# PRODUCTION OF XYLOSE REDUCTASE AND XYLITOL BY *Candida guilliermondii* USING WHEAT STRAW HYDROLYSATES

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**ABSTRACT**

**The objective of this study is to evaluate the production of Xylose reductase (XR) and Xylitol by *Candida guilliermondii* using wheat straw hydrolysates (WSH) supplemented with 2.0 g/l of (NH4)2SO4 and 0.1 g/l of CaCl2.2H2O as fermentation media . Wheat straw hydrolysis run at 121°C for 15 min. by diluted sulfuric acid . The fermentation process was conducted on a shaker bath (150 rpm) at 30C for 20 h in three separate flasks using different concentration of Xylose being , 30% Xylose , WSH(27.13 g/l Xylose)and WSH plus 30 g/l Xylose. The best concentration( WSH plus 30 g/l Xylose ) was chosen to run the fermentation process for XR production at different incubation temperature ( 20, 25, 30, 35, 40, 45 C, different pH values (5, 5.5, 6, 6.5 and 7) and different fermentation period (5 , 10 , 15,20 ,25 ,30 h ). The results indicated that the optimum condition for XR production was using 30%Xylose plus WSH at pH 6 over 20h incubation at 30 C. The crude extract of Xylose reductase was used to reduce Xylose into Xylitol with simultaneous oxidation of NADPH. The crude extract of XR was able to convert about 90 % 0f the Xylose to Xylitol through 24 h. incubation at 30°C.According to these findings WSH can be used as a promising source for Xylose to produce Xylose reductase enzyme by *Candida guilliermondii* and the crude extract could be used successfully in conversion of Xylose to Xylitol.**

**Keywords: Wheat waste hydrolysates, fermentation , lignocelluloses, xylose , NADPH.**

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## أنتاج الزايلوز ردوكتيز و الزايليتول باستعمال خميرة *Candida guilliermondii* من متحلل قش القمح

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**المستخلص**

**هدفت هذه الدراسة إلى تقييم إنتاج الزايلوز ردوكتيز و الزايليتول من خلال عمليات التقانة الحيوية باستخدام الخميرة *Candida guilliermondii* ومتحلل قش القمح(WSH) المدعم. أجريت عملية التحلل ألحامضي لقش القمح(WS) باستعمال حامض الكبريتيك المخفف لمدة 15 دقيقة وعلى 121 درجة مئوية وتم استخدامه كوسط تخمير بعد تدعيمه بأملاح كبريتات الامونيوم (2 g/L) و كلوريد الكالسيوم (0.1 g/L). أجريت عملية التخمير لإنتاج الزايلوز ردوكتيز بواسطة خميرة *Candida guilliermondii* باستعمال حمام هزاز عند 150 دورة في الدقيقة وعلى درجة حرارة 30 مئوي ولمدة 20 ساعة وباستعمال تراكيز مختلفة من الزايلوز تمثلت في 30% زايلوز ، ومتحلل قش القمح(27.13 غم/لتر زايلوز) و ومتحلل قش القمح مع 30% زايلوز لغرض تحديد التركيز الأمثل ثم تم استخدام التركيز الأمثل في وسط التخمير وعلى درجات حرارة مختلفة ( 20, 25, 30 , 35 , 40, 45 م) وعند أرقام هيدروجينية مختلفة (5 ، 5.5 ، 6 و 6.5 و 7) ولفترات تخمر مختلفة. تم استخدام متحلل قش القمح الخام لتحويل الزايلوز إلى الزايليتول مع أكسدة متزامنة لـ NADH . أظهرت النتائج أن متحلل قش القمح(WSH) يحتوي على 27.13% زايلوز ، وكان أعلى إنتاج لـXR 61 وحدة/ مل عند استخدام (30 ٪ + متحلل قش القمح(WSH) في وسط التخمير عند درجة حرارة التحضين 30 مئوية ورقم هيدروجيني 6 .وعليه يمكننا القول إن متحلل قش القمح يعد كمصدر واعد لسكر الزايلوز و إنتاج أنزيم الزايلوز ردوكتيز بواسطة *Candida guilliermondii*.**

**الكلمات المفتاحية : متحلل مخلفات القمح، التخمير، ليكنوسليلوز، زايلوز.**

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## INTRODUCTION

Xylose reductase (XR) (EC 1.1.1.21) is one of the main enzymes for Xylitol production, it assist the reduction of D- Xylose to Xylitol with concomitant oxidation of NADPH ( 14 ) . It is not available commercially despite the several studies found in the literature on the potential usage of Xylose reductase as well as there being a description of downstream procedures regarding its separation from the yeasts (6). Xylitol is a sugar alcohol obtained from Xylose with wide range industry application in food and pharmaceutical industries (2 , 4 , 10). Besides a chemical process which is industrially applied, Xylitol can be produced by a biotechnological means in which a strain of *Candida guilliermondii* is used. The yeast, as any pentose-fermenting or pentose-utilizing microorganism , has the Xylose reductase in the cytoplasm, which catalyses the conversion of Xylose into Xylitol(2). Consequently, the enzyme must be obtained by cultivating the yeast *Candida guilliermondii*, which is reported to be one of the most efficient Xylitol producers (9) through an aerated batch process, (16). This intracellular enzyme occurs in the cytoplasm of Xylose-assimilating microbes, where it mediates the first step of Xylose metabolism by reducing Xylose to Xylitol(2 , 17) The use of high-priced commercial Xylose limits the large-scale production of XR as well as its industrial application for manufacturing Xylitol and other value-added bio products (3 , 14) . Xylose reductase has a molecular weight (MW) of 36 KDa and requires NADPH as cofactor (8). Its use, either in a continuous or a discontinuous bioreactor, constitutes an alternative of economic interest regarding both the catalytic hydrogenation of pure Xylose and the fermentation of the Xylose present in lignocelluloses hydrolysates (obtained from agricultural waste hydrolysates, such as sugar cane bagasse, wheat or rice straw, etc.) rich in Xylose (1). Wheat straw is a widely available source for hemicelluloses which can easily hydrolyse by enzyme to release Xylose for Xylitol production. The aim of this study was to evaluate the production of Xylose reductase and Xylitol through microbial processes using the yeast *Candida guilliermondii* and hemicelluloses hydrolysates from wheat straw. *Candida guilliermondii* yeast was chosen because it has the ability to synthesize Xylose reductase enzyme, involved in the process of converting Xylose into Xylitol.

## MATERIALS AND METHODS

**Chemicals**: Reduced nicotinamide adenine dinucleotide phosphate (NADPH), β-mercaptoethanol, and Xylose were purchased from Sigma Chemical Company (St. Louis, MO, USA). All other chemicals were of analytical grade

**Microorganisms**: *Candida guilliermondii* was obtained from Microbiology Laboratory (University of Baghdad - College of Science ). The culture was stored at 4°C after inoculation on Yeast Extract Peptone Dextrose (YEPD) agar slant composed from 10 g L-1 yeast extract; 20 g L-1 peptone; 20 gl-1 dextrose and 20 g L-1 agar) at 4 °C.

**Preparation of wheat straw hydrolysates**: Wheat straw hydrolysates(WSH) was prepared according to ( 2 ). WS (10 g) with total volume ( 100 ml)of diluted sulphuric acid (0.5 %) was mixed in Erlenmeyer flask, placed in autoclave at 121 °C for 15 min. , then the mixture was filtered under vacuum ,pH of the filtrate was adjusted to 6.0 ± 0.2 with NaOH, and re- filtrated to exclude the precipitate. The obtained hydrolysates was detoxified by activated charcoal (3% w/v) under agitation ( 200 rpm) at 30 °C for 2 h. The detoxified hydrolysates were recovered by filtration under vacuum, and sterilized at 121 °C for 15 min in autoclave.

**Inoculums preparation:** A medium containing 3.0 g/l of Xylose supplemented with 20.0 g/l of rice bran extract, 2.0 g/l of (NH4)2SO4 and 0.1 g/l of CaCl2.2H2O was used for the inoculums cultivation. Erlenmeyer flasks (125 mL), each containing 50 mL of medium (initial pH 5.5)and inoculums ( 6 $X10^6$ ), were incubated on a shaker bath (150 rpm) over 24 h at 30 $^oC$.

**Fermentation conditions**: The fermentation process was carried out at three separate flasks , each one contained the fermentation medium that has been described in inoculums preparation section with some modification being (30%Xylose in first flask, WSH in the second one and 30%Xylose + WSH). All these medium were inoculated with $6 \times 10^6$ viable

cells/ml . The fermentation was carried out at 30 $^{o}$C over 24 h using shaker bath (150 rpm).

**Preparation of Crude Enzyme Extract**: Yeast cells were harvested by centrifugation at 6000 rpm at 4°C and washed in phosphate buffer (50 mM, pH 7.2) and the cell pellets were stored at (-4C). For enzyme assays, cell extracts were thawed and disrupted by sonicator (SONICS, USA) using ultrasound waves at 4°C for 20 min. Cell homogenates were centrifuged at 10000 rpm for 15 min. at 4°C and the supernatant was used for enzymatic assays.

**XR Activity Determination:** The enzyme activity was determined spectrophotometrically by UV-Visible (ELICO Double Beam SL-210) spectrophotometer at 340 nm. The final mixture (1 ml) contained 600 µl of 250 mM potassium phosphate buffer (pH 7.0), l00 µl of 100 mM mercaptoethanol, 50 µl of 0.5 M D-Xylose, 100 µl of distilled water, 50 µl of 3.4 mM NADPH and the mixture was kept for 1 min. then 100 µl of enzyme solution were added in order to initiate the reaction(30°C /24 h. pH 6). One unit of XR was defined as amount of enzyme required to produce one µmol of Xylitol in one minute.

**Analytical methods**: Cellulose, hemicelluloses and lignin were determined according to (20) methods. Glucose and Xylose were determined using DNSA according to ( 11 ). Xylose and Xylitol were quantified using a HPLC device (Shimadzu, Kyoto, Japan), equipped with RI detector and ion-exclusion column Amines HPX-87H (Bio-Rad Laboratories, Redmond, WA, USA).

**Statistical analysis**: Data analysis for statistical significance was conducted by one-way analysis of variance (ANOVA) at a significance level of 95%.

## RESULTS AND DISCUSSION

Table 1. illustrate the chemical composition of wheat straw. The percentages of cellulose, hemicelluloses, lignin, and ashes were 35.47 %, 31.97 % 20.53 % and 4.50 % respectively. Sun ( 25 ) mentioned that WS composed of cellulose (34-43%) hemicelluloses (25-35%) and lignin ( 14-21 % ) . Bohden and Yaser ( 5) reported that WS consist of cellulose (30-40 %) , hemicelluloses ( 20 - 25 %) and lignin (15-25 % ). The recent study results are within the same range of above studies. The differences in WS composition could be attributed to variations in geographical location, local temperature, and heterogeneity of feedstock of the WS samples .

**Table 1. Composition of wheat straw(WS).**

| Wheat Straw components | (%) |
|---|---|
| Dry mater | 93.35 |
| Cellulose | 35.46 |
| Hemicelluloses | 31.97 |
| Lignin | 20.53 |
| Ash | 4.43 |

,the chemical analysis of WSH after detoxification shows that the glucose and Xylose amount were 40.68 g $l^{-1}$ and, 27.13 g $l^{-1}$ respectively (Fig. 1).

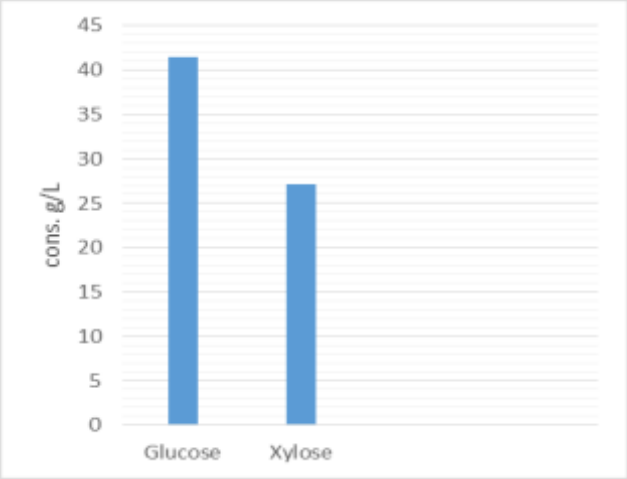



**Figure 1. concentration of glucose and Xylose in wheat straw hydrolysates**

Acid hydrolysis of WS was capable in releasing Xylose and glucose from the hemicelluloses and the cellulose present in the WS, these carbohydrates became available for microbial assimilation and Xylitol production. The suitable detoxification process to improve the fermentation of hemicelluloses hydrolysates depends on the source of hydrolysates. WSH alkalization, during the pH adjustment, also considered as a method of detoxification,. This method advantages are low cost, partial removal of acetic and Phenolic acid compounds, precipitation of toxic metal ions such as Fe, Ni, Cr, and converting furfural to less toxic compounds (such as furfural acid Which can be metabolized by fermenting microorganisms (12). The proper method for detoxification should be efficient to remove the most toxic compounds with a slight loss in fermentable sugars. Additionally, the effective

fermentation should result in a total or partial reduction of the inhibitory compounds with no negative effect on the cellular metabolism of the yeast.

**Effect of Xylose concentration in fermentation medium on XR production**

The effect of the Xylose concentration on Xylose reductase production was investigated in order to determine the optimum substrate concentration (figure 2). It has been noticed that the maximum Xylose reductase productivity( 61 U/ml) was obtained with WSH+ Xylose 30 g/L treatment, considerable fraction of Xylose is consumed. While at low concentration (WSH) the production of Xylose reductase dropped to (41 U/ml). Effect of the initial substrate concentration is particularly relevant in the design of a fermentation strategy, because this factor directly influences Xylose metabolism and Xylose reductase production. Fermentation in a complex medium, such as a WSH, is critical since the hydrolysates contains various substances that interfere with microbial metabolism. These results seem to contradict previous findings reported in Yokoyama *et al.* ( 21 ), where maximum yields of Xylitol obtained on initial Xylose concentration of 50–70 g/L were been 38%–42% respectively.

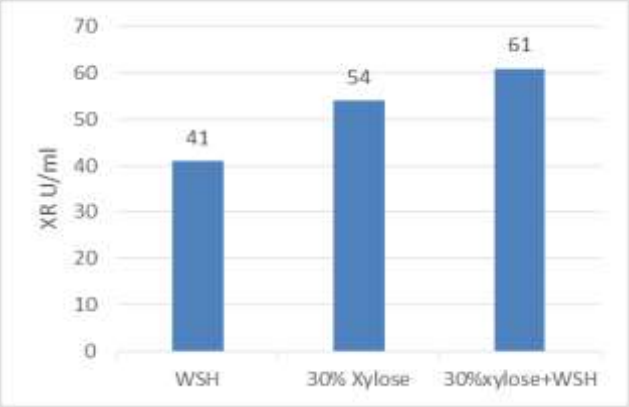


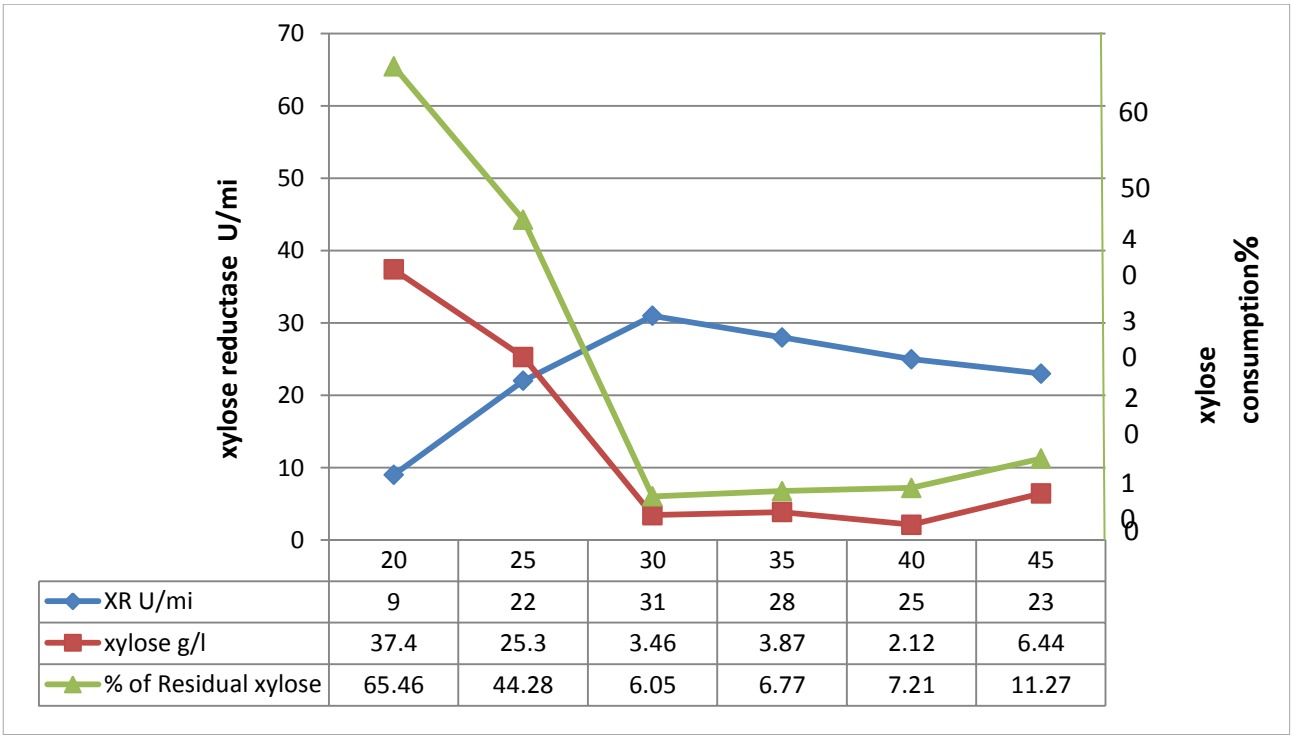

**Figure 2. Effect of Xylose concentration on Xylose reductase activity.**

**Effect of temperature on Xylose reductase production**

Figure 3 shows the effect of incubation temperature (20, 25, 30, 35, 40, 45 °C) on XR production through WSH fermentation by *Candida guilliermondii* , over 20 h . It has been noticed that the highest value for XR units ( 8 , 23 ) appeared at 30 C, and as the temperature rises Xylose consumption increases. Above 25 °C the Xylose residue in the fermentation media is sharply decreased, became increasingly less available for *Candida guilliermondii*. The percentages for Xylose consumption were more than 90% at 30, 35 and 40 C , this coincides with increasing in XR production which reached to 31, 28, 25 U/ml respectively**.** These results mean that the fermentation was temperature-dependent at 30- 40 °C**.** These finding were similar to that reported by ( 16 ) who found that the highest production of Xylitol by Xylose reductase was between 25-35 °C.

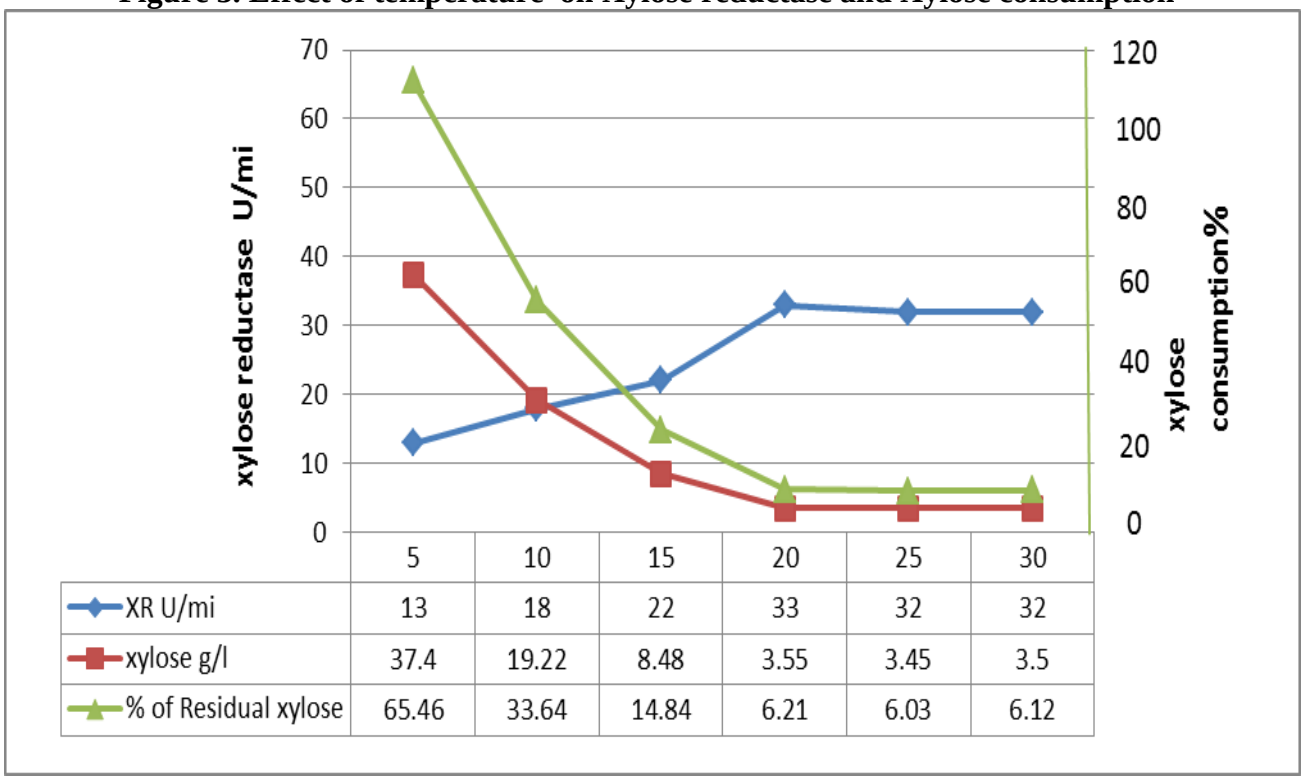
**Effect of fermentation duration on Xylose reductase production**

Fig. 4 , illustrates the effect of fermentation duration on XR production, the maximum values of enzyme units ( 33, 32, 32 U/ml) were achieved by *Candida guilliermondii* after 20, 25, and 30 h) of fermentation at 30 °C. The percentages for Xylose residue in fermentation media were (65.46, 33.64, 14.84, 6.21, 6.03, and 6.12 %) after (5, 10, 15, 20, 25, and 30 h ) respectively .XR U/ml after 5 , 10 , 15 h were 13, 18,and 22 respectively. After 20 h. XR activity in fermentation media reached the highest value (32 U/ml) , and the activity stayed at the same level even after 25 and 30 h fermentation. This could be attributed to NADPH depletion or/and to Xylose availability in the fermentation mixture.




| | 20 | 25 | 30 | 35 | 40 | 45 |
|---|---|---|---|---|---|---|
| XR U/mi | 9 | 22 | 31 | 28 | 25 | 23 |
| xylose g/l | 37.4 | 25.3 | 3.46 | 3.87 | 2.12 | 6.44 |
| % of Residual xylose | 65.46 | 44.28 | 6.05 | 6.77 | 7.21 | 11.27 |

**Figure 3. Effect of temperature on Xylose reductase and Xylose consumption**




| | 5 | 10 | 15 | 20 | 25 | 30 |
|---|---|---|---|---|---|---|
| XR U/mi | 13 | 18 | 22 | 33 | 32 | 32 |
| xylose g/l | 37.4 | 19.22 | 8.48 | 3.55 | 3.45 | 3.5 |
| % of Residual xylose | 65.46 | 33.64 | 14.84 | 6.21 | 6.03 | 6.12 |

**Figure 4. Effect of fermentation duration on Xylose reductase production and Xylose consumption**

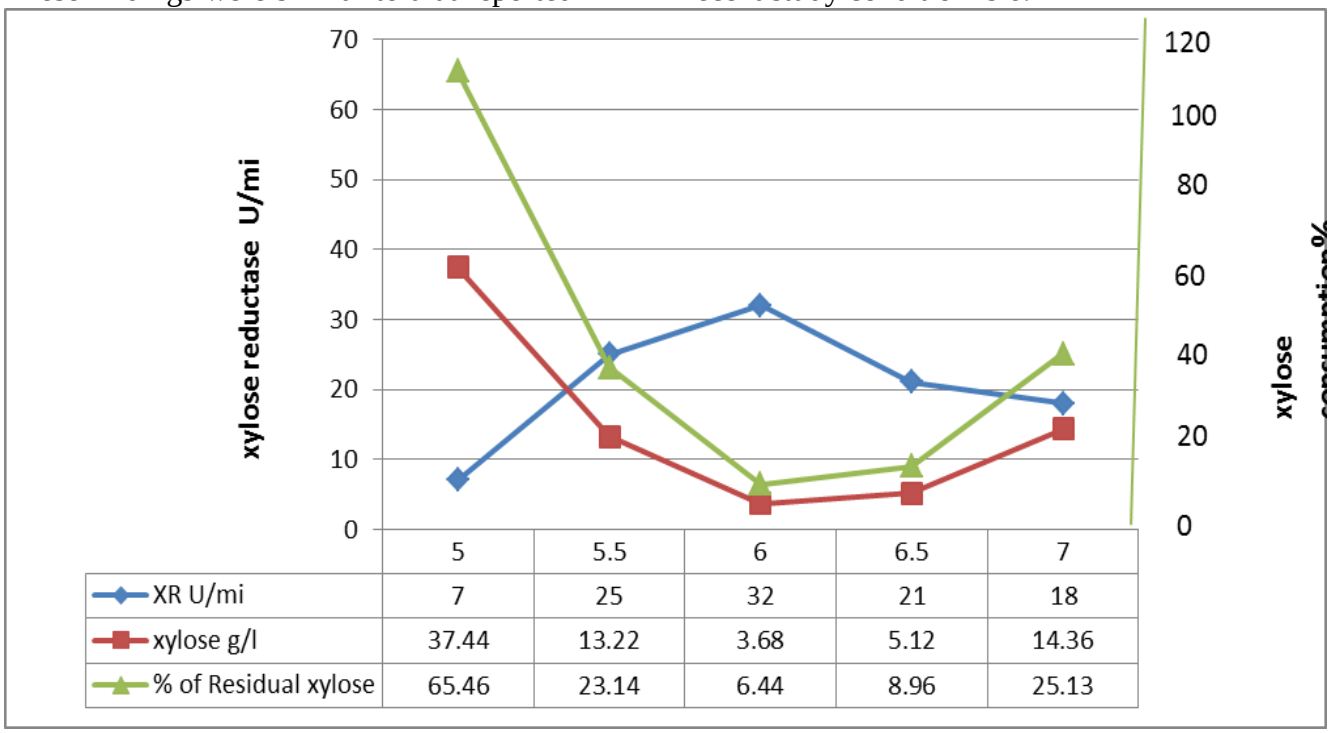
Mushtaq et al. (12) evaluated the production of Xylitol from bean husk for by *Candida guilliermondii*, Xylitol production was 7.5 g/L at 24 h. consumed 80% of substrate (Xylose and glucose) during fermentation only at 27°C and 30°C.

**Effect of pH on Xylose reductase production**

The fermentation process was run in a number of flasks at various pH values ( 5 , 5.5 , 6 , 6.5, 7) at 30 °C to examine the effect of fermentation media pH on Xylose production by *Candida guilliermondii* . In each

experiment, the initial Xylose concentration was 57.13 g/L.

Figure 5 summarizes the percentage of Xylose recovered in each flask at each pH. At pH 6, 6.5 more than 90 % of the total Xylose was consumed while at pH 5.5 , 7 about 75% of Xylose was consumed . At pH 5 only 34 % was consumed , the consumption of Xylose was positively proportional to the amount of Xylose reductase in the fermentation media. These findings were similar to that reported in the published literature, the optimum pH was 6.0 for *Candida tropicalis* (24 ) and 5.5 and 6.0 for *Candida guilliermondii* FTI 20037 (23) . Elena *et al.* (6), reported that the highest value for Xylitol units achieved at pH 5.5 by *Candida tropicalis* . The highest activity for XR was recorded at pH 6 , followed by pH 5.5 . whereas the lowest value for enzyme activity was at pH 5. Accordingly , the optimum pH for Xylose reductase productivity under the recent study condition is 6.




| | 5 | 5.5 | 6 | 6.5 | 7 |
|---|---|---|---|---|---|
| XR U/mi | 7 | 25 | 32 | 21 | 18 |
| xylose g/l | 37.44 | 13.22 | 3.68 | 5.12 | 14.36 |
| % of Residual xylose | 65.46 | 23.14 | 6.44 | 8.96 | 25.13 |

**Figure 5. Effect of fermentation media pH on XR production and Xylose consumption**

HPLC analysis (6) indicated that the obtained Xylose reductase was effective in converting approximately more than 80 % of Xylose present (from WSH) in fermentation media to Xylitol. The percentages of Xylitol and Xylose in fermentation media after 20 h under the optimum condition were 86.68 and 10 % respectively.

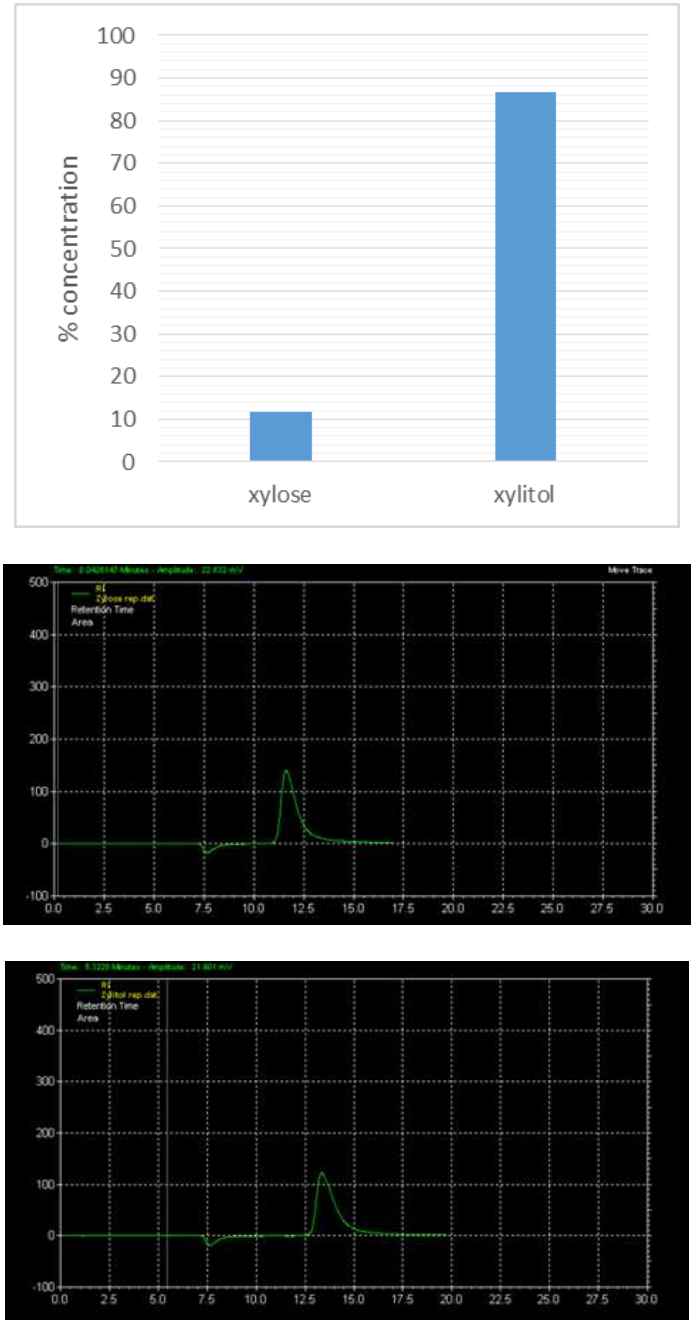



**Figure 6 Xylose and Xylitol concentration in fermentation medium after 20 h under optimum conditions as determined by HPLC.**

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