

# Production of Xylitol by the Thermotolerant *Kluyveromyces marxianus* IMB Strains

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## Abstract

Xylitol is a fermentation product of xylose that can be used as a sweetener by diabetics. Five novel *Kluyveromyces marxianus* yeast strains, IMB1, IMB2, IMB3, IMB4 and IMB5, have shown the ability to grow on xylose and produce xylitol. In comparison to chemical synthetic xylitol production, biological production can be more cost effective since no expensive metal catalysts and high temperatures are needed. The five *K. marxianus* IMB strains were tested at temperatures ranging from 25°C to 45°C with xylose as the sole carbon source. IMB2 was found to be the best xylitol producer among the IMB yeast. Product yields ( $Y_{P/S}$ ) up to 0.90 g/g at 40°C with 1.0 g/l initial cell mass and 50 g/l initial xylose concentration were observed for IMB2.

## Keywords

Bioproducts; Sweeteners; Xylose; Fermentation

## Introduction

Xylitol is a by-product of xylose fermentation and is a sugar alcohol [1]. Xylitol contains 40% fewer calories than sucrose on a mass basis and has similar sweetness [2]. It is odorless and has no aftertaste. Researchers have found that xylitol prevents dental cavities [3] and has an anti-ketonic [4] and an anti-infection effect [5]. Xylitol is produced by catalytic hydrogenation of xylose [6], which is costly and needs harsh reaction conditions. Biological xylitol production could be more cost effective, since no expensive catalysts are needed and the reaction takes place at ambient temperatures. Yeasts are well-known for their potential in industrial applications. *Candida mogii* was found to produce xylitol with a high yield ( $Y_{P/S} = 0.62$ ) [7]. The researchers observed that initial xylose concentration, co-factor regeneration and oxygen transfer were key factors for xylitol production [7]. *Debaryomyces hansenii* was observed to produce xylitol with product yields from 0.76 g/g to 0.78 g/g at 30°C and 200 rpm using Erlenmeyer flasks [8-10]. In a study using corn cob hemicellulose hydrolyzate, xylitol yields from xylose up to 0.73 g/g were observed using *Candida tropicalis* W103 at 35 °C [11].

In this study five *K. marxianus* strains, IMB1, IMB2, IMB3, IMB4, and IMB5, were investigated for their ability to produce xylitol from xylose. The yeast were isolated from an Indian distillery and showed the ability to grow at high temperatures using glucose (52°C) and ferment xylose as a carbon source at temperatures up to 45°C [12,13]. The thermotolerant nature of the IMB yeasts allows them to be used in tropical locations while reducing the need for fermenter cooling systems. The optimum growth rates of IMB strains were found at 40°C and a value of 0.93 h<sup>-1</sup> with glucose as substrate [13]. Wilkins et al. carried out tests by using *K. marxianus* IMB2, IMB4, and IMB5 with different pH values and different temperatures under anaerobic conditions [14]. IMB5 had the greatest xylitol production at 40°C and an initial pH of 4.5 with a value of 1.33 g/l and a product yield of 0.25 g/g [14].

The enzymes necessary for xylose fermentation, xylose reductase (XR) and xylitol dehydrogenase (XD) must be activated by the presence of xylose. XR and XD are not available during glucose fermentation [15]. Xylose is reduced to xylitol, catalyzed by XR, and further oxidized to xylulose, catalyzed by XD. Xylulose enters the pentose phosphate cycle and is eventually converted to ethanol [16-18]. Some yeast tend

to produce mainly xylitol and others ethanol. This is caused by different co-factor regeneration systems. It is known that yeast like *Pichia stipitis*, which produce ethanol, can use NADH as a co-factor for XR; whereas, xylitol producers primarily use NADPH [19]. XD uses NAD<sup>+</sup> as a co-factor in all xylose fermenting yeast [19]. In ethanol producing yeast, a regeneration cycle for NADH is established and xylulose production is favored. In xylitol producing yeast, NADPH is regenerated with the subsequent pentose phosphate cycle, but NADH accumulates and XD is inhibited, resulting in xylitol accumulation. This process only occurs under anaerobic or microaerobic conditions since NAD<sup>+</sup> is regenerated by oxygen [19].

In this study all five *K. marxianus* IMB yeast strains were screened at 25, 30, 35, 40, and 45°C at microaerobic conditions to ascertain their ability to produce xylitol using xylose as the sole carbon source. Growth rates, xylitol and ethanol concentrations, conversion efficiencies, as well as product and biomass yields were determined. Also, XR and XD activities at 40°C for all IMB strains were analyzed. In addition, the effect of initial substrate concentration on fermentation was analyzed.

## Materials and Methods

### Organisms

The organisms used were five *K. marxianus* yeast strains called IMB1, IMB2, IMB3, IMB4, and IMB5. These yeast were first discovered in an Indian distillery and are maintained at the University of Ulster [13]. Cultures were stored on slants of solid media in a refrigerator. The slant media contained 3 g/l yeast extract, 3 g/l malt extract, 5 g/l peptone, 20 g/l xylose, and 20 g/l agar.

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## Preparation of seed culture

Prior to use all glassware, stoppers, and other equipment used in direct contact with the organisms were sterilized by autoclaving at 121°C for 30 min. Cell transfer and sample collection were done in a biological safety cabinet (Coy, Grasslake, MI, USA). A seed culture was prepared in media containing 10 g/l yeast extract, 20 g/l peptone and 20 g/l xylose (YPX media). Sterilization of media was achieved by using a filter assembly with a 0.45 µm filter. A loopful of cells for each *K. marxianus* IMB strain was added to 100 ml of YPX media in 250 ml baffled flasks. The flasks were closed with a stopper containing a filter to allow air exchange and prevent microbial contamination (Bug Stopper, Whatman Inc., Florham Park, NJ, USA). The seed culture was incubated for 18 h at 45°C. Aeration was achieved by shaking at 220 rpm by using an incubator with an orbital shaker (MaxQ Mini 4450, Barnstead International, Dubuque, IA, USA).

## IMB yeast screening

The inoculum media contained 10 g/l yeast extract, 20 g/l peptone, and 20 g/l glucose. The media was sterilized with a filter assembly using a 0.45 µm filter. Baffled flasks (250ml) were filled with 100 ml of inoculum media, inoculated with 1ml of seed culture containing one of the *K. marxianus* strains and closed with the stopper previously described. Conditions were the same as for the seed culture (45°C, 18 hours, 220 rpm). To maintain pH during fermentation, a 50 mM sodium citrate buffer at pH 5.5 was used to dissolve the media components. The fermentation media contained 0.50 g/l yeast extract, 2.0 g/l  $\text{KH}_2\text{PO}_4$ , 1.0 g/l  $(\text{NH}_4)_2\text{SO}_4$ , 1.0 g/l  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.10g/l  $\text{MnSO}_4$ , and 20.0 g/l xylose [12].

After 18 h the optical density (OD) of the inocula at a wave length of 660 nm was determined using a UV visible spectrophotometer (Varian, Palo Alto, CA, USA). An initial OD of 0.5 (representing 0.14 g dry cells/l) was set for each fermentation culture by mixing centrifuged (5 min, 4°C, and 6,100 g) (Sorvall Legend RT, Thermo Scientific, Waltham, MA, USA), DI water washed cells from the inocula with 10 ml DI water. Ten ml inoculum was added to 90 ml fermentation media [20]. The flasks were enclosed with the previously described stoppers. Growth conditions were 25, 30, 35, 40 or 45°C with shaking at 100 rpm. Constant temperature and shaking was achieved by using the incubator/shaker. According to Yablochkova et al. [19], shaking at 100 rpm on an orbital shaker like the one used in this study resulted in an oxygen transfer rate of 5 mmol/(l\*h) and led to a microaerobic environment for ethanol and xylitol production from xylose. For the IMB screening experiment, samples were taken at 0, 1, 2, 3, 4, 5, 6, 24, 48, 72, 96, 120, and 144 h.

OD was measured via the UV-Vis spectrophotometer and samples were filtered through a 0.2 µm filter from Fisher Scientific (Pittsburgh, PA., USA). The permeate was used for high performance liquid chromatography (HPLC) analysis, which is described below. Every IMB strain was tested twice at each temperature.

## Effect of initial cell mass concentration on xylitol production

To investigate the effect of different initial cell mass concentrations, a test with IMB2 at 40°C and high xylose concentration was performed. The inoculum was prepared the same as the inoculum for the screening experiment except the incubation temperature was 40°C and xylose was the carbohydrate source. Xylose was used since it was found that xylitol production was better for cells produced from medium containing

xylose as opposed to cells produced from medium containing glucose (data not shown). The fermentation media was prepared as the fermentation media for the screening test containing a xylose concentration of 50 g/l. The fermentation media was inoculated with cells to achieve 0.5, 1.0, and 1.5 g/l of dry cell mass using the same procedure for cell harvest and wash as described above [20]. Samples were taken at 0, 6, 12, 24, 48, 72, 96, 120 and 144 hours and analyzed via HPLC as described later.

## HPLC analysis

Concentrations of xylose, xylitol, ethanol, acetic acid, and glycerol were analyzed on a HPX-87H column (Bio-Rad, Sunnyvale, CA, USA). The eluent was 0.01 N  $\text{H}_2\text{SO}_4$  with a flow rate of 0.6 ml/min at 60°C, and a refractive index detector (1100 Series, Agilent, Santa Clara, CA, USA) was used for detection [21].

## Assay for XR and XD Activity

The procedure was modified from the one described by Smiley and Bolen [15]. All IMB yeast strains were grown in YPX medium containing 10 g/l yeast extract, 20 g/l peptone, and 20 g/l xylose for 18 h at 40°C and 220 rpm. The cells were centrifuged at 4°C and 6,100 g for 10 min. After the liquid was discarded, the cells were washed with DI water and centrifuged again under the same conditions. The cells were re-suspended with 1 ml 0.1 M potassium phosphate buffer per gram cell pellet. The cells were stored until usage at -18°C. After the cells were thawed, 0.3 ml of 0.1 M 2-mercaptoethanol per ml cell suspension was added. A cell disruption was achieved via sonication (Misonix Sonicator 3000, Misonix Incorporated, Farmingdale, NY, USA) for 35 min in 10 sec pulses with 10 sec intervals on level 5. The mass was centrifuged at 16,060 g for 20 min (AccuSpin Micro, Fisher Scientific, Pittsburgh, PA, USA). The following solutions were added into a 1 ml spectrophotometer cuvette: 0.05 ml of 1.0 M potassium phosphate buffer pH 7.4, 0.1 ml of 0.1 M 2-mercaptoethanol, 0.1 ml of 0.5 M D-xylose, 0.1 ml of cell free extract, and 0.55 ml DI water. At time zero 0.1 ml of 3.4 mM NADH was added. The absorption was measured in a spectrophotometer (Varian, Palo Alto, CA, USA) at 340 nm for 6 min. The same procedure was repeated with NADPH as cofactor. NADH and NADPH were purchased from Sigma-Aldrich (St. Louis, MO, USA) and EMD Chemicals (Gibbstown, NJ, USA), respectively.

## Statistical analysis

Two level factorial tests were performed for both the yeast screening and effect of carbohydrate sources tests. Yeast strain and temperature were the independent variables and xylitol concentration was the dependent variable. An analysis of variance was calculated using SAS Release 9.1 (SAS, Carey, NC) and means were separated for each variable by Fisher's protected least significant difference analysis at 95% confidence level.

## Results and Discussion

### IMB Yeast screening

Table 1 presents the product and substrate yields, maximum xylitol concentrations, conversion efficiencies, and growth rates for each strain at each temperature. Table 2 summarizes the mean xylitol concentration for 72 to 144 h, including p-values for strain, temperature, and the interaction of strain and temperature. Values with the same letter are not significantly different ( $p > 0.05$ ). The growth rates differed among temperatures. In general the growth rate was highest at 40°C. At 40°C

Temp. [°C]	IMB	YP/S (g xylitol/g xylose)	YP/X (g xylitol/g cells)	CE [%]	Maximum Xylitol Concentration	Growth rate [h <sup>-1</sup> ]
25	1	0.06	0.18	3.7	0.75	0.08
	2	0.09	0.26	5.8	1.18	0.08
	3	0.03	0.09	2.0	0.41	0.08
	4	0.08	0.20	5.0	1.01	0.07
	5	0.18	0.68	10.3	2.09	0.07
30	1	0.26	1.13	18.6	3.77	0.10
	2	0.15	0.54	10.1	2.05	0.11
	3	0.12	0.45	8.3	1.68	0.11
	4	0.19	0.60	13.3	2.70	0.11
	5	0.17	0.61	11.5	2.33	0.10
35	1	0.26	1.18	24.0	4.86	0.12
	2	0.26	1.18	23.6	4.78	0.13
	3	0.26	1.35	23.5	4.76	0.11
	4	0.23	0.96	20.9	4.24	0.14
	5	0.24	1.24	21.8	4.42	0.12
40	1	0.41	3.37	35.8	7.26	0.15
	2	0.42	3.42	38.8	7.86	0.16
	3	0.34	2.18	26.1	5.29	0.14
	4	0.40	2.06	30.2	6.12	0.20
	5	0.32	2.27	27.3	5.53	0.16
45	1	0.36	3.45	27.4	5.55	0.11
	2	0.42	4.18	38.1	7.72	0.12
	3	0.31	3.21	21.5	4.36	0.08
	4	0.41	3.42	33.3	6.75	0.08
	5	0.29	2.76	21.8	4.42	0.09

**Table 1:** Xylitol yields from xylose and cells (YP/S, YP/X), conversion efficiency (CE) and growth rates of *K. marxianus* IMB strains at 100 rpm in 250 ml baffled flasks.

Time (h)		72	96	120	144
Strain	IMB1	2.63 <sup>AB</sup> ± 0.47	3.74 <sup>A</sup> ± 0.63	4.01 <sup>A</sup> ± 0.60	3.37 <sup>A</sup> ± 0.48
	IMB2	2.85 <sup>A</sup> ± 0.59	3.90 <sup>A</sup> ± 0.77	4.10 <sup>A</sup> ± 0.75	3.50 <sup>A</sup> ± 0.61
	IMB3	2.32 <sup>C</sup> ± 0.47	2.74 <sup>C</sup> ± 0.51	2.58 <sup>C</sup> ± 0.49	2.07 <sup>C</sup> ± 0.51
	IMB4	2.50 <sup>B</sup> ± 0.52	3.23 <sup>B</sup> ± 0.57	3.35 <sup>B</sup> ± 0.53	2.86 <sup>B</sup> ± 0.55
	IMB5	2.75 <sup>AB</sup> ± 0.43	3.17 <sup>B</sup> ± 0.42	3.07 <sup>B</sup> ± 0.31	2.63 <sup>B</sup> ± 0.35
Temp (°C)	25	0.73 <sup>A</sup> ± 0.14	0.83 <sup>A</sup> ± 0.12	0.99 <sup>A</sup> ± 0.14	1.09 <sup>A</sup> ± 0.17
	30	1.42 <sup>B</sup> ± 0.10	2.04 <sup>B</sup> ± 0.17	2.40 <sup>B</sup> ± 0.22	2.36 <sup>B</sup> ± 0.23
	35	3.03 <sup>C</sup> ± 0.14	4.31 <sup>C</sup> ± 0.16	4.57 <sup>C</sup> ± 0.16	3.74 <sup>C</sup> ± 0.23
	40	5.71 <sup>E</sup> ± 0.12	6.24 <sup>E</sup> ± 0.40	4.73 <sup>C</sup> ± 0.66	2.42 <sup>B</sup> ± 0.72
	45	3.48 <sup>D</sup> ± 0.32	4.80 <sup>D</sup> ± 0.44	5.57 <sup>D</sup> ± 0.45	5.55 <sup>D</sup> ± 0.37
P values	Strain	0.0001	<.0001	<.0001	<.0001
	Temp	<.0001	<.0001	<.0001	<.0001
	Str. +Temp.	<.0001	<.0001	<.0001	<.0001

**Table 2:** Mean values with standard error of xylitol concentration [g/l] during *K. marxianus* IMB strains screening. Factor strain shows the mean values over all temperatures and factor temperature shows the mean values over all strains. Values with the same letter for each column were not significantly different ( $\alpha = 0.05$ ).

the highest growth rate of 0.2 h<sup>-1</sup> was achieved with IMB4 Table 1. Banat and Marchant [12], who first investigated the IMB strains, found growth rates on xylose at 45°C between 0.18 h<sup>-1</sup> and 0.26 h<sup>-1</sup> for IMB5 and IMB4, respectively. The higher growth rates in their study can be explained by the difference in aeration. In this study, microaerobic conditions were used; whereas, Banat and Marchant [12] determined growth rates for aerobic conditions. Microaerobic condition is defined by aeration with oxygen concentrations below the oxygen concentration of air. Yablochkova et al. [19] determined an oxygen transfer rate of 5 mmol O<sub>2</sub>/(l\*h) as microaerobic conditions leading to optimum xylose

fermentation. Low growth rates are favorable, since more of the substrate can be used for product formation.

Xylose consumption varied with temperature as well. At 40°C all strains consumed almost all xylose; whereas, at all other temperatures xylose remained after 144 h. The highest xylose concentrations remained in the media at 25°C with values ranging from 8.2 g/l with IMB5 to 6.6 g/l with IMB3 (data not shown). Similar characteristics were shown in a previous study that was conducted with IMB2, IMB4, and IMB5 [14]. According to the figures shown in their work, IMB4 did not utilize xylose completely at 45°C with a value of about 10 g/l after 120 h. In that study it was concluded that IMB4 produced the highest ethanol concentration. However, that study was under anaerobic conditions; whereas, this study was conducted under microaerobic conditions [14].

The IMB yeast showed a greater capability for xylitol production in comparison to ethanol production. Ethanol production was negligible. Temperature has a strong influence on xylitol production with the highest mean concentration at 40°C (6.24 g/l after 96 h) and the lowest at 25°C (1.09 g/l after 144 h) Table 2. This is in alignment with the higher xylose utilization at 40°C. The results indicate the thermotolerant nature of the *K. marxianus* IMB yeast, which was, to the authors' knowledge, not found in any other publication on xylitol producing yeast. Yeast strain also affected xylitol production. IMB2 had the highest mean maximum xylitol concentration of all strains with the same value at 40 and 45°C (4.1 g/l), but it was not significantly different than IMB1. The lowest production was achieved by IMB3 with a mean value of 2.74 g/l Table 2.

The greatest xylitol yield from xylose ( $Y_{P/S}$ ) and initial cell mass ( $Y_{P/X}$ ) was achieved by IMB2 with 0.42 g xylitol/g xylose and 4.18 g xylitol/g cells at 45°C Table 1. The highest conversion efficiency (maximum xylitol concentration/theoretical xylitol concentration) was 38.1%. In general, the yeast with the greatest YP/S also had the greatest YP/X. The same was true for the lowest YP/S and YP/X with the exception of 40°C, where the lowest YP/S and YP/X was shown by IMB5 and IMB4, respectively. It should be noted that the xylitol concentration for all IMB strains at 40°C decreased rapidly after reaching the maximum concentration (data not shown). At all other temperatures, the maximum xylitol concentrations were observed at the end of fermentation. Within xylose metabolism xylitol is an intermediate that can be further converted to ethanol. Indeed, ethanol concentration increased while xylitol concentration decreased after 96 h at 40°C (data not shown). However, ethanol production was very low with the greatest observed concentration of 1.64 g/l by IMB1 at 40°C after 144 h. It can be assumed that the decrease in xylitol concentration is caused by the usage of xylitol for other metabolites and/or cell maintenance.

### Effect of initial cell mass concentration on xylitol production

During the test with three different initial cell mass concentrations, similar cell mass concentrations were observed after 120 h, ranging between 2.22 and 2.45 g/l for 1.5 g/l and 0.5 g/l, respectively. Cultures initially containing 0.5 g/l and 1.5 g/l cell mass showed an increase in cell mass during the last 24 h (between 120 and 144 h) to 3.68 and 2.95 g/l, respectively Figure 1. The cultures initially containing 1.0 g/l cell mass did not grow in the last 24 h. It can be assumed, that during the 120 h sampling, the 0.5 g/l and 1.5 g/l cultures were introduced to higher oxygen concentration, which led to elevated growth rates.

The cultures with 0.5 and 1.5 g/l initial cell mass concentration consumed the most xylose after 144 h with a final xylose concentration of

4.90 and 6.28 g/l, respectively Figure 1A. Cultures initially containing 1.0 g/l cell mass had a final xylose concentration of 15.3 g/l.

The highest xylitol concentration for all tests was achieved after 144 h. Cultures initially containing 0.5 g/l cell mass produced the highest xylitol concentration with 34.6 g/l Figure 1B, which represents an YP/S of 0.76 g/g Tables 4 and 5. Cultures initially containing 1.0 g/l cell mass produced a lower concentration with 29.9 g/l of xylitol, but had a product yield of 0.86 g/g since less xylose was used. Cultures initially containing 1.5 g/l cell mass produced a xylitol concentration of 30.6 g/l and a product yield of 0.70 g/g. There was no significant dif-

ference between the three initial cell mass concentrations in terms of 144 h xylitol concentration ( $p>0.05$ ). Comparing YP/S values at 120 h, which excludes the great increase in cell mass in the last 24 h, shows that cultures with 1.0 g/l initial cell mass had the highest yield with 0.90 g/g compared to 0.80 and 0.76 g/g with cultures with 0.5 g/l and 1.5 g/l initial cell mass, respectively. Xylitol concentrations after 120 h were different among the initial cell mass concentrations Table 3. The greatest mean xylitol concentration produced was 29.76 g/l by cultures with 0.5 g/l initial cell mass.

Cultures with 1.0 g/l initial cell mass still had the potential to produce more xylitol after 144 h, since 18 g/l of xylose remained in the media. Comparing the YP/X values, 0.5 g/l initial cell mass showed the highest yield of 71.16 g xylitol/g initial dry cell mass after 144 h Table 4. Cultures initially containing 1.0 g/l and 1.5 g/l cell mass have lower yields since less xylitol was produced with higher initial cell mass concentrations. The yields are 30.20 and 20.64 g/g for 1.0 g/l and 1.5 g/l, respectively Table 4.

Initial substrate and cell mass concentrations have an effect on the xylitol production of the IMB yeast. The yields of all three tests with elevated initial cell mass concentrations are higher than the yields achieved during the IMB screening and the inoculum carbohydrate test. Compared to the literature, the yields achieved with IMB2 at 40°C with 50 g/l initial substrate concentration and 1.0 g/l initial dry cell mass concentration (0.86 g/g) are the highest with natural occurring yeast we have found (e.g. *D. hansenii* with 0.78 g/g) [8], [10]. However, the maximum observed productivity of 0.24 g/L/h is much lower than observed for *C. tropicalis* W103, which was 1.07 g/L/h [11]. Increased productivity is necessary for utilizing *K. marxianus* IMB2 for industrial xylitol production.

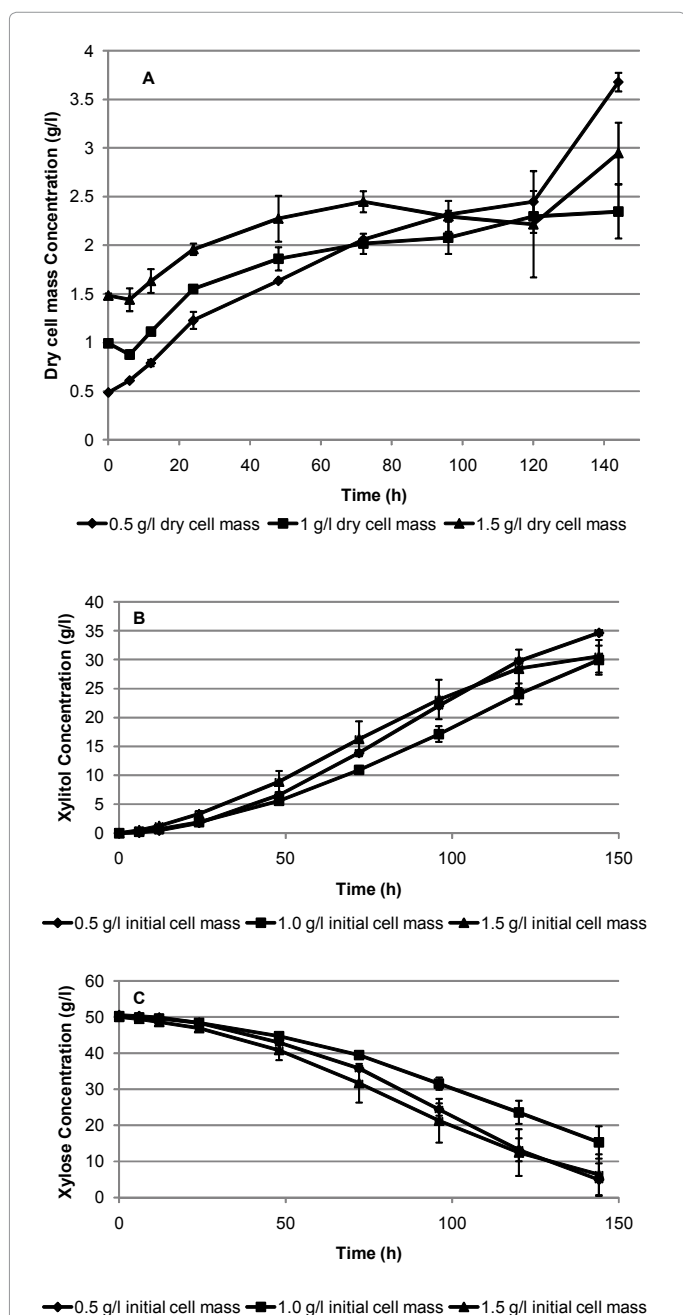


Figure 1: Dry cell mass concentration (A), xylitol production (B) and xylose consumption (C) by *K. marxianus* IMB2 at 40°C three different initial cell mass concentrations (0.5, 1.0, 1.5 g/l). Error bars represent standard error.

Time (h)		120	144
Initial cell mass concentration [g/l]	0.5	29.76 <sup>A</sup> ± 0.19	34.64 ± 0.20
	1.0	24.09 <sup>B</sup> ± 1.04	29.92 ± 1.44
	1.5	28.43 <sup>AB</sup> ± 1.92	30.59 ± 1.62
P value	Cell mass	0.0439	0.0745

Table 3: Mean values and standard errors for xylitol concentration after 120 and 144 hours with different initial cell mass concentrations ( $\alpha = 0.05$ ). Values with different letters are significant different (t-test (LSD)).

Time [h]	Cell mass [g/l]	YP/S (g xylitol/g xylose)	YP/X (g xylitol/g initial cells)	CE [%]	Maximum Xylitol Concentration [g/l]
120	0.5	0.80	61.15	58.4	29.76
	1.0	0.90	24.32	47.5	24.09
	1.5	0.76	19.19	56.3	28.43
144	0.5	0.76	71.16	67.9	34.64
	1.0	0.86	30.20	58.9	29.92
	1.5	0.70	20.64	60.6	30.59

Table 4: Xylitol yields from xylose and cells (YP/S, YP/X), conversion efficiency (CE) and growth rates of IMB2 with different initial cell mass concentrations (Test parameters: 40°C, 100 rpm, and 50 g/l initial xylose concentration).

Yeast	XR activity [IU/ml]	
	NADH	NADPH
IMB1	n.d.	2.85
IMB2	n.d.	2.68
IMB3	n.d.	0.51
IMB4	n.d.	0.26
IMB5	n.d.	2.91

Table 5: XR activity of IMB yeast strains at 40°C (n.d. = non-detectable). The units are in IU/ml ( $\mu\text{mol}$  of cofactor oxidized / (ml cell free extract \* min)).

## Determination of XR Activity

To understand why the IMB yeast produce high amounts of xylitol and low amounts of ethanol, the XR activity of all IMB strains was tested with NADH and NADPH as cofactors. Table 5 lists the activities of XR in IU/ml cell free extract. The resulting XR activities using NADH versus NADPH support the proposed cofactor imbalance being responsible for high xylitol and low ethanol yields. No activity was detected using NADH as cofactor for all IMB strains. By using NADPH the activity ranged from 0.26 to 2.91 IU/ml. No correlation between XR activities and maximum xylitol concentrations or product yields was established. However, IMB1 and IMB2, which were observed to produce the greatest xylitol concentrations during the yeast screening, had two of the higher activities observed.

NADPH cannot be regenerated with the second step and an imbalance of cofactors is caused by microaerobic conditions in the yeast. This confirms the study by Yablochkova et al. (2004) [20] which presented that *K. marxianus* has a low xylose reductase activity while using NADH as a cofactor. The researchers reported <0.01 and 0.16  $\mu\text{mol}/(\text{mg}\cdot\text{min})$  as the activities for XR with NADH and NADPH, respectively [20]. It should be noted that their assay was conducted at 30°C, while ours was conducted at 40°C.

## Conclusion

The results indicate that IMB yeast have potential for biological xylitol production. IMB1 and IMB2 were the best xylitol producers ( $p < 0.05$ ). Xylitol production until 96h was greatest at 40°C, and from 96 to 144 h it was greatest at 45°C. The greatest xylitol yield achieved from xylose was 0.90 g/g with IMB2 at 40°C using 1.0 g/l initial cell mass and 50 g/l initial xylose concentration. Based on the present study, *K. marxianus* IMB2 was the most promising strain in this study for biological xylitol production with the following advantages: high product yields, no genetic modification, and a thermotolerant nature; however, productivity must be improved.

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