Xylitol production by *Debaryomyces hansenii* and *Candida guilliermondii* from rapeseed straw hemicellulosic hydrolysate

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Abstract

This study evaluated the possibility of using rapeseed straw hemicellulosic hydrolysate as a fermentation medium for xylitol production. Two yeast strains, namely *Debaryomyces hansenii* and *Candida guilliermondii,* were used for this bioconversion process and their performance to convert xylose into xylitol was compared. Additionally, different strategies were evaluated for the hydrolysate detoxification before its use as a fermentation medium. Assays in semi-defined media containing different combinations of sugars were also performed in order to verify the influence of hexose sugars on xylose metabolism by the yeasts. *C. guilliermondii* exhibited higher tolerance to the toxic compounds than *D. hansenii*. Not only the toxic compounds present in the hydrolysate affected the yeast´s performance to convert xylose into xylitol, but glucose also had a negative impact on the bioconversion process. It was not necessary to completely eliminate the toxic compounds to obtain an efficient conversion of xylose into xylitol, mainly by *C. guilliermondii*.

***Keywords*:** rapeseed straw; hemicellulosic hydrolysate; detoxification; semi-defined media; xylitol

1. Introduction

Xylitol ($C_5H_{12}O_5$) is a natural food sweetener that has been used as a sucrose substitute due to the similar sweetening power and lower caloric value. In addition, several benefits to the health have been associated to the ingestion of xylitol. One of the most important is in oral health, reducing and/or preventing tooth decay. However, xylitol can also be used for the treatment of several diseases such as diabetes, hemolytic anemia, inflammatory processes, colon diseases and parenteral and renal injuries, as well as to prevent respiratory infections, acute otitis media and osteoporosis. Due to these properties, the xylitol market, including applications in food and pharmaceutical industries, has significantly increased in the last decades (Mussatto, 2012; Mussatto and Roberto, 2002).

Xylitol is traditionally produced by chemical synthesis (xylose hydrogenation using chemical catalysts), but it can also be produced by fermentation using microorganisms with ability to convert xylose into xylitol, mainly yeasts. Xylose-to-xylitol bioconversion by yeasts is considered an attractive alternative to the currently large-scale commercial chemical process since the biological route has lower energy requirements (milder conditions of pressure and temperature are needed), is more sustainable and environmentally-friendly (Cortez et al., 2016; Mussatto, 2012).

Different feedstocks have been tested as carbon source for xylitol production by fermentation; the efficiency of the bioconversion process is clearly dependent on the feedstock and pretreatment applied to release xylose, as well as on the microorganism and cultivation conditions. Rapeseed straw is an agricultural residue with a high content of cellulosic and hemicellulosic sugars - greater than 55% (Choi et al., 2013); however,

with no relevant application currently. The generation of this residue has significantly increased in the last years due to the growing interest on rape oil for biodiesel production (Pinkowska et al., 2013). In 2014, for example, 9.1 million and 36.1 million hectares of rapeseed were cultivated in Europe and worldwide, respectively (FAOSTAT, 2017).

Different types of pretreatment can be used to release sugars from lignocellulose biomass, among of which, diluted acid pretreatment is one of the most efficient to remove hemicellulose sugars (Mussatto, 2016). However, the generation of toxic compounds such as furan derivatives (furfural and hydroxymethylfurfural), acetic acid, and phenolic compounds, may also occur during this process, and such compounds affect the cell metabolism during fermentation. For this reason, the generation of these compounds must be avoided as much as possible during biomass pretreatment (Mussatto and Dragone, 2016). Some detoxification strategies can also be used in order to reduce the concentration of toxic compounds to levels that do not affect the microbial metabolism, which include vacuum evaporation, overliming, adsorption in activated charcoal or in ion-exchange resins, and extraction with organic solvent, among others (Mussatto and Roberto, 2004a).

The present study evaluated the possibility of producing xylitol from rapeseed straw hemicellulosic hydrolysate. The performance of two yeasts, namely *Debaryomyces hansenii* and *Candida guilliermondii,* to convert xylose into xylitol from hydrolysate medium was evaluated and compared. In addition, different detoxification strategies were tested to reduce the negative effect of toxic compounds on fermentation performance. To the best of our knowledge, this is the first study on the use of rapeseed straw as a feedstock for xylitol production.

2. Materials and methods

2.1. Raw material

Rapeseed straw was collected from the fields of the province of Seville, Spain, after seed harvesting. As soon as collected, the feedstock was air dried, milled to particle size smaller than 0.4 cm using a laboratory hammer mill (Retsch, SM-100, Haan, Germany), homogenized and stored in a dry place until use. The feedstock (94% w/w dry matter) presented the following composition (% w/w): glucan 31.5 ± 0.3, hemicellulose 17.4 ± 0.1 (xylan 13.2 ± 0.1, galactan 1.9 ± 0.1, arabinan 1.2 ± 0.02, mannan 1.2 ± 0.04), acid insoluble lignin 16.2 ± 0.5, acid soluble lignin 1.6 ± 0.05, acetyl groups 3.4 ± 0.07, ashes 6.7 ± 0.26, and extractives 15.4 ± 1.3 (López-Linares et al., 2015).

2.2. Hemicellulosic hydrolysate

Rapeseed straw hemicellulosic hydrolysate (RH, raw hydrolysate) was produced by dilute acid pretreatment of the feedstock (10% w/v feedstock load) with 2% (w/v) sulfuric acid at 130 ºC for 1 h. After pretreatment, the liquid fraction (hemicellulosic hydrolysate) was recovered by vacuum filtration and the contents of sugars (glucose, xylose, arabinose, mannose, and galactose) and toxic compounds (furfural, hydroxymethylfurfural (HMF), acetic acid, formic acid, and phenolic compounds) were determined.

2.3. Hydrolysate detoxification

Different strategies were used for detoxification of the rapeseed straw hemicellulosic hydrolysate in order to decrease the concentration of toxic compounds present (**Fig. 1**). Initially, the raw hydrolysate (RH) was treated with activated charcoal (100 mesh particle size - Sigma-Aldrich) in a 3.5% (w/v) ratio and the suspension was incubated in a rotary shaker (Certomat-R, B-Braun, Germany) at 45 ºC, 150 rpm for 1 h (Mussatto and Roberto, 2004b). The liquid fraction was recovered by vacuum filtration in Whatman no. 1 filter paper (ACH, activated charcoal-treated hydrolysate) and 4.5-fold concentrated by evaporation at 60 ºC under vacuum conditions. The concentrated liquid fraction thus obtained (CH, ACH 4.5-fold concentrated) had the pH adjusted to 6.0 by addition of solid KOH. Then, an ion-exchange resin treatment was carried out by passing the liquor through a copper column (1.2 cm wide × 50 cm length) filled with the resin Microionex MB200 (Rohm Haas, Copenhagen, Denmark) in a ratio of 0.2 g resin per mL liquor. At the end of this process, the pH of the liquor was corrected to 6 using solid KOH. The hydrolysate obtained after this procedure was denominated DH (detoxified hydrolysate). An aliquot of each hydrolysate sample was collected and used for sugars and toxic compounds determination.

To decrease even more the concentration of toxic compounds present in DH, some additional strategies were applied. 1) A portion of the DH was two-fold diluted to decrease the level of inhibitory compounds and then the diluted hydrolysate was supplemented with sugars up to the original level present in DH. The hydrolysate obtained after this step was denominated DDH (diluted detoxified hydrolysate). 2) A portion of DH was submitted to an additional detoxification step using organic solvents. In this case, the hydrolysate had the pH adjusted to 3 by addition of H_2SO_4 (98% w/v) and was then mixed with ethyl acetate or chloroform in a 1:3 (v/v) ratio. The mixture

was maintained in a rotary shaker at 200 rpm, 30 °C for 45 min. Finally, the residual organic solvent was eliminated by evaporation. The hydrolysates obtained after this step were denominated EA-DH (DH further treated with ethyl acetate) and CL-DH (DH further treated with chloroform).

2.4. Microorganisms and inocula

Debaryomyces hansenii (NRRL Y-7426) and *Candida guilliermondii* FTI 20037 (ATCC 201 935) were the yeast strains used in the experiments. The strains were maintained at 4 ºC on malt extract agar slants containing (g/l): malt extract 30, peptone 5, and agar 15.

The inocula were grown in 250-mL Erlenmeyer flasks containing 100 ml of medium composed of (g/l): xylose 30, malt extract 3, yeast extract 3, and peptone 5. For preparation of this medium, a concentrated solution containing all the nutrients was prepared and sterilized at 121 ºC for 20 min, while the xylose solution was autoclaved at 112 ºC for 15 min. The cells were grown in a rotary shaker at 30 ºC and 200 rpm for 24 h, being subsequently recovered by centrifugation (3000 *g*, 15 min) and resuspended in the fermentation medium.

2.5. Fermentation media and conditions

2.5.1. Hydrolysate-based fermentation media

The detoxified hydrolysate (DH) and its variations (DDH, EA-DH, and CL-DH) were used as fermentation medium for xylitol production. To be used as fermentation medium, the hydrolysates had the pH adjusted to 5.5 by addition of solid KOH and were subsequently autoclaved at 112 ºC for 15 min.

Fermentation assays were performed in 100-mL Erlenmeyer flasks containing 50 ml of hydrolysate inoculated with an initial cell concentration of 3 g/l. The flasks were maintained in a rotary shaker at 200 rpm, 30 ºC for 72 h. During the experiments, samples were withdrawn each 24 h, centrifuged at 8,000 *g* for 10 min, and used to determine cell, sugars, xylitol and ethanol concentration. All the experiments were carried out at least in duplicate.

2.5.2. Semi-defined fermentation media

A semi-defined medium with the same composition of sugars present in DH (g/l: glucose 11.7, xylose 40.6, galactose 8.7, arabinose 6.8, and mannose 2.2), was prepared and used for xylitol production. Such medium was denominated DM1. Additionally, fermentation media with the same composition of DM1 but without glucose (DM2), without galactose (DM3), and without glucose and galactose (DM4) were also formulated and used for fermentation. All the semi-defined media were supplemented with the same nutrients used for inoculum preparation. The nutrients solution was sterilized at 121 ºC for 20 min, while the sugars solutions were autoclaved at 112 ºC for 15 min. The pH of the semi-defined media was adjusted to 5.5 by addition of 2M KOH solution.

Fermentation assays were carried out in 100-mL Erlenmeyer flasks containing 50 ml of medium inoculated with 3 g/l of cells concentration, and were maintained under the same conditions used for the assays in hydrolysate-based medium (200 rpm, 30 ºC for 72 h). Samples were periodically withdrawn for sugars, xylitol, ethanol, and cell growth determinations. All the experiments were carried out at least in duplicate.

2.6. Analytical methods

Glucose, xylose, arabinose, mannose and galactose concentrations in the hydrolysates were determined by high performance liquid chromatography (HPLC) in a Waters 2695 liquid chromatograph (Mildford, MA, EEUU) equipped with a refractive index detector (Waters 2414). A Transgenomic CHO-782 column operating at 70 ºC with ultrapure water as a mobile-phase (0.6 ml/min) was used. Acetic acid, formic acid, furfural and HMF concentrations were also determined by HPLC using a refractive index detector, but, in these cases, a Bio-Rad HPX-87H column operating at 65 ºC with 5 mM H_2SO_4 as mobile phase at a flow rate of 0.6 ml/min, was used. Phenolic compounds were estimated by the Folin-Cicalteau method (Singleton and Rossi, 1965) using gallic acid as standard.

Sugars, xylitol and ethanol concentrations from fermentation samples were determined by HPLC using a Bio-Rad HPX-87H column under the same conditions described for acetic acid determination. Cell concentration was estimated by dry weight. For analysis, fermentation samples were filtered through cellulose nitrate filter with 0.2 μm pore size (Sartorius Stedim Biotech, Germany), which were previously dried to constant weight. The cell concentration was calculated as the ratio between the mass of dried biomass and filtered inoculum volume.

All analytical determinations were performed in triplicate and average results are shown. Relative standard deviations were below 3%.

2.7. Fermentation parameters

Cell yield ($Y_{X/S}$, g/g) was defined as the ratio between formed cells and total sugars consumed. Xylitol yield ($Y_{PX/S}$, g/g) was calculated as the ratio between xylitol

produced and xylose consumed, while the ethanol yield ($Y_{PE/S}$, g/g) was considered as the ratio between the ethanol produced and total sugars consumed during the fermentation. Xylitol and ethanol productivities (Q_{PX} and Q_{PE} respectively, g/l.h) were determined as the ratio between the xylitol or ethanol concentration (g/l) and the fermentation time (h), respectively.

3. Results and discussion

3.1. Hydrolysate composition and detoxification strategies

Rapeseed straw pretreatment with dilute sulfuric acid was very efficient for solubilization of the feedstock´s hemicellulose fraction. In total, 72.3% of the hemicellulosic sugars were recovered in the hydrolysate, xylose being the most representative corresponding to 58% of the total sugars present in the hydrolysate (**Table 1**, RH). Acetic acid was the most abundant among the inhibitor compounds. This organic acid is commonly found in hemicellulosic hydrolysates since it is also released from the hemicellulose structure during pretreatment (Mussatto and Roberto, 2004a). Besides acetic acid, other inhibitor compounds including formic acid, furfural and HMF (sugar´s degradation products), and phenolic compounds (from the partial breakdown of lignin) were also present in the hydrolysate. The low tolerance of xylitol-producing microorganisms to the toxicity of furans, acetic acid and phenolic compounds is well-known (Carvalheiro et al., 2005; Felipe et al., 1995; Morita and Silva, 2000; Mussatto and Roberto, 2004b). For this reason, a detoxification step is required to at least partially remove the toxic compounds present and generate a hydrolysate more suitable for use as fermentation medium.

Unfortunately, there is not a specific method of detoxification able to efficiently remove all the toxic compounds and a combination of strategies is usually required. In the present study, the original hydrolysate (RH) was initially treated with activated charcoal as this method has been shown to be efficient to remove furans and phenolic compounds from hydrolysates produced from different feedstocks (Carvalheiro et al., 2005; Lee et al., 2011; Mussatto and Roberto, 2001). In agreement with that, the use of activated charcoal for detoxification of rapeseed straw hemicellulosic hydrolysate resulted in an almost complete removal of furans and 68% removal in the content of phenolic compounds (**Table 1**, ACH). However, the efficiency of this detoxification method to remove formic and acetic acids was very limited (27% and 14%, respectively).

Vacuum evaporation process has been suggested as an alternative to remove weak organic acids like acetic and formic acids from hydrolysate as such compounds are partially volatilized under these conditions (Converti et al., 2000; Mussatto and Roberto, 2004a). Besides removing organic acids, this method is also convenient since it promotes an increase in the concentration of sugars in the medium, which, in turn, is beneficial to obtain higher xylitol yield by fermentation (Mussatto and Roberto, 2003). As can be seen in **Table 1** (CH), the hydrolysate concentration step promoted an increase in the content of total sugars to 75.5 g/l (58% xylose, 17% glucose), which was proportional to the concentration factor employed (4.5-fold). Formic and acetic acids were partially volatilized during this process, as their concentration increased less than 4.5-fold. On the other hand, the vacuum evaporation process did not remove phenolic compounds, which is in agreement with other authors (Chandel et al., 2011; Parajó et al., 1997).

Phenolic compounds are usually very toxic to microorganisms even when present in low concentrations in the fermentation medium (Mussatto and Roberto, 2004a). These compounds affect cell growth and metabolism decreasing the product formation. For this reason, additional efforts were done to decrease even more the concentration of phenolic compounds in rapeseed straw hemicellulosic hydrolysate, by submitting this liquor to a treatment with ion-exchange resins, which has been suggested to be effective for phenolic compounds removal (Taherzadeh and Karimi, 2011). When applied for the detoxification of CH, this method resulted in phenolic compounds and formic acid reductions of 41% and 53%, respectively (**Table 1**, DH).

Finally, the detoxified hydrolysate (DH) was submitted to extraction with organic solvents as a final strategy to reduce the concentration of toxic compounds, mainly acetic acid. Two different organic solvents, ethyl acetate and chloroform, were used in this step. Acetic acid was removed by 62% when ethyl acetate was used and only 12% when chloroform was used. However, these treatments resulted in an increase in the level of phenolic compounds probably due to their concentration during the distillation step to eliminate the residual organic solvent. Overall, extraction with ethyl acetate was more efficient than with chloroform, generating a hydrolysate (EA-DH) with lower level of toxic compounds (**Table 1**).

3.2. Xylitol production from rapeseed straw hemicellulosic hydrolysates

Rapeseed straw detoxified hydrolysate (DH) was used as fermentation medium for xylitol production by *D. hansenii* and *C. guilliermondii*. Fermentation assays were also performed using the hydrolysate after extraction with ethyl acetate (EA-DH) and with chloroform (CL-DH). Additionally, fermentation assays were carried out using the

DH diluted with water (1:1) and supplemented with sugars up to the original level present in DH, in order to have another medium with a different composition in terms of toxic compounds (DDH). Besides evaluating and comparing the performance of the yeasts in the different hydrolysate-based media, these assays made also possible to estimate the level of toxic compounds that the yeasts are able to tolerate without affecting the efficiency of xylose conversion into xylitol. The differences in the composition of the hydrolysates used as fermentation medium can be seen in **Table 1**.

Both yeasts, *D. hansenii* and *C. guilliermondii* had a similar behavior when cultivated in DH (**Fig. 2**). Likewise, the time course of the fermentation from DDH was also very similar for both yeasts. However, when comparing the fermentation of DH and DDH, it is evident the faster consumption of xylose by both yeasts when cultivated in the medium containing lower concentration of toxic compounds, DDH (**Figs. 2a,c**). From DDH, xylose was almost exhausted after 72 h, while from DH, more than 13 g/l of xylose remained in the medium at the same time and about 5 g/l after 144 h. Xylitol production was also higher and faster from DDH (**Figs. 2b,d**), resulting in productivities four-fold higher when compared to DH (**Table 2**).

It is worth mentioning that a gradual consumption of acetic acid was observed during the fermentation of DDH by both yeasts, being this acid completely exhausted after 144 h (data not shown). Previous studies also reported consumption of acetic acid during hydrolysate fermentation by *C. guilliermondii* (Morita and Silva, 2000) and during semi-defined medium fermentation by *D. hansenii* (Duarte et al., 2006). According to Felipe et al. (1995), acetic acid can be potentially toxic for *C. guilliermondii* in concentrations above 3 g/l, while lower concentrations of this acid may have a positive effect on xylose uptake, xylitol yield and cell growth. Under these

conditions, yeasts are able to assimilate and use acetic acid as an additional source of nutrients for growth (Camargo et al., 2015). DDH hydrolysate contained 2.65 g/l of acetic acid, while the concentration of this acid in DH was double (**Table 2**). This fact would explain the consumption of acetic acid during the fermentation of DDH, but not during the fermentation of DH.

When comparing the hydrolysates further treated with solvents (EA-DH and CL-DH) with DH, it is evident that the extraction with organic solvents improved the fermentability of the hydrolysate. Similar concentration of residual xylose was observed in the media after 144 h of fermentation (**Figs. 2a,c**). However, higher xylitol concentration was obtained in the fermentation of extracted hydrolysates by both yeasts, mainly by *C. guilliermondii* (**Figs. 2b,d**). Fermentation of EA-DH and CL-DH by *C. guilliermondii* surpassed by more than 25% the xylitol yield obtained in DH medium (**Table 2**). Similar improvement in xylitol yield was not observed by *D. hansenii*. Since the distillation after DH extraction with organic solvents resulted in an increase in the concentration of phenolic compounds in these media and the content of acetic acid in the extracted media was even lower than in DH (**Table 1**), it can be concluded that phenolic compounds in concentrations above 3 g/l were toxic for *D. hansenii*. Moreover, a synergistic effect between acetic acid and phenolic compounds on the growth of *D. hansenii* has been previously reported (Domínguez et al., 1999).

When comparing the performance of the yeasts in the extracted hydrolysates, the best results in terms of xylitol yield and productivity were obtained with *C. guilliermondii* (**Table 2**) independently of the solvent used for extraction. Xylitol yield, in particular (0.53-0.54 g/g) was very close to the value obtained in DDH fermentation (0.57 g/g). However, an important difference was observed in the productivity values,

since maximum xylitol production by *C. guilliermondii* in EA-DH and CL-DH media occurred at 144 h, while for DDH medium it occurred at 48 h only. Phenolic compounds could have been the main responsible for such differences since they were presented in much lower amount in DDH than in EA-DH and CL-DH, while the concentration of acetic acid was in the same range of values observed for EA-DH and CL-DH (**Table 1**).

Similar to DDH, a gradual consumption of acetic acid was also observed during the fermentation of the EA-DH by both yeasts, reaching approx. 0.2 g/l after 144 h (data not shown). It should be noted that the assimilation of acetic acid by *C. guilliermondii* and *D. hansenii* was only observed during the fermentation of DDH and EA-DH, which contained less than 3 g/l of this acid in the composition, and not during the fermentation of DH and CL-DH (acetic acid concentration > 3 g/l). This fact is in agreement with the studies reported by Felipe et al. (1995) and Camargo et al. (2015) in terms of threshold level for acetic acid to act as a toxic compound or as a nutritional source for the microorganism. Moreover, both media where acetic acid was assimilated by the yeasts resulted also in the highest productions of xylitol.

In brief, the assays in hydrolysate media revealed that rapeseed straw hemicellulosic hydrolysate can be used as fermentation medium for xylitol production. However, a detoxification step is required to reduce the concentration of toxic compounds and improve the xylitol yield and productivity. The best results of xylitol production ($Y_{PX/S}$ = 0.54 g/g and Q_{PX} = 0.14 g/l.h) were achieved when using the detoxified hydrolysate submitted to extraction with solvents as fermentation medium, and *C. guilliermondii* as yeast strain. These results, mainly the productivity, can be

further improved by decreasing the concentration of toxic compounds present, especially phenolic compounds, as demonstrated through the assays in DDH.

3.3. Xylitol production from semi-defined media vs hydrolysate media

It is well known that other sugars present in the composition of hemicellulosic hydrolysates, mainly hexoses, may affect the xylose conversion to xylitol by yeast (Mussatto et al., 2006). In order to evaluate the influence of the other sugars present on rapeseed straw hemicellulosic hydrolysate composition on xylose metabolism by *D. hansenii* and *C. guilliermondii,* different semi-defined media (DM) were formulated containing the same concentration of sugars present in DH, but in different combinations. DM1 contained all the five sugars identified in DH (xylose, glucose, arabinose, mannose and galactose), while DM2 was absent in glucose, DM3 was absent in galactose, and DM4 was absent in glucose and galactose. These assays made possible to identify the influence of glucose and galactose on xylose metabolism by both yeasts and revealed also the maximum values of xylitol yield and productivity able to be achieved in a medium containing the mixture of sugars present in DH but without toxic compounds.

As can be seen in **Figs. 3a** and **c**, both yeasts were able to assimilate all the xylose present in the different media after 48 h of fermentation; only a minimal amount (< 10%) was still present at that time in DM3 and DM4 cultivated by *C. guilliermondii* (**Fig. 3c**). Besides xylose, all the other sugars present in the different media were also completely assimilated by both yeasts after 48 h. On the other hand, xylitol production was favored in the absence of glucose, DM2, achieving 27.5 g/l with *D. hansenii* (**Fig. 3b**) and 24.7 g/l with *C. guilliermondii* (**Fig. 3d**). These results suggest inhibition of

glucose on xylose metabolism by both yeasts. Such effect is probably associated to a catabolic repression caused by glucose on the enzymes responsible for xylitol production. According to Tochampa et al. (2005), glucose/xylose ratios lower than 10% can even improve the activity of xylose-reductase increasing the xylitol volumetric productivity. However, higher glucose/xylose ratios inhibit the xylose transport into the cell and repress induction of relevant enzymes. In the present study, the glucose/xylose ratio present in rapeseed straw hemicellulosic hydrolysate (as well as in the semi-defined media) was above 30%. Therefore, it can be concluded that not only the toxic compounds present in the hydrolysate medium affected the yeast´s performance to convert xylose into xylitol, but glucose also had a negative impact on the bioconversion process.

When compared to DM1, which contained all the sugars present in DH, xylitol production by both yeasts was also improved in the absence of glucose and galactose (DM4) (**Figs. 3b,d**). However, in this case the maximum xylitol production was lower than in DM2 (absent in glucose only), probably because in DM4 part of the xylose was used for cell growth and not for xylitol production. As can be seen in **Table 2**, cell yield was practically the same in DM2 and DM4, by both yeasts, supporting the theory that the yeasts used part of the xylose to keep the same growth level during the cultivations. Assays in absence of galactose only (DM3) revealed that this hexose did not have a negative impact on xylose metabolism by both yeasts, since the maximum xylitol production in DM3 was practically the same as that achieved in DM1 by *D. hansenii*, and even a bit lower than that achieved in DM1 by *C. guilliermondii* (**Figs. 3b,d**). These assays confirm that glucose was the hexose with the most significant negative impact on xylose metabolism by both yeasts.

Assays in DM1 revealed also the maximum production of xylitol able to be achieved by both yeasts when cultivated in a medium with the same composition of sugars present in DH but without toxic compounds. Such values corresponded to xylitol yield and productivity of 0.57 g/g and 0.80 g/l.h, respectively by *D. hansenii*, and 0.55 g/g and 0.43 g/l.h respectively, by *C. guilliermondii* (**Table 2**). These values were in close agreement with those achieved during the fermentation of DDH by both yeasts, with exception only for xylitol productivity by *D. hansenii*, which was better in fermentation from semi-defined medium. These results show that it is not necessary to completely eliminate the toxic compounds from the hydrolysate medium to obtain an efficient conversion of xylose into xylitol. As can be seen in **Table 1**, DDH still contained acetic acid (2.65 g/l), formic acid (0.60 g/l), and phenolic compounds (0.85 g/l) in its composition and resulted in xylitol yield and productivity similar to DM1 (**Table 2**).

When comparing the yeasts, *C. guilliermondii* showed higher tolerance to the toxic compounds present in DDH than *D. hansenii,* since its performance to convert xylose into xylitol was very similar in DDH and DM1 ($Y_{PX/S}$ = 0.57 and 0.55 g/g respectively, and Q_{PX} = 0.41 and 0.43 g/l.h, respectively), while xylitol productivity by *D. hansenii* in DDH was only half the value achieved in DM1 (**Table 2**). On the other hand, *D. hansenii* was able to produce xylitol faster than *C. guilliermondii* from semi-defined medium. However, to achieve this productivity from hydrolysate medium, additional detoxification steps would be required to reduce even more the level of toxic compounds present in DDH.

The strategy used in the present study for hydrolysate detoxification followed by extraction with solvents (EA-DH and CL-DH) was not enough to decrease the

concentration of toxic compounds to the same level present in DDH (**Table 1**). Even so, xylitol yield was significantly improved (~ 30%) by *C. guilliermondii* when compared to the DH fermentation (independent of the solvent used for extraction), achieving values similar to those obtained from DM1 and DDH (0.55-0.57 g/g) (**Table 2**). Similar improvement in xylitol yield was not observed for *D. hansenii* cultivated in EA-DH and CL-DH. However, xylitol productivity was not improved from EA-DH and CL-DH by any of the strains, confirming that both require a lower level of toxic compounds in the medium for a faster conversion of xylose into xylitol.

3.4. By-product formation

Ethanol was detected as a by-product in all the fermentations in hydrolysate and semi-defined media. The formation of this compound is related to the metabolism of hexoses by yeasts. For this reason, ethanol production was lower in DM4 when compared to the other cultivation media (**Table 2**), as mannose was the only hexose sugar present in this medium. Maximum concentration of ethanol was achieved after 24 h of fermentation in all the media containing glucose in the composition. During this time, all the glucose present in the media was consumed by the yeasts, while galactose and mannose were only partially consumed at that time. However, assays in semi-defined media DM2 and DM4 revealed that galactose and mannose were also used for ethanol production by both yeasts (**Table 2**).

Another interesting fact observed during the cultivations was a simultaneous consumption of xylose and glucose by both yeasts with simultaneous formation of xylitol and ethanol, although xylitol was indeed the product produced in higher amount. On the other hand, arabinose was only slightly consumed by the yeasts after exhaustion

of xylose from the media, being not observed simultaneous consumption of pentose sugars. Xylitol and ethanol were also partially consumed by the yeasts after exhaustion of the main carbon sources (xylose and glucose), being used for the cell´s growth. Similar behavior was reported by Gírio et al. (2000) during the cultivation of *D. hansenii* in mixed substrates. According to these authors, cell growth increased during the fermentation and was also associated to the consumption of ethanol and xylitol by the yeast when xylose concentration in the medium was lower than 3 g/l.

4. Conclusions

Rapeseed straw hemicellulosic hydrolysate can be used as fermentation medium for xylitol production by D. *hansenii* or *C. guilliermondii*. However, *C. guilliermondii* exhibited higher tolerance to the toxic compounds present in the hydrolysate being therefore more attractive for use in this bioconversion process since it would require less detoxification steps. As the detoxification steps result in more expenses, other alternatives including adaptive laboratory evolution of the strains could be an interesting option to overcome the hydrolysate toxicity as well as the negative impact of glucose on xylose metabolism. These results open up new possibilities for the valorization of rapeseed straw.

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Figure Captions

Figure 1. Schematic representation of the steps performed for the production and detoxification of rapeseed straw hemicellulosic hydrolysate. RH: raw hydrolysate (obtained by dilute acid pretreatment of rapeseed straw); ACH: activated charcoal-treated hydrolysate; CH: ACH 4.5-fold concentrated; DH: detoxified hydrolysate (CH further treated with ion-exchange resin); DDH: DH diluted with water (1:1) and supplemented with sugars up to the same concentration present in DH; EA-DH: DH further treated with ethyl acetate; CL-DH: DH further treated with chloroform.

Figure 2. Xylose consumption (A) and xylitol production (B) by *Debaryomyces hansenii* and xylose consumption (C) and xylitol production (D) by *Candida guilliermondii.* Fermentation assays in rapeseed straw hemicellulosic hydrolysate based media. DH: detoxified hydrolysate; DDH: DH diluted with water (1:1) and supplemented with sugars up to the same concentration present in DH; EA-DH: DH further treated with ethyl acetate; CL-DH: DH further treated with chloroform.

Figure 3. Xylose consumption (A) and xylitol production (B) by *Debaryomyces hansenii* and xylose consumption (C) and xylitol production (D) by *Candida guilliermondii.* Fermentation assays in semi-defined media. DM1: medium formulated with the same concentration of sugars present in the detoxified hydrolysate (DH); DM2: same composition of DM1 but without glucose; DM3: same composition of DM1 but without galactose; DM4: same composition of DM1 but without glucose and galactose.

Table 1. Composition of rapeseed straw hemicellulosic hydrolysate (g/l) in the original form and after each detoxification step.

	Sugars					Inhibitor compounds				
Hydrolysate	Glucose	Xylose	Galactose	Arabinose	Mannose	Formic acid	Acetic acid	HMF	Furfural	Phenolic compounds
RH	2.96 ± 0.04	10.30 ± 0.07	2.20 ± 0.04	1.72 ± 0.03	0.66 ± 0.03	1.20 ± 0.02	3.84 ± 0.02	0.50 ± 0.00	0.41 ± 0.01	1.93 ± 0.27
ACH	2.81 ± 0.12	9.91 ± 0.38	2.09 ± 0.11	1.66 ± 0.06	0.62 ± 0.03	0.88 ± 0.11	3.31 ± 0.31	0.02 ± 0.01	0.00 ± 0.00	0.61 ± 0.09
CH	12.49 ± 0.19	43.82 ± 0.79	9.13 ± 0.19	7.31 ± 0.08	2.76 ± 0.06	2.65 ± 0.03	6.54 ± 0.24	0.08 ± 0.02	0.00 ± 0.00	2.94 ± 0.13
DH	11.68 ± 0.09	40.59 ± 0.21	8.67 ± 0.12	6.79 ± 0.09	2.22 ± 0.06	1.25 ± 0.02	5.34 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	1.74 ± 0.01
EA-DH	14.83 ± 0.41	49.66 ± 0.90	10.88 ± 0.40	8.41 ± 0.37	2.79 ± 0.43	1.22 ± 0.11	2.02 ± 0.09	0.00 ± 0.00	0.00 ± 0.00	3.74 ± 0.12
CL-DH	13.04 ± 0.25	43.62 ± 0.49	9.57 ± 0.11	7.64 ± 0.14	2.45 ± 0.12	1.49 ± 0.18	4.71 ± 0.04	0.00 ± 0.00	0.00 ± 0.00	3.43 ± 0.17
DDH	11.82 ± 0.09	41.51 ± 0.10	8.79 ± 0.13	6.83 ± 0.08	2.25 ± 0.05	0.60 ± 0.02	2.65 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.85 ± 0.01

RH: raw hydrolysate (obtained by dilute acid pretreatment of rapeseed straw);
ACH: activated charcoal-treated hydrolysate
CH: ACH 4.5-fold concentrated
DH: detoxified hydrolysate (CH further treated with ion-exchange resin)
EA-DH: DH further treated with ethyl acetate
CL-DH: DH further treated with chloroform
DDH: DH diluted with water (1:1) and supplemented with sugars up to the same concentration present in DH

Table 2. Fermentation parameters obtained during the production of xylitol from rapeseed straw detoxified hydrolysates (DH) and semi-defined media (DM) by *Debaryomyces hansenii* and *Candida guilliermondii*.

Fermentation medium	Yeast strain	Time (h) *	Xylitol yield ($Y_{PX/S}$, g/g)	Xylitol productivity (Q_{PX}, g/l.h)	Cell yield ($Y_{X/S}$, g/g)	Ethanol yield ($Y_{PE/S}$, g/g)	Ethanol productivity (Q_{PE}, g/l.h)
DH	D	72	0.42 ± 0.01	0.16 ± 0.00	0.02 ± 0.00	0.18 ± 0.00	0.13 ± 0.00
	C	144	0.42 ± 0.01	0.10 ± 0.00	0.06 ± 0.00	0.12 ± 0.01	0.05 ± 0.00
DDH	D	48	0.55 ± 0.01	0.42 ± 0.00	0.07 ± 0.00	0.19 ± 0.01	0.24 ± 0.01
	C	48	0.57 ± 0.01	0.41 ± 0.06	0.06 ± 0.00	0.19 ± 0.00	0.23 ± 0.03
EA-DH	D	144	0.45 ± 0.01	0.12 ± 0.01	0.13 ± 0.01	0.10 ± 0.00	0.04 ± 0.01
	C	144	0.54 ± 0.01	0.14 ± 0.00	0.12 ± 0.01	0.13 ± 0.00	0.06 ± 0.01
CL-DH	D	144	0.38 ± 0.00	0.09 ± 0.00	0.04 ± 0.00	0.11 ± 0.00	0.05 ± 0.00
	C	144	0.53 ± 0.03	0.13 ± 0.01	0.05 ± 0.00	0.13 ± 0.01	0.06 ± 0.00
DM1	D	24	0.57 ± 0.01	0.80 ± 0.04	0.08 ± 0.00	0.11 ± 0.00	0.23 ± 0.04
	C	48	0.55 ± 0.01	0.43 ± 0.01	0.06 ± 0.00	0.21 ± 0.00	0.25 ± 0.00
DM2	D	24	0.78 ± 0.01	1.15 ± 0.01	0.08 ± 0.01	0.13 ± 0.02	0.23 ± 0.03
	C	48	0.64 ± 0.01	0.51 ± 0.02	0.08 ± 0.00	0.15 ± 0.02	0.15 ± 0.01
DM3	D	48	0.57 ± 0.03	0.41 ± 0.00	0.14 ± 0.01	0.20 ± 0.01	0.17 ± 0.00
	C	48	0.47 ± 0.00	0.35 ± 0.00	0.09 ± 0.00	0.22 ± 0.01	0.21 ± 0.01
DM4	D	48	0.65 ± 0.02	0.50 ± 0.01	0.10 ± 0.00	0.06 ± 0.00	0.05 ± 0.00
	C	48	0.67 ± 0.01	0.49 ± 0.02	0.09 ± 0.01	0.06 ± 0.01	0.05 ± 0.00

* Time at which maximum xylitol production was achieved.
D: *Debaryomyces hansenii;* C: *Candida guilliermondii;* Fermentation time: time of maximum xylitol production;
DH: detoxified hydrolysate; DDH: DH diluted with water (1:1) and supplemented with sugars up to the same concentration present in DH;
EA-DH: DH further treated with ethyl acetate; CL-DH: DH further treated with chloroform.
DM1: semi-defined medium formulated with the same concentration of sugars present in DH; DM2: same composition of DM1 but without glucose;
DM3: same composition of DM1 but without galactose; DM4: same composition of DM1 but without glucose and galactose.

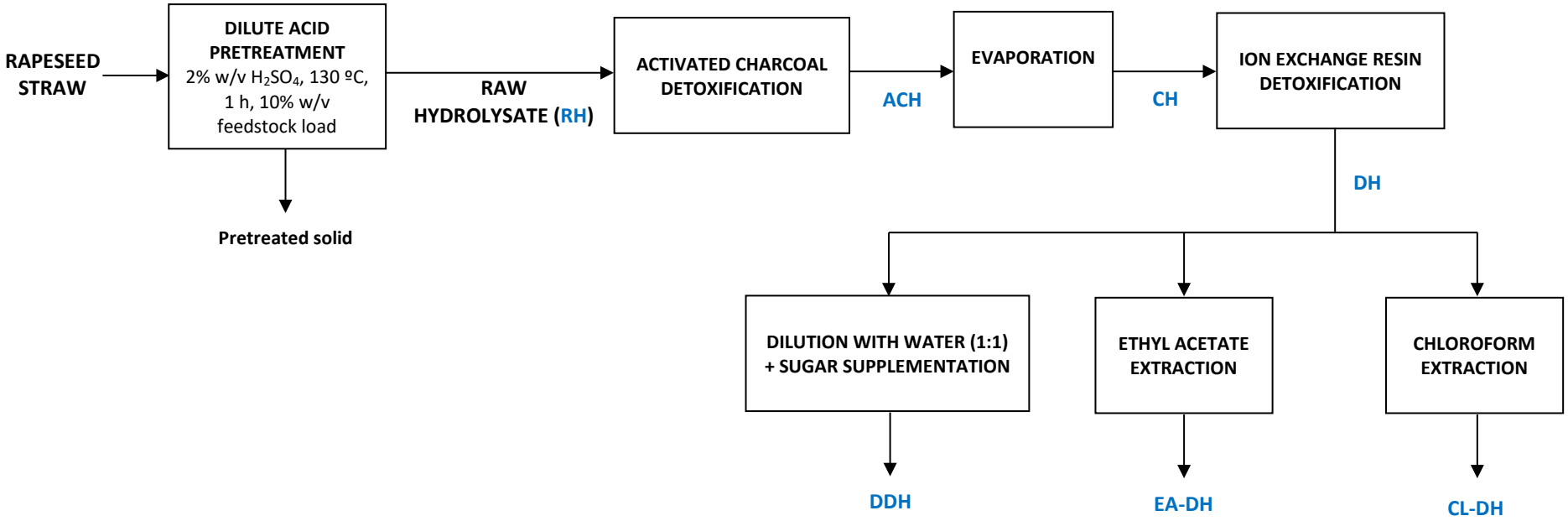
RAPESEED STRAW
DILUTE ACID PRETREATMENT
2% w/v H_2SO_4, 130 ºC, 1 h, 10% w/v feedstock load
Pretreated solid
RAW HYDROLYSATE (RH)
ACTIVATED CHARCOAL DETOXIFICATION
ACH
EVAPORATION
CH
ION EXCHANGE RESIN DETOXIFICATION
DH
DILUTION WITH WATER (1:1) + SUGAR SUPPLEMENTATION
DDH
ETHYL ACETATE EXTRACTION
EA-DH
CHLOROFORM EXTRACTION
CL-DH

Figure 1

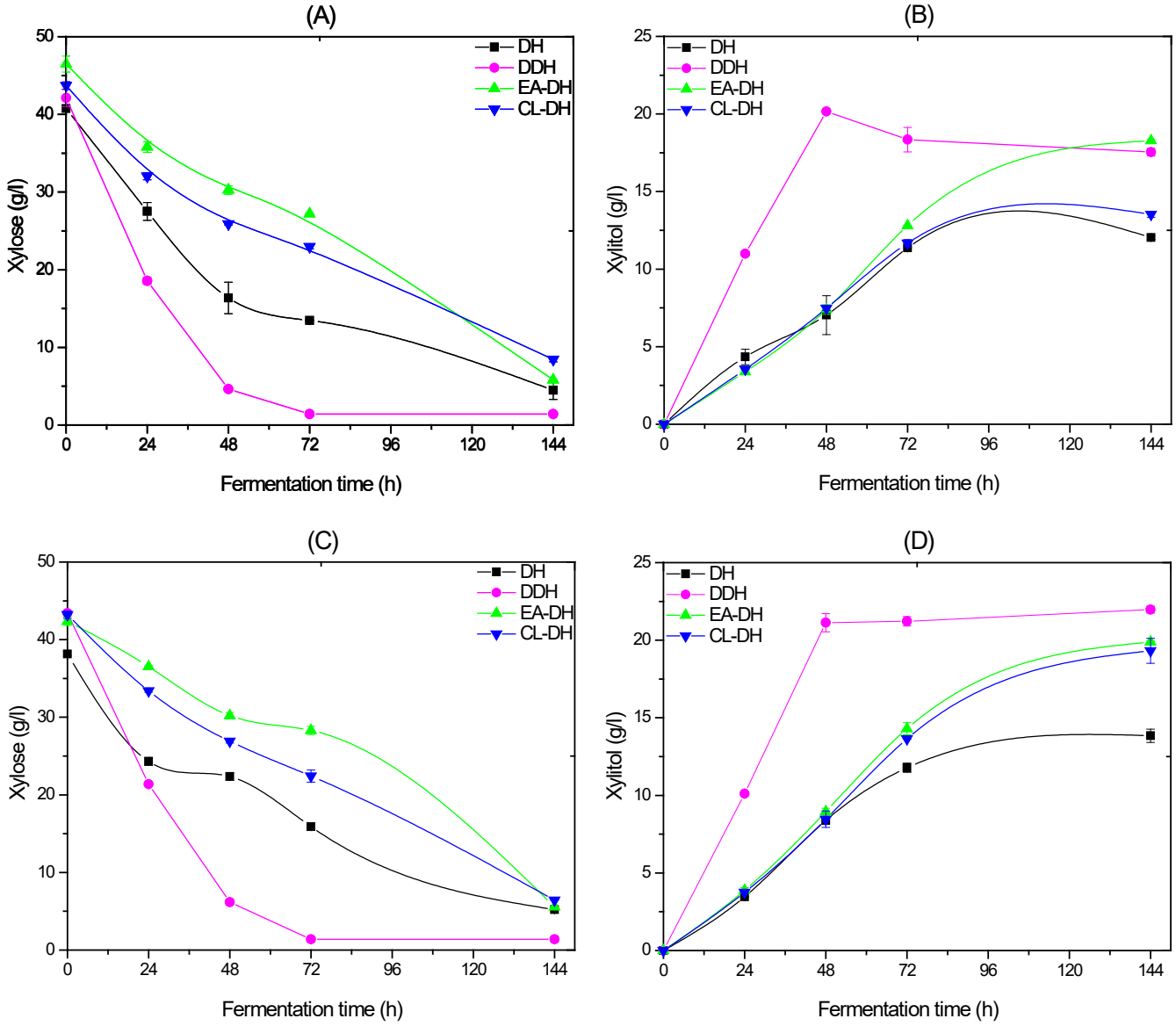
(A)
Xylose (g/l)
Fermentation time (h)
DH
DDH
EA-DH
CL-DH
(B)
Xylitol (g/l)
Fermentation time (h)
DH
DDH
EA-DH
CL-DH
(C)
Xylose (g/l)
Fermentation time (h)
DH
DDH
EA-DH
CL-DH
(D)
Xylitol (g/l)
Fermentation time (h)
DH
DDH
EA-DH
CL-DH

Figure 2

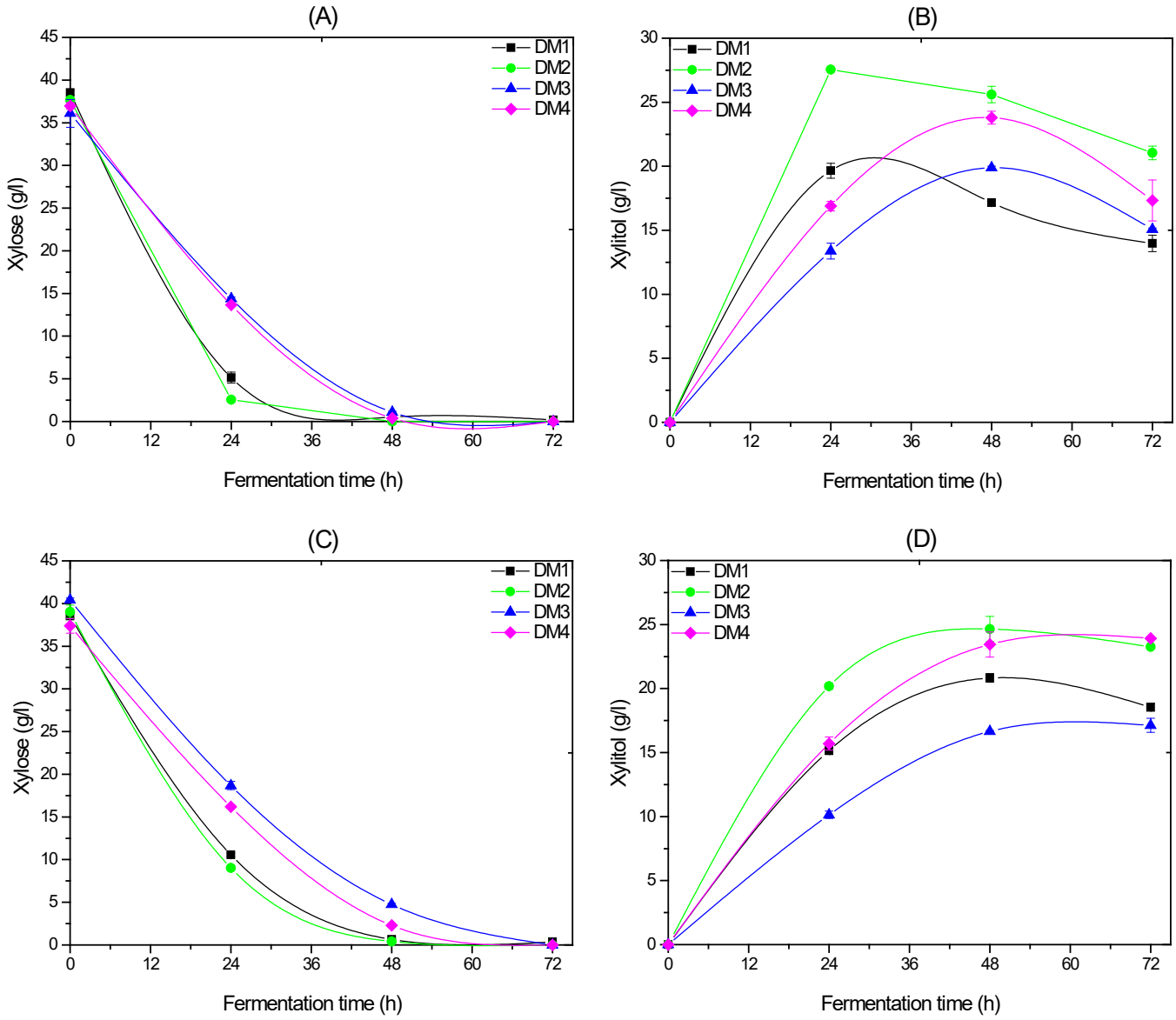
(A)
Xylose (g/l)
Fermentation time (h)
DM1
DM2
DM3
DM4
(B)
Xylitol (g/l)
Fermentation time (h)
DM1
DM2
DM3
DM4
(C)
Xylose (g/l)
Fermentation time (h)
DM1
DM2
DM3
DM4
(D)
Xylitol (g/l)
Fermentation time (h)
DM1
DM2
DM3
DM4

Figure 3