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Xylitol Production from Corn Cobs Hemicellulosic Hydrolysate by *Candida tropicalis* Immobilized Cells in Hydrogel Copolymer Carrier

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ABSTRACT

The ability of five yeast strains (locally isolated) to ferment xylose to xylitol were screened using a corn cobs hydrolysate. *Candida tropicalis* proved to be the best producer. The effects of culture conditions, namely initial pH, nitrogen source and yeast extract concentration on xylitol production were evaluated. The conditions for batch production of xylitol, using *C. tropicalis* immobilized growing yeast cells in hydrogel copolymer carrier have been optimized. The effects of supplementation of fermentation medium with different concentrations of metabolic inhibitors (monofluoroacetic acid or monochloroacetic acid) and methanol as an activity modifier, on xylitol production by immobilized cells were studied. In repeated batch fermentation (4 days for each run), the immobilized cells retained their ability to produce the superior xylitol yield (48.5 gL^{-1}) in presence of 10 mg % of monofluoroacetic acid and 1.5 ml % methanol supplemented together to fermentation medium. The results also demonstrated that during about 5 weeks of repeated batch mode under optimized conditions, the final xylitol concentrations decreased gradually in nine consecutive runs.

Key Words: Xylitol; *Candida tropicalis;* Hemicellulostic hydrolysate; Immobilization

INTRODUCTION

Xylitol, a five-carbon sugar alcohol, has similar sweetening power as sucrose. It has unique pharmacological properties such as prevention of tooth decay and ear infection in children. It is used as a sugar substitute for diabetic patients and in parental application to trauma patients. Xylitol is increasingly being used in chewing gums, candy, soft drinks, ice cream and oral hygiene products. It occurs naturally in low levels in fruits and vegetables but it is impractical to extract xylitol from these sources. Xylitol can be produced from the catalytic hydrogenation of xylose or xylose-rich hemicellulose hydrolysates. The chemical production route requires extensive purification to meet food and pharmaceutical standards and therefore the product is very expensive. Microbial production is lately becoming more attractive since the downstream processing is expected to be cheaper (Rodrigues *et al.,* 1998; Winkelhausen & Kuzmanova, 1998).

Bioconversion can be carried out with fungi, bacteria, yeast, or purified enzymes from these microorganisms. The most studied xylitol producers are yeasts, with strains of the genus *Candida* and *Debaryomyces hansenii* being the best natural producers. However, the microbial route to xylitol production is environmentally friendly and research in this area is growing. The key enzymes for xylitol production in yeasts are D-xylose reductase which, using either NADH or NADPH, reduces D-xylose to xylitol, and predominantly, NAD-linked xylitol dehydrogenase which reoxidizes xylitol to D-xylulose. Xylitol accumulation in yeasts is sensitive to environmental conditions such as nutrition, temperature, pH, inoculum, substrate and aeration, with the last two being critical for yeast growth and fermentation. Hemicellulosic hydrolysates derived from hardwood and particularly from agricultural residues, such as sugar cane bagasse, corn cobs, wheat and rice straw, are used as feedstock for xylitol production. Due to the presence of inhibitory components, some hydrolysates have to be treated prior to microbial utilization (Parajo *et al.,* 1998a; Rodrigues *et al.,* 1998; Sene *et al.,* 2000). Corn is a major crop of Egypt. Corn cobs, however, are mainly used as a fuel in furnaces or used as manure in the soil. Modern biotechnology allows the use of such lignocellulosic substrates as feedstock for the production of chemicals and fuel using microorganisms. Chemical pretreatments can be accomplished by using strong acids or bases to render the crystalline structure of these substrates amenable for the microbial enzyme production and saccharification into fermentable sugars.

Cell immobilization by means of biomass entrapment within various hydrogels is one of the progressive approaches for the creation of immobilized biocatalysts. Extensive work on the immobilization of biofunctional components by radiation polymerization and their applications has already been performed. Recently, the use

of polyvinyl alcohol (PVA) for cells immobilization has been investigated (Zhaoxin & Fujimura, 1993; El-Batal *et al.,* 1997; Lozinsky & Plieva, 1998; Dominguez *et al.,* 1999). Ethanol production, by *Kluyveromyces lactis* immobilized cells in PVA copolymer hydrogel carrier produced by radiation polymerization, and the conditions for batch and continuous production have been optimized (El-Batal *et al.,* 2000).

The goal of this research is to use corn cobs hemicellulosic hydrolysate as the feedstock for microbial production of xylitol by immobilized growing yeast cells in hydrogel copolymer carrier, also to develop efficient optimized cultural conditions in presence of some metabolic inhibitors and activity modifier in batch mode.

MATERIALS AND METHODS

Microorganisms and culture conditions. The yeast cultures (locally isolated) *Candida boidinii, C. guilliermondii, C. tropicalis, Pichia farinosa* and *Rhodotorula glutinis,* were tested for xylitol production and classified taxonomically as being able to assimilate D-xylose (Barnett *et al.,* 1990). The growth medium was modified YM medium containing 1% glucose, 1% pure xylose, 0.3% yeast extract, 0.3% malt extract and 0.5% peptone. Adapted yeasts (maintained on agar slants made from corn cobs hydrolysate instead of pure glucose and xylose solutions) were used for fermentation of corn cobs hydrolysate. Fermentation medium was made from neutralized treated corn cobs hydrolysate which was supplemented with 0.5% yeast extract, 0.5% KH_2PO_4, 0.5% $(NH_4)_2$ SO_4 and 0.04% $MgSO_4$. $7H_2O$ as described by Oh *et al.* (1998) and Dominguez *et al.* (1999). Yeast cells from growth medium pH 5.0 incubated for 3 days at 30°C on a shaking incubator (150 rpm), were harvested by centrifugation and transferred into fermentation medium under the same conditions containing 50 ml medium per 125 ml Erlenmeyer flasks incubated for 4 days at 150 rpm (semi- aerobic conditions) at 30°C. All tests were carried out in duplicate, and the results shown are average values.

Dilute acid hydrolysis of corn cobs hemicellulose. Ground corn cobs (10% moisture) with an average particle diameter of 2.8-3.6 mm, were treated with ammonia for dissolving and removal of lignin. Dry corn cobs, after ammonia treatment, were mixed with 2% HCl in a 250 ml Erlenmeyer flask with solid to liquid ratio of 1:4. Hemicellulose hydrolysis was performed at 100°C for 2h (Dominguez *et al.,* 1997). For treatment and preparation of neutralized hemicellulose hydrolysate, the pH obtained after acid hydrolysis was adjusted to 5.0 by adding $CaCO_3$ and after neutralization the solids were removed from the supernatant by centrifugation. Anion exchange resin (Amberlite weak-base) treatment was performed and the collected hydrolysate was treated with activated charcoal then the pH was adjusted to 5.0 (Dominguez *et al.,* 1997; Dominguez *et al.,* 1999). Charcoal adsorption removed about 86% of the phenolic compounds.

Immobilization procedure. Immobilized growing cells in PVA and HEMA (7%: 10%, w/w) hydrogel copolymer carrier was prepared and the immobilization procedure of yeast cells was described by El-Batal *et al.* (2000).

Analytical methods. Xylitol concentration was determined by colorimetric assay (Sanchez, 1998). The method involves mild oxidation of alditol (xylitol sugar alcohol) by sodium periodate under acidic conditions followed by reaction of the resulting formaldehyde with ammonia and acetylacetone for 2 min in boiling water bath, to yield a colored heterocycle amenable to spectrophotometer measurement at λ = 412 nm. Ethanol concentration was determined using a gas chromatograph as described by El-Batal *et al.* (2000). Glucose sugar was estimated by kits. Xylose, arabinose, acetic acid, lignin and phenolic compounds were determined by AOAC methodology (AOAC, 1995) and Dominguez *et al.* (1997). Cell dry weight was determined after drying the collected yeast cells at 80°C. The growth was determined in terms of the protein content of whole cells immobilized in copolymerized hydrogel carrier, which were extracted by the method of Freeman *et al.* (1982). Colorimetric assays of the extracted proteins of free and immobilized cells, were performed according to Bradford's procedure (1976). Also, the numbers of viable cells (colony forming unit, CFU) were counted, using seed agar medium.

Statistical analysis. Each experiment was performed in three replicates and analyses were carried out in duplicate. The data given here are the averages of the measurements. The standard deviation of the duplicate never exceeded ± 10% of the mean through out this work .

RESULTS AND DISCUSSION

Selection of yeasts for production of xylitol from corn cobs hemicellulosic hydrolysate. Fermentation in a complex medium, such as a corn cobs hydrolysate, is always critical since the hydrolysate contains various substances that interfere with microbial metabolism. In present study ground corn cobs which have a relative high hemicellulose (31.5%) were steeped in dilute ammonia solution (10%) which removes over 90% of corn cobs lignin. After that they were hydrolysed to their sugar constituents (xylose 70.5, glucose 7.1 and arabinose 5.0 gL^{-1}) using dilute HCl under milder conditions of temperature and time which limited the hydrolysis of cellulose. The acetic acid (2.8 gL^{-1}) was obtained in the hydrolysate from the deacetylation of hemicellulose. On the other hand, lignin-degradation products (phenolic compounds) were removed by charcoal adsorption and were found as (1.2 gL^{-1}). It is difficult to say in advance whether the organism will accept the mixture of nutrients, salts and toxic substances (acetic acid and lignin-degradation products such as furfural and hydroxymethyl furfural),

which are difficult to remove before conducting a fermentation.

Table I presented the fermentation batches carried out with corn cobs hydrolysate under limited oxygen supply conditions for five different yeasts chosen. All the yeasts tested for xylitol production were classified taxonomically as being able to assimilate D-xylose and produce xylitol. In contrast, *Candida guilliermondii* and *C. tropicalis* were better xylitol prod- ucer than the other yeasts, accumulating respectively 13.4 and 16.5 gL^{-1}. They adapted and grew on fermentation medium of corn cobs hydrolysate and produced with *R. glutinis* strain more biomass, than the other yeast strains. It is worthy to mention that the final pH values increased constantly than the initial pH (5.0), suggesting that all the tested yeasts used the acetic acid present as a carbon source as well. According to Morita and Silva (2000), acetic acid at high concentrations strongly inhibit xylose metabolism and cell growth which was caused by its penetration into cells resulting in intracellular acidification which leads to a decoupling of energy production and of the transport of various nutrients. On the basis of xylitol productivity it was concluded that *C. tropicalis* the most potent adapted and tolerated superior producer yeast strain in this study and was chosen to perform the further experimentation.

Effect of cultural conditions on xylitol production by *C. tropicalis.* It is clear that the toxic effect of acetic acid which was found in corn cobs hydrolysate, on the xylitol metabolism depends on the fermentation conditions and the yeast strain employed. To handle this problem, it is necessary to determine the effect of initial pH of fermentation medium, on xylitol production by *C. tropicalis* (Fig.1). The highest xylitol production (17.9 gL^{-1}) was obtained at pH 6.0. The results presented in Fig.1 show that xylitol production was low at initial pH 3.0 and its values increased with an increase in initial pH up to 6.0. In fact, Pfeifer *et al.* (1996) and Rodrigues *et al.* (1998) have reported that the toxic effect of acetic acid is increased by a low pH value in the medium because of the acid's entry into the cell in its nondissociated form. Acetic acid inside the cell, especially under these conditions, may induce cytoplasm acidification. According to data in Fig.1, the initial pH strongly influenced the xylitol yield. In this context, Morita and Silva (2000) reported that at acetic acid concentrations (> 1.0 gL^{-1}) as in this study (2.8 gL^{-1}), part of the acid continues to be directed towards the Krebs cycle and the remainder may be utilized by another energy-consuming metabolic pathway. This may result in the lack of energy for the maintenance of the overall metabolism of the yeast, leading to reduce cell growth. The inhibitory effect of the acetic acid depends not only on fermentation conditions (such as initial pH) and yeast strain (*C. tropicalis* the most potent tolerant strain), but also on the presence of other compounds in the hydrolysate culture medium such as phenolic compounds (1.2 gL^{-1}). According to our results the inhibitory effect of the acetic acid can be overcome by a simple and economical corn cobs hydrolysate treatment (by mild acid hydrolysis after deligninfication by ammonia solution and treatments by activated charcoal), and by the use of suitable initial pH (6.0) at which the xylose reductase activity may be improved as reported by Sene *et al. (*2000). Therefore, the following experiments were carried out with initial pH 6.0.

The choice of nitrogen source for the fermentation medium can be an important factor influencing the subsequent fermentation. All nitrogen sources tested singly at concentration 1.06 g nitrogen per liter favoured xylitol production (Table II). Furthermore, use of KNO_3 resulted in the highest xylitol production (19.1 gL^{-1}) and 52.4% xylitol yield from theoretical value compared with the other nitrogen sources. On the other hand, use of urea resulted in a small xylitol production (16.2 gL^{-1}) and the highest increase in dry cell mass (12.1 gL^{-1}) with cell mass yield coefficient (0.33) as compared with 0.26 in using KNO_3. This may suggest a metabolic activation in using KNO_3 and metabolic repression in using urea for xylitol production.

As can be seen in Fig. 2, xylitol formation was affected by different concentrations of yeast extract (1 up to 10 gL^{-1}) added to corn cobs hydrolysate medium in presence of KNO_3 as nitrogen source and at initial pH 6.0. Yeast extract is an important nutrient for xylitol production which provides all the necessary vitamins. Yeast extract at a concentration 2.5 gL^{-1} was optimum and sufficient for *C. tropicalis* and maximum xylitol production (21.5 gL^{-1}) was obtained. Increased concentrations of yeast extract (more than 2.5 gL^{-1}) decreased its xylitol productivity. These results are in accordance with those of Horitsu *et al.* (1992) and Silva and Afschar (1994) for *C. tropicalis* IFO 0618 and DSM 7524, in spite of their being related to other microorganisms. They found that increased yeast extract concentration higher than 15 gL^{-1} blocked the conversion of D-xylose to xylitol and yeast extract at a maximum concentration of 1.0 gL^{-1} was sufficient. On the other hand, Parajo *et al.* (1998a) reported that supplementation of the fermentation medium with yeast extract and peptone improved growth, cell yield and productivity of xylitol. Some yeasts require vitamins and oligoelements found in yeast extract, for growth or for enhancing productivity of xylitol. From the results obtained, it is quite clear that KNO_3 is a good nitrogen source with 2.5 gL^{-1} yeast extract for high xylitol production from corn cobs hydrolysate with initial pH 6.0, by free cells of *C. tropicalis.*

Xylitol production from corn cobs hydrolysate by entrapped immobilized *C. tropicalis* growing cells in hydrogel copolymer carrier produced by radiation polymerization. Ionizing gamma radiation is a very useful tool to create hydrogels of different shapes, different degrees of cross-linking and different composition. In previous studies (El-Batal *et al.,* 2000) the highest ethanol production was obtained by immobilized growing *Kluyveromyces lactis* yeast cells in copolymer carrier (PVA: HEMA) system with optimum composition ratio of (7% :

Table I. A survey of xylitol production by various yeasts from corn cobs hydrolysate after 4 days and at initial pH 5.0

Yeast strain	Final pH	Ethanol gL^{-1}	Consumed xylose (s) gL^{-1}	Xylitol conc. (x) gL^{-1}	Xylitol yield coefficient Yx/s (gg^{-1})	% Xylitol yeild from theoretical value (0.9) Yx/t	Dry cell mass [Cm] gL^{-1}	Cell mass yield coefficient (YCm/s) gg^{-1}
Candida boidinii	6.6	3.5	37.2	11.7	0.32	34.9	10.2	0.27
C. guilliermondii	6.4	4.6	38.5	13.4	0.35	38.7	12.6	0.33
C. tropicalis	6.5	2.9	37.4	16.5	0.44	49.0	11.6	0.31
Pichia farinosa	6.5	5.0	40.7	10.6	0.26	28.9	9.3	0.23
Rhodotorula glutinis	6.8	2.8	39.3	12.8	0.33	36.2	15.7	0.40

Table II. Effect of nitrogen source on xylitol production by *C. tropicalis* after 4 days at initial pH 6.0

Nitrogen source (N=1.06 gL^{-1})	Final pH	Consumed xylose (s) gL^{-1}	Xylitol conc. (x) gL^{-1}	Xylitol yield coefficient Yx/s (gg^{-1})	% Xylitol yeild from theoretical value (0.9) Yx/t	Dry cell mass [Cm] gL^{-1}	Cell mass yield coefficient (Ycm/s) gg^{-1}
$(NH_4)_2 SO_4$ (0.5%)	6.6	38.1	17.7	0.45	51.6	11.4	0.30
NH_4Cl (0.405%)	6.4	39.7	18.5	0.47	51.8	10.3	0.26
$NH_4 NO_3$ (0.303%)	6.5	38.8	17.6	0.45	50.4	11.8	0.30
KNO_3 (0.765%)	6.8	40.5	19.1	0.47	52.4	10.5	0.26
Urea (0.227%)	6.3	36.3	16.2	0.45	49.6	12.1	0.33

Table III. Effect of initial immobilizing growing yeast cells density expressed as cell loading (14.7 mg dry cells per g support hydrogel) on xylitol production by *C. tropicalis* free and immobilized cells at initial pH 6.0 after 4 days incubation (50 ml culture / 125 ml flask).

Parameter	Free cells batch	Initial immobilized growing yeast cells loading (g hydrogel per 50 ml culture)			
		5	10	15	20
Consumed xylose (s) (gL^{-1})	42.1	33.1	48.5	53.2	56.7
Xylitol Conc. (x)	21.2	16.0	26.2	30.7	27.8
Xylitol yield coefficient Yx /s (gg^{-1})	0.50	0.48	0.54	0.58	0.49
% Xylitol yield from the theoretical value (0.9) Yx/t	56.0	53.7	60.0	64.1	54.5
Final dry cell mass (mg dry cell per g hydrogel)	--	32.3	30.8	30.5	30.4
Final free cells or (escaped cells from hydrogel) mg dry cell per ml culture	10.4	0.03	0.08	0.13	0.19

10%, w/w). In present study, to test the growth of yeast cells in (PVA: HEMA) hydrogel carrier, *C. tropicalis* yeast cells were grown in the prepared hydrogel beads with incubation in the mixture of precultured yeast cells and nutrient growth medium. The changes in populations of yeast cells in the hydrogel copolymer carrier beads were investigated after incubation periods (12, 24, 36, 48, 60 and 72 h) which resulted in growing immobilized cells populations expressed as dry yeast cells per gm hydrogel carrier of (0.53, 1.4, 3.5, 8.1, 14.7 and 14.5 mg dry cells g^{-1} hydrogel), respectively. The results indicated that, the immobilized yeast cells multiplied with increasing the incubation period and the population of yeast cells filled the almost whole hydrogel carrier [7.8 x 10^8 (CFU) cells g^{-1} hydrogel equal 14.7 mg dry cells per gm hydrogel] after 60 h incubation. The process of yeast cells immobilization was described as the cells at first adsorbed on the surface of copolymer carrier by the interaction of cells and carrier, especially in a dent or fold of the copolymer surface, then the cells on the surface intruded or entered in the interior of the copolymer carrier by brisk multiplication and multiplication through the pores of copolymer carrier. Nutrient availability (mainly glucose and xylose) due to diffusion may be greatest near the surface of the hydrogel. The yeast cells, therefore, by taking these nutrients, grow in the lattice of the hydrogel and form colonies which expand into a continuous dense layer of cells near the surface of the hydrogel beads as described by Zhaoxin and Fujimura (1993) and El-Batal *et al.* (2000). It is worthy to mention that the substrates to product conversion can be characterized by the metabolic activity of the cells and in general, culture age is strongly related to this parameter.

Fermentation assays were performed to discern the effect of the experimental conditions (inoculum cells concentration and oxygen limitation). In order to evaluate the improvements derived from increased initial inoculum of biomass concentrations, experiment was performed exploring four concentrations of inoculum expressed as cell loading (14.7 mg dry cells per gm support hydrogel) of (5, 10, 15 and 20 g hydrogel loaded by yeast cells) in 50 ml fermentation culture put in 125 ml flask under oxygen limiting (150 rpm). The results presented in Table III show that the increase in both xylose consumption and xylitol production was achieved when the initial inoculum

Fig. 1. Effect of initial pH on xylitol production by C. tropicalis after 4 days incubation

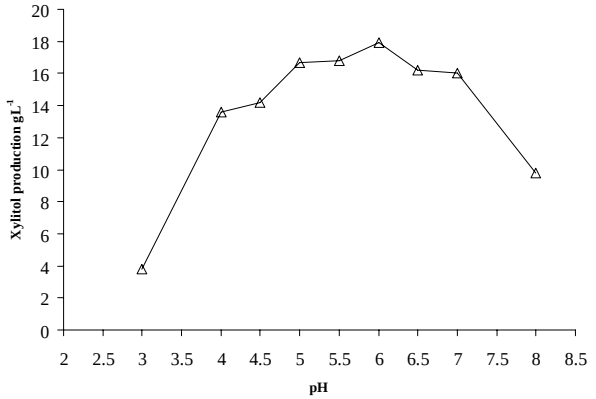


Fig. 2. Effect of different concentrations of yeast extract added to fermentation medium after 4 days incubation and initial pH 6.0.

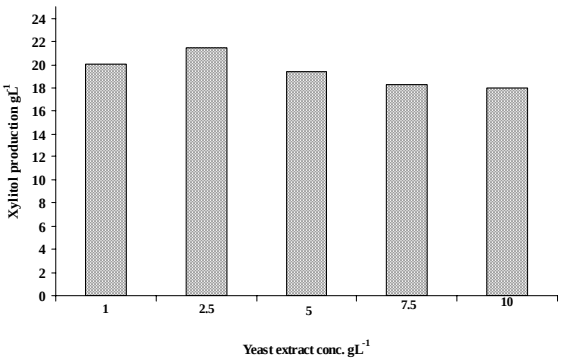


Fig. 3. Effect of different aeration (oxygen) on xylitol production by C. tropicalis immobilized yeast (initial inoculum 15 g hydrogel per 125 ml volume flask) incubated for 4 days. Culture medium volume per flask volume (CMV/FV) ratios correspond [aerobic < 0.26 ratio, 0.26< semiaerobic < 0.52 ratios and microaerobic > 0.52 ratios]

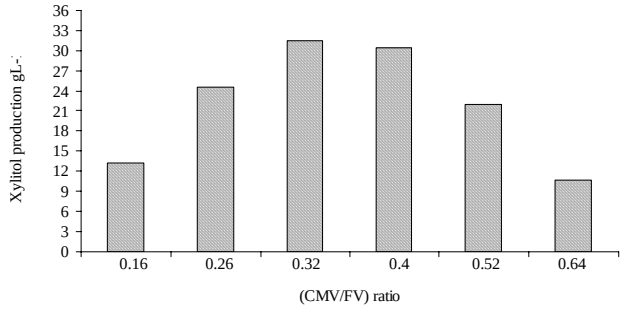
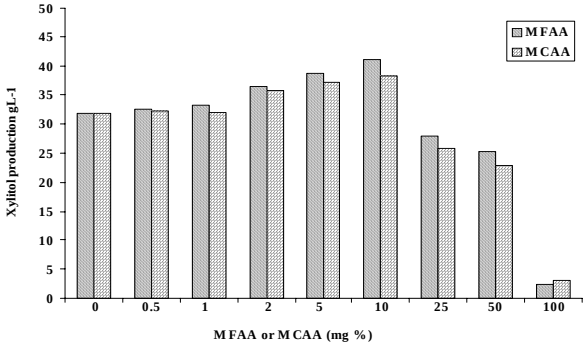


Fig. 4. Effect of different concentrations of metabolic inhibitors as monofluoroacetic acid (MFAA) or monochloroacetic acid (MCAA) added to fermentation medium on xylitol production by C. tropicalis immobilized cells (15 g hydrogel per 40 ml culture) incubated for 4 days and initial pH 6.0.



concentration was increased. On the basis of xylitol production, it was concluded that good xylitol yield (30.7 gL^{-1}) was obtained in medium supplemented with initial inoculum concentration of 15 g hydrogel carrier contained (220.5 mg dry cells) of initial entrapped growing yeast cells and the resulted final yeast mass accumulated was 459.0 mg dry cells or 9.18 mg ml^{-1} after 4 days of fermentation. This may be due to the presence of glucose in the hydrolysate which improved the cell growth in the hydrogel and the high density of the yeast cells in the hydrogel was maintained at steady state for long periods due to growth and loss of cells. The escaped free cells from hydrogel in the medium were ranged from 0.9% up to 1.6% as compared to the final number of the immobilized growing cells in the hydrogel carrier after 4 days of incubation, which showed the efficiency of the hydrogel matrix for immobilization process. The lost yeast cells were replaced in the hydrogel by cells growing on the nutrient.

In present study (Table III), higher initial immobilized growing yeast cells density resulted in marked improvements in xylose uptake and utilization and xylitol production. With this procedure, the lag phase was avoided, simultaneous consumption of glucose and xylose was allowed and also overcame (at least in part) the toxic effect of inhibitors (acetic acid and phenolic compounds) limiting the cell death caused by their assimilation or degradation, which was close to the results of Parajo *et al.* (1998b). On the other hand, the results in Table III revealed that at initial inoculum with (20 g hydrogel loaded cells) the xylitol production decreased. This could be explained as in media containing high cell densities, a reduction in the oxygen availability in the medium could be responsible for

decreased yields and productivity. This idea suggests the existence of an optimum cell concentration for given operational conditions (15 g hydrogel loaded cells). It is worthy to mention that the xylitol production (30.7 g L^{-1}) from (15 g hydrogel loaded cells) cultures was higher than that produced (21.2 gL^{-1}) from free cells culture containing approximately the same cell dry mass at the end of fermentation (Table III). These results could be explained by the fact that the gradient of the toxicant concentration found in hydrolysate, would allow immobilized cells to tolerate the toxic substrate under soft conditions than during direct contact of free cells in liquid medium. In fact Fedorov *et al.* (1999) have reported that immobilization of cells by incorporation in a matrix is known to affect significantly their physiology and intensify their metabolism e.g. changes in properties of outer membranes, protein composition and permeability. Such deviations are hypothesized to be induced by changes in parameters of physicochemical medium during immobilization.

A complementary experiment was performed in order to evaluate the influence of aeration on xylitol production by immobilized growing cells in hydrogel carrier of 15 g hydrogel per different levels of medium volume. In order to simulate different aeration conditions, different ratios of culture medium volume (CMV) per flask volume (FV) in the ranges of [0.16, 0.26 (aerobic); 0.32, 0.4 (semiaerobic) and 0.52, 0.64 microaerobic)] and shaking speed of 150 rpm as described by Walther *et al.* (2001). The results of this experiment (Fig. 3) indicated that maximum xylitol production (31.5gL^{-1}) was attained in semiaerobic condition at (CMV/FV) ratio of 0.32 with relative increase of (2.4 and 2.9 folds) more than in aerobic (0.16 ratio) and microaerobic condition (0.64 ratio), respectively. In fact, the behaviour of free and immobilized yeasts can differ significantly. For example, an oxygen transfer rate corresponding to microaeration with free cells can correspond to anaerobic conditions with immobilized cells due to diffusional limitation in mass transfer. The use of lower aeration in immobilized cultures (microaerobic) caused dramatic reduction in xylitol concentration (22.1 and 10.7 gL^{-1}) as illustrated in Fig.3. In this context, Oh *et al.* (1998) reported that in xylitol-producing yeasts, oxygen supply affects the rate and yield of xylitol production, and determines the partitioning of the carbon flux from xylose between cell growth and xylitol formation. Excessive oxygen conditions (aerobic) lead to NADH being oxidized to NAD^+, and a high NAD^+ / NADH ratio lead to oxidation of xylitol to xylulose, which is further metabolized to cell material, and as a result, less xylitol and more cells are accumulated. Under a limited oxygen supply (semiaerobic), the electron transfer system is unable to reoxidize all of the produced NADH by respiration and/or fermentation, the intracellular NADH level increases and the reaction of xylitol to xylulose decreases, and consequently more xylitol accumulates. Therefore, in our study for effective xylitol production, oxygen must be carefully controlled at low or limited oxygen condition (15 g hydrogel loaded cells per 40 ml culture medium in 125 ml flask at 150 rpm shaking speed).

Fig. 5. Effect of different concentrations of activity modifier as methanol added to fermentation medium, on xylitol production by C. tropicalis immobilized cells (15 g hydrogel per 40 ml culture) incubated for 4 days and initial pH 6.0.

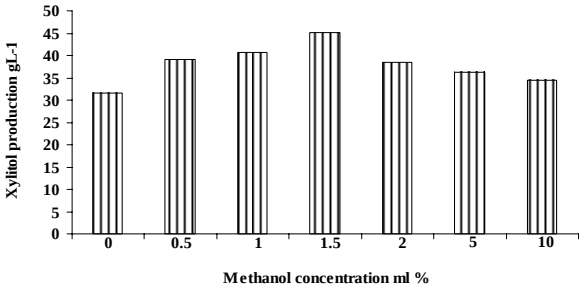
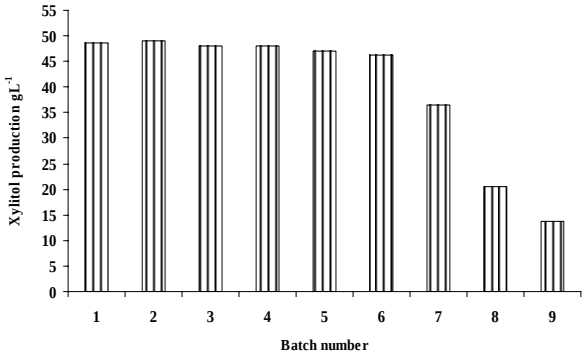


Fig. 6. Repeated batch fermentation (4 days for each run) of xylitol production from corn cobs hydrolysate medium in presence of 10 mg % monofluoroacetic acid and 1.5 ml% methanol, by C. tropicalis immobilized cells initial pH 6.0.



Effects of some metabolic inhibitors (monofluoroacetic acid and monochloroacetic acid) on xylitol production by immobilized *C. tropicalis* cells. It is well known that various compounds, such as monofluoroacetic acid (MFAA) and monochloroacetic acid (MCAA) are metabolic inhibitors and were used to increase product formation. To channel the xylose utilization for fermentation, MFAA or MCAA of different concentrations (0.0 up to 100 mg%) were added to the corn cobs hydrolysate nutrient fermentation medium in presence of immobilized growing yeast cells in hydrogel carrier. The results presented in Fig. 4 show that with the increase in MFAA or MCAA concentrations from 0.0 to 10mg%, xylitol production increased from 31.7gL^{-1} (control) to 41.0 or 38.2 gL^{-1} with

addition of MFAA or MCAA, respectively. Further increase in both MFAA or MCAA from 25.0 to 100mg%, inhibited xylitol production dramatically. The addition of 10 mg % of MFAA or MCAA to the medium improved xylitol yield with maximum relative production increase (1.3 or 1.2 folds), respectively than that of control. These results can be attributed to the increase of channeling of the carbon source for fermentation. Previous reports suggested that MFAA inhibited cisaconitase activity in *Candida* sp. Y_2 (Tani *et al.*, 1990). Monochloroacetic acid might have acted, in a similar way to fluoroacetic acid. In fact, inside the yeast cell, xylose is reduced to xylitol by NADH dependent xylose reductase. Xylitol either secreted from the cell or oxidized to xylulose, by NAD dependent xylitol dehydrogenase, which utilized by the central catabolic pathways via Embden-Meyerhof Pathway for biosynthesis of pyruvate (Winkelhausen & Kutzmanova, 1998).

However, in present study (Fig. 4) when 10 mg % of MFAA or MCAA were incorporated into the nutrient medium, complete oxidation of pyruvate through the tricarboxylic acid cycle might have been inhibited (Arasaratnam & Balasubramaniam, 1998). On the other hand, MFAA and MCAA are structural analogs of acetyl CoA (Mayes, 1993) and might condense with oxaloacetate to form fluorocitrate or chlorocitrate which would then inhibit cisaconitase. This block in the tricarboxylic acid cycle could have lead to the accumulation of pyruvate which may cause metabolic imbalance (feedback inhibition) and channeling for xylitol production. Above 10 mg % concentration of MFAA or MCAA seem to affect the metabolism of the yeast adversely. Also, it is worthy to mention that MFAA or MCAA may inhibit or uncouple the respiratory chain and help in the channeling of the carbon source (xylose) to xylitol production by increasing NADH accumulation and subsequent inhibition of NAD-linked xylitol dehydrogenase. This phenomena, known as the Custer effect, results from incapability of the yeasts to compensate the accumulation of excess NADH and this consequently stimulates the accumulation of xylitol in the culture medium under oxygen limitation (Winkelhansen & Kutzmanova, 1998).

Effect of an activity modifier (methanol) on xylitol production by immobilized *C.tropicalis* cells. Xylitol production was carried out by varying the methanol concentration from 0.5 to 10 ml % supplemented to fermentation medium in presence of immobilized growing yeast cells in hydrogel carrier for enhancement the reduction of xylose to xylitol by xylose reductase in presence of NADH. As shown in Fig. 5, the addition of methanol to the fermentation medium resulted in an increase in xylitol production in all concentrations studied in comparison to control (without addition). The most favorable concentration of methanol was 1.5 ml % yielding the maximum amount of xylitol (45.2 gL^{-1}) which was higher than that in the absence of methanol (31.5 gL^{-1}). However, increasing methanol concentration from 2 up to 10 ml % decreased slightly and gradually xylitol production as compared to (1.5 ml % concentration). In fact, yeasts grown on methanol make 2 mole of NADH from oxidation of one formaldehyde. On the other hand, the oxidation of methanol supplies NADH, this coenzyme being utilized for the reduction of either D-xylulose or D-xylose to xylitol by the NADH-dependent xylose reductase (Parajo *et al.*, 1998b). Our results are in general agreement with the observation of Vongsuvanlert and Tani (1988) who reported that methanol and xylose could induce the synthesis of enzymes of a methanol oxidation system.

To elucidate some aspects of the induction effects of monofluoroacetic acid (10 mg %) and methanol (1.5 ml %), selected from previous experiments for their maximum influence on the induction and promoting capacities for xylitol production, they were supplemented to fermentation medium together. The use of MFAA and methanol improve xylitol production. The highest yield (48.3 gL^{-1}) was attained (which is higher than both MFAA or methanol separately), suggesting a possible synergistic effect of MFAA and methanol on activation of xylitol production after 4 days of cultivation. The time course of xylitol production using optimized cultural conditions of MFAA and methanol addition resulted in xylitol production of (12.7, 29.6, 40.2, 48.5, 41.8 and 30.0 gL^{-1}) after incubation of (1, 2, 3, 4, 5 and 6 days), respectively. Therefore, a low level of xylitol appeared in the early stages of incubation and reached the maximum level by 4 days. A prolonged incubation period beyond this did not help further increase in yield.

Production of xylitol using repeated batch fermentation by *C. tropicalis* immobilized cells in presence of monofluoroacetic acid and methanol. Repeated batches fermentation for xylitol production were carried out with immobilized growing cells of *C. tropicalis* in hydrogel carrier, from corn cobs hydrolysate medium in presence of monofluoroacetic acid (10 mg %) and methanol (1.5 ml %) under optimized conditions. The fermentation was carried out for 4 days for each run and the production medium was removed and the immobilized cells in hydrogel were washed with sterile medium and fresh production medium was added to the flasks. As shown in Fig. 6, the immobilized cells were able to produce xylitol for long time and could be reused successfully for about cosecutive runs (24 days), without detected activity loss. However, the production of xylitol was decreased gradually after the 6^{th} run with no sign of breakage or disintegration of the carrier beads. It is worthy to mention that, after 36 days the immobilized growing cells in hydrogel carrier beads were dramatically became unstable and softened which may be possible due to the effects of various stresses of ingredients of the corn cobs hydrolysate fermentation medium.

The inference from these data is that the operational stability of the xylitol production indicated the possibility of the application of immobilized yeast cells for long time use in consecutive batch mode. The experiments in this work

show that, the long term cells activity, productivity, the operational stability of xylitol production and the durability of the hydrogel copolymer carrier are feasible for successful full-scale operation for xylitol production which is economically very attractive.

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