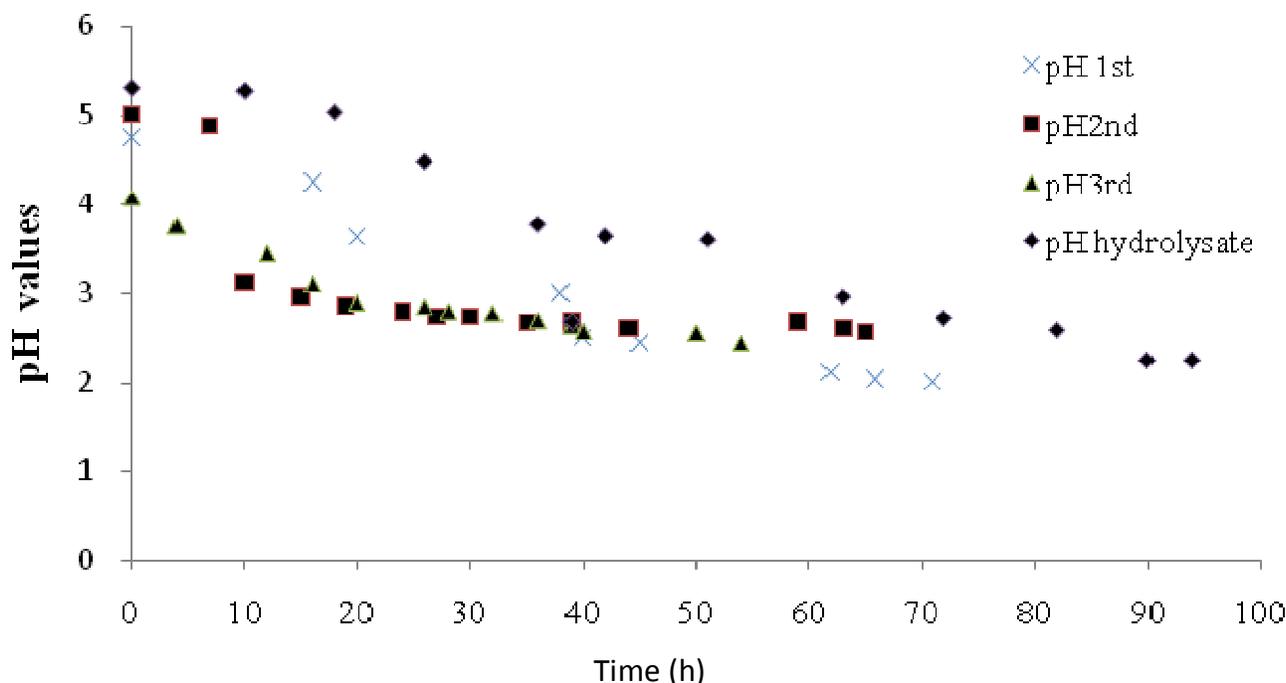


Figure 3. pH profile of the four batch fermentation runs of *Candida tropicalis*.

runs (Figure 3). According to Nolleau et al. (1993), a fall in pH values can be attributed to the liberation of H^+ ions from the utilization of ammonium sulfate as a nitrogen source. This is important since it may be possible to conduct this bioprocess in non sterile conditions, which has an economic advantage (Silva et al., 1998).

Such results demonstrated that under appropriate conditions, *C. tropicalis* can efficiently convert xylose from bagasse hydrolysates into xylitol. Kinetic parameters were enhanced under reduced aeration conditions. Hemicellulose hydrolysate was significantly utilized with a higher $Y_{p/s}$ value more than that of the semi synthetic media. Further studies, which take into account increasing the bagasse hydrolysate concentration using a high inoculum under fed batch conditions, are necessary to optimize the yeast kinetic behavior.

Nomenclatures

Q_p: dp/dt , Xylitol volumetric productivity (g/L/h); **Q_s**, ds/dt , xylose volumetric consumption (g/L/h); **Q_g**: dg/dt , rate of glucose consumption (g/l/h); **Y_{p/x}**, xylitol yield coefficient with respect to cell mass (g/g); **Y_{x/s}**, biomass yield coefficient (g/g); **q_g**, specific glucose uptake rate (g/g/h); **q_s**, specific xylose uptake rate (g/g/h); **η₁**, theoretical xylitol coefficient (g/g) considering 0.917 g/g the theoretical value; **S₀**, initial xylose concentration (g/L); **v_{vm}**, volume air /volume medium / minute; **s**, xylose; **p**, biomass; **x**, xylitol; **g**,

glucose; **t**, time.

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