RESEARCH ARTICLE

Isolation and screening of wild yeasts for maximum xylitol production

Londhe Madhavi Navnit and Padalia Unnati

Department of Microbiology, K.J. Somaiya College of Science and Commerce, Vidhyavihar, India

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ABSTRACT

The sugar xylitol is a five carbon sugar alcohol that has beneficial health effects. Xylitol represents an alternative to current dominant sweeteners for diabetic people. The microbial production of xylitol is an alternative for the catalytic hydrogenation of xylose in wood hydrolysates. The bioconversion of xylose to xylitol is efficiently brought about by yeasts. A total of 20 yeasts strains form natural environments were screened to check their capacities for3% xylose utilization within 72hours by DNSA method. The best xylose utilizing isolate of all was thus selected for xylitol production from 5% xylose as substrate. A garden soil isolate (Sx) appeared to be a promising strain producing xylitol with good yield in a optimized medium containing Yeast Nitrogen Base (0.58g of xylitol per gram of xylose consumed). This isolate was identified to be Candida tropicals strain. Xylitol yield by the immobilized cells was also studied for this isolate. The percentage efficiency of xylose to xylitol conversion was significant. All the samples were analyzed by HPLC for determination of xylitol production. Xylitol yield in immobilized condition was also studied. The present work deals with experimental investigation for the production of xylitol from natural isolates.

Keywords: HPLC, Candida tropicals, xylitol, xylose, yeast nitrogen base.

INTRODUCTION

Xylitol was first popularized in Europe as a safe sweetener for people with diabetes which would not affect insulin levels. This tolerance is attributed to the lower effect of xylitol on a person's blood sugar compared to that of regular sugar. Its dental significance was researched in Finland in the early 1970s when scientists at Turku University showed it had significant dental benefits. Thus xylitol production gains importance (Tom *et al*., 2007).

MATERIAL AND MATERIALS

Presently, xylitol is manufactured by reducing pure xylose, obtained from hard-wood hydrolysates, in the presence of a Raney nickel catalyst. However the overall xylitol yield is relatively low from the total xylan content of the wood hemicelluloses. Thus microbial xylitol production is an alternative to chemical method of xylitol production. The use of metabolically engineered yeasts, *Saccharomyces cerevisiae* or *Candida*, has been studied as an alternative for industrial production of xylitol (Tom *et al*., 2007). *Candida species* are an excellent set of model organisms for xylitol production, and can grow on xylose as a sole carbon and energy source. *Candida* yeasts have been extensively studied with regards to their biotechnological application in the production of xylitol. This is due to the fact that they have an advantage over the metabolically engineered *S. cerevisiae* for being natural D-xylose consumers and maintaining the reduction–oxidation balance during xylitol accumulation (Raluca *et al*., 2010).

Screening of naturally occurring xylose utilizing yeast is an effective method for obtaining xylitol producing yeast with industrial applications (Guo and Zahao, 2005).The fermentation process that produces xylitol in yeasts is controlled by a series of factors such as substrate concentration, carbon source, inoculum, aeration degree, temperature or pH. Although high xylitol yields from D-xylose have been frequently reported, a lots of research work has been devoted to optimization conditions, with particular concern to the effect of oxygen level (Deigo *et al*., 2007)(Walter*et al*., 2002).Among nitrogen sources, the yeast extract, urea and the yeast nitrogen base are the nutrients preferred by the yeasts producing xylitol(Raluca *et al*., 2010).

The present work deals with experimental investigation for the isolation, screening, optimization and production of xylitol from natural yeast isolate (Kock

2.1 Sample collection for isolation of xylose utilizing yeasts (Guo and Zahao, 2005)

Several soil and fruit samples were collected which are source of xylose themselves. These samples included black grapes, garden soil, honey, strawberries, dates, sugarcane juice and stem washings etc. These samples were cleanly washed and processed.

2.2 Enrichment of 3% xylose utilizing yeast (Kumar *et al*., 2007; Kumar *et al*., 2007; Srivani and Setty, 2007)

All the above samples were selected for enrichment of xylose utilizers. One g of each sample was inoculated in 100ml sterile 3% xylose broth containing xylose 3 %, peptone 0.25% , 100 ml distilled water (pH 6.5) and incubated at 30^0C for 48h on rotary shaker at 100 rpm.

2.3 Isolation of 3% xylose utilizers

Serial dilutions of the enriched sample were made in sterile saline. An aliquot of 0.1ml of each dilution was spread on 3% xylose agar medium and the cultured plates were incubated at 30^0C for 48h. All the colonies of yeasts were selected and examined under microscope .The cultures were re-streaked and pure cultures were maintained on 3% xylose agar medium.

2.4 Screening of xylose utilizing yeasts

(Guo and Zahao, 2005)**,**

The ability to utilize 5% xylose was checked for all the 35 yeast isolates obtained from various sources. Each isolate was grown for 24h in broth containing xylose 1%, glucose 1%, peptone 0.5% (pH6.5) on shaker and incubated at 30^0C.Ten ml of pre-grown cultures were inoculated in medium containing xylose 5%, peptone 1.2%, NH_4Cl 0.4%, $MgSO_4$ 0.0075%, $ZnSO_4$ 0.00075%, KH_2PO_4 1.2% (pH 6.5) and incubated on rotary shaker at 30^0C for 72h.

The samples were withdrawn periodically and the concentration of xylose was estimated by standard DiNitro Salicylic Acid (DNSA) method.

2.5 Optimization of fermentation medium.

(Srivani and Setty, 2007; Timothy, 2003; Timothy, 2003; Solange *et al*., 2006; Rodrigues, 2003) Out of all 35 isolates, the garden soil isolate (Sx) which showed rapid utilization of xylose within 72 was selected for shake flask fermentation.

Fermentation was carried out in 500ml flasks containing 100ml sterile fermentation medium. Two Fermentation media were optimized for enhancing xylitol production. Medium A was containing xylose 5%, peptone 0.5%, yeast extract 0.5%,NH_4Cl 0.4%, $MgSO_4$ 0.0075%, $ZnSO_4$ 0.00075%, KH_2PO_4 1.2% (pH 6.5) and Medium B was containing xylose 5%, peptone 0.5%, yeast extract 0.5%,NH_4Cl 0.4%, $MgSO_4$ 0.0075%, $ZnSO_4$ 0.00075%, KH_2PO_4 1.2% , glycerol 0.5g% ,biotin 2µg/L (pH 6.5) (Maria *et al*., 1988). Yeast nitrogen base was filter sterilized using a 0.22µ sterile syringe filter, glycerol was separately autoclaved and used. The culture was pre-grown and a 10% inoculum was used. These flasks were incubated at 30^0C on rotary shaker at 100rpm. Samples were withdrawn periodically after every 24h up to 48h. Samples were centrifuged at 10,000rpm and supernatant was used for estimating xylose concentration by DNSA method (Miller, 1959). The 48h samples were analyzed for xylitol production by HPLC.

The xylose utilization and xylitol production was studied in these two fermentation media under shaker condition. Also, the effect of various nitrogen sources on xylose utilization was studied (Deigo *et al*., 2007; Walter *et al*., 2002).

2.6 Identification of the isolate by standard bio-chemicals and CHROMagar Medium

Aragao and Azeredor , 1998; Walter , 1994)

The soil isolate (Sx) producing xylitol was identified by its cultural and morphological characteristics. Gram staining and wet mount were performed and observed under microscope .The CHROM agar *Candida* medium, majorly contributed towards the identification of the isolates.

A loopful of saline suspension of the culture was isolated on sterile CHROMagar *Candida* medium and incubated at 30^0C for 48h. The colony appearance and colour on CHROMagar was compared with the Pantone Colour Guide, thus helped in identification of this isolate (Kock *et al*., 2008; Frank and Chromagar, 1994).

2.7 Production of xylitol by immobilization of selected strains in calcium-alginate gel capsules (Deigo *et al*., 2007; Walter*et al*., 2002)

The yeasts cells were immobilized by entrapment in calcium alginate bead method. The concentrations of sodium alginate 20g/L, and calcium chloride 11g/L was used. An adequate volume of cell suspension of 0.1 OD was added to a solution of sodium alginate previously heated at 121^0C for 15min. Cell-gel beads were produced by dripping this suspension in calcium chloride solution, using 1ml pipette. The beads were maintained in calcium chloride solution at 4^0C for 2h. Afterwards they were washed with sterile distilled water and 10g of beads were introduced into the fermentation flasks.

Under same parameters as free cells, fermentation was carried out. Samples were withdrawn periodically, centrifuged and DNSA test was performed. The 72h samples were analyzed by HPLC for xylitol production.

RESULTS AND DISCUSSION

In all 20 different yeast isolates were obtained from all the sources. These isolates were purified and maintained on sterile xylose agar slants and refrigerated for further studies. Their ability to utilize xylose was screened in xylose peptone medium. The garden soil isolate was able to utilize 5% xylose within 72h as compared to other isolates. This soil isolate was named as “Sx”. This isolate was further employed for xylitol production. Identification of this isolate was carried out by morphological (Fig No.1) cultural (Fig No.2) biochemical tests (Table No. 1).

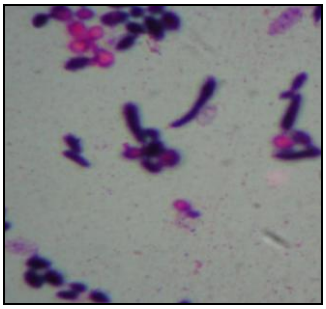
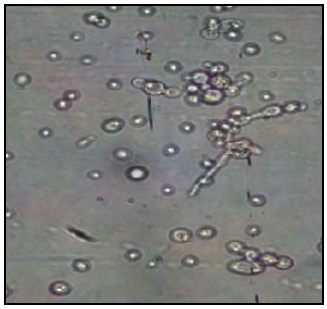



Fig. 1: Gram staining (100X) and wet mount (40X) of Soil isolate (Sx) from 3% xylose agar plate

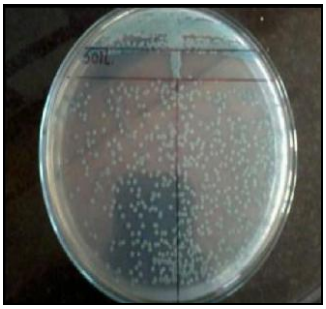
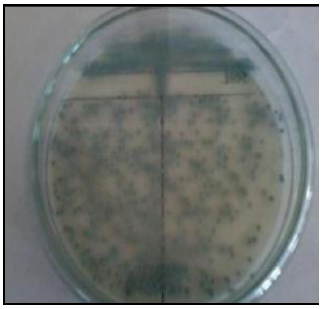
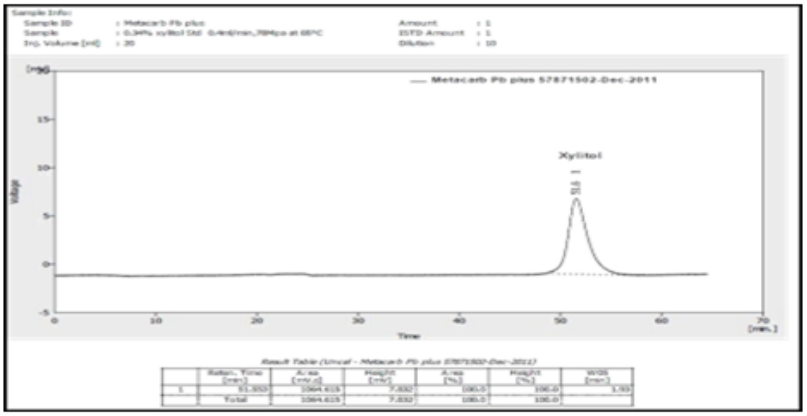



Fig. 2: Isolation on CHROMagar medium. Blue-gray colonies, reverse and front view of Sx.



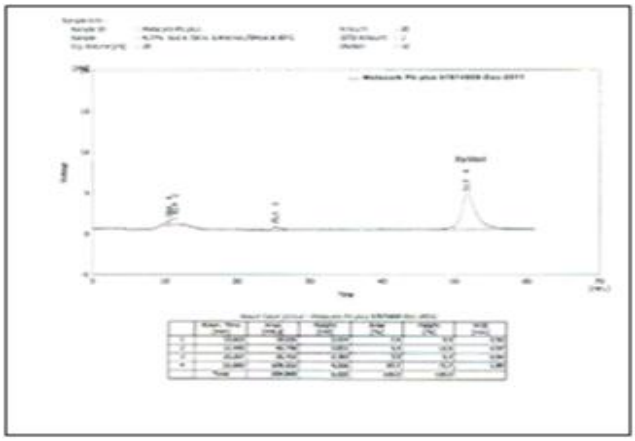
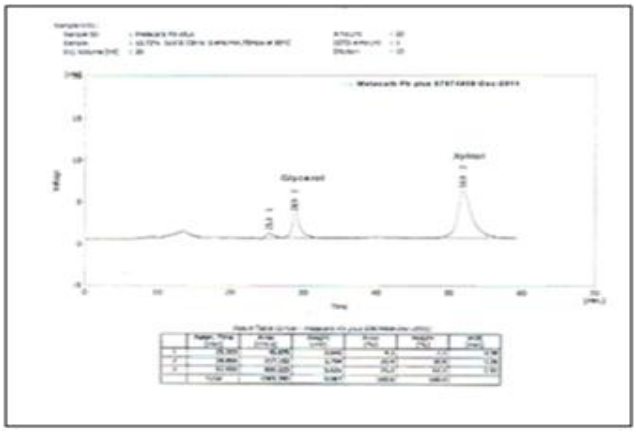



Fig. 3: HPLC analyses: Standard Xylitol

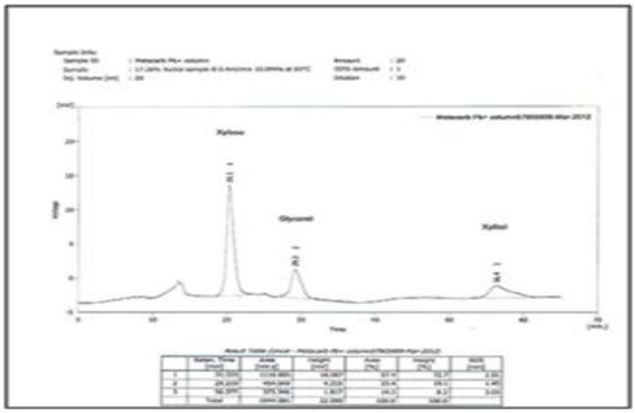


Fig.4: HPLC analyses Fermentation Medium A, Medium B of free cells.

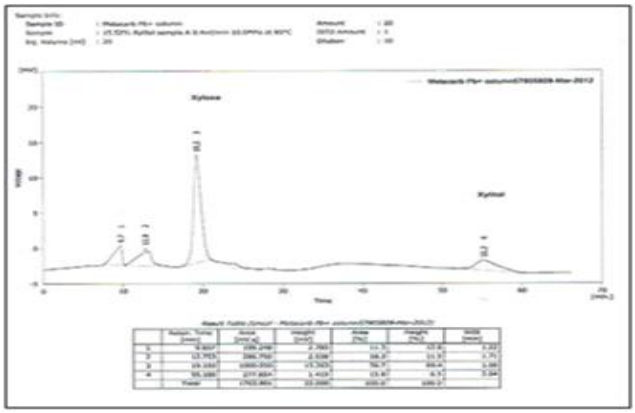


Fig.5: HPLC analyses Fermentation Medium A, Medium B of immobilized cells.

Table 1: Biochemical identification of Sx isolate.

Test	P. anmola	C. Succiphila	C. guilliermon	C. tropicalis	S. cerevesiae	C. intermedia	Isolate (Test) Sx
Dextrose	+	+	+	+	+	+	+
Xylose	+	-	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+
Maltose	+	+	-	+	+	+	+
Lactose	+	-/+	-	-	-	-	-
Mannitol	+	-	+	+	-	+	+
Raffinose	+	-	-	-	+	+	-
Starch	+	-	-	-	+	-	-
Inositol	+	-/+	-	-	-	-	-
Citrate	+	+	+	+	-	+	+

Table2: Xylitol yield by free cells and immobilized cells

Parameters	Sx (immobilized cells)			Sx (free cells)	
	Media A	Media B		Media A	Media B
€ (gL^{-1})	50	50		50	50
Time (hr)	48	48		48	48
Xylose Consumption (%)	80.56	79.65		100	100
ά (%)	16.35	13.95		64.12	51.90
$Y_{p/s}$ (gg^{-1})	0.157	0.128		0.58	0.47
B (g)	-	-		Shaker : 3.9 Static :0.195	Shaker : 2.8 Static :0.17

This isolate was identified to be a *Candida tropicalis* strain on the basis of morphological cultural and biochemical tests. Strains of this genus can carry out the xylose to xylitol conversion rapidly and efficiently.

Furthermore, there was a rapid utilization of xylose in both the optimized media by Sx isolate. It utilized 5% xylose completely within 48 hour in both the optimized media. The HPLC analyses of samples are depicted in Fig No. 3, 4 and 5 as follows.

This indicates that xylitol production is a relatively common feature among xylose utilizing yeasts, as suggested also by other workers (Raluca *et al*., 2010; Kumar *et al*., 2007; Kumar *et al*., 2007). The xylose to xylitol conversion was sensitive to nitrogen source.

Key : € (gL^{-1}): initial xylose concentration, ά (%): percentage efficiency of xylose to xylitol conversion
$Y_{p/s}$ (gg^{-1}): yield of xylitol, B (g): Biomass, cell dry weight.

Also a notable aspect of the present study was that a relative high yield was obtained in the Medium A specifically 0.58gg^{-1} of xylose with yeast nitrogen base as nitrogen source (Table 2: free cells).

Medium A containing yeast nitrogen base gave higher yields of xylitol as compared to medium B containing glycerol and biotin. This could be because yeast nitrogen base (YNB) contains all essential nutrients and vitamins necessary for the cultivation of yeasts (Maria *et al*., 1988). Although in medium B, xylitol was produced but the yield

was comparably low. Complete xylose consumption was shown by the Sx within just 48 hours. The highest xylose to xylitol conversion efficiency was for soil isolate which was 64.12% (free cells). On immobilization of the soil isolate by calcium-alginate bead method, it was observed that the highest yield of xylitol was 0.157 gg^{-1} of xylose in medium A (Table 2: immobilized cells). Also the overall yield was maximum in the medium A as compared to medium B. The immobilization strategy using calcium- alginate beads gave a very low yield as compared to the free cells. The immobilization of *Candida guilliermondii* for xylitol production gave a yield of 0.53gg^{-1} of xylose in the work carried out by (Walter *et al*., 2002) by using sugarcane bagasse as biomaterial for immobilization. Thus this strategy could be applied to the present study for improving the yield.

The soil isolate showed higher production of biomass in shaker condition in comparable with static conditions in the 5% xylose medium. Under aerobic conditions, there was rapid utilization of 5% xylose. The biomass production was high under shaker condition as compared to static condition (Table 2: free cells). There was rapid xylose utilization by free cells than immobilized cells in the fermentation media. Therefore, there was very slow utilization of xylose in both the media with altered nitrogen sources by immobilized soil isolate as compared to free cells of soil isolate (Fig 6).

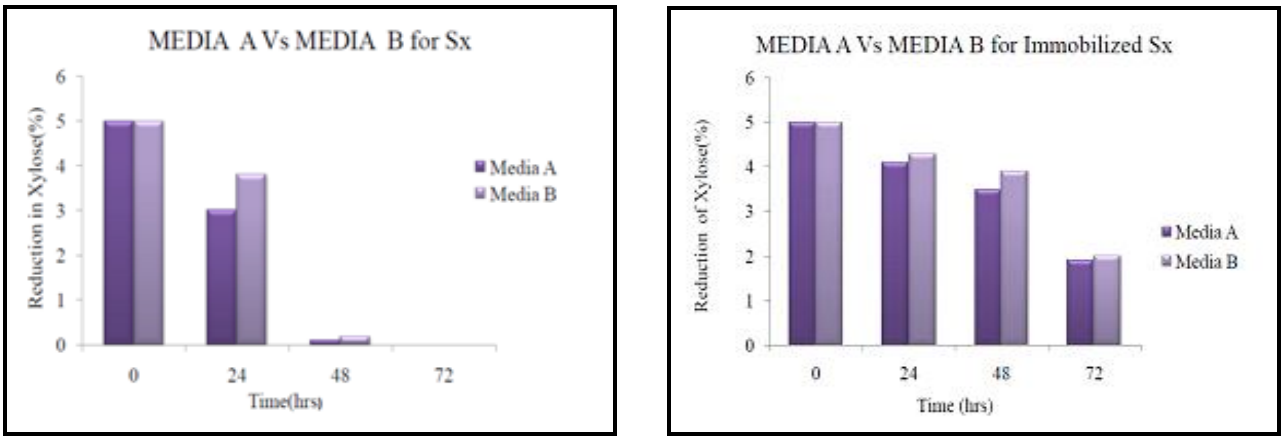


Fig. 6: Xylose utilization by free cells and immobilized cells in Medium A and Medium B

Key: Sx- Soil isolate, Media A- xylose medium containing yeast nitrogen base; Media B – xylose medium containing glycerol and biotin

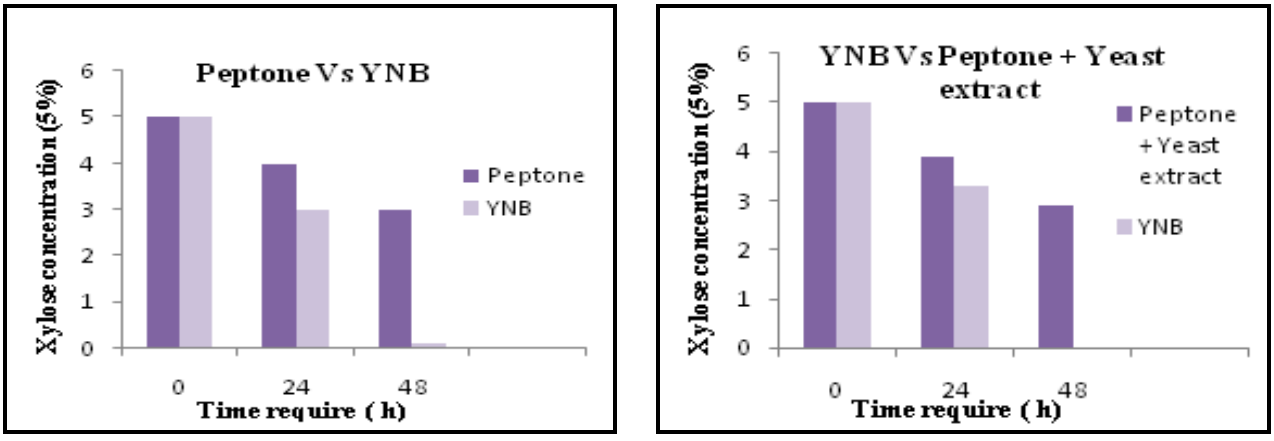


Fig. 7: Xylose utilization pattern with different nitrogen source by free cells.

Key: Sx- Soil isolate, Peptone- xylose medium containing peptone as nitrogen source, YNB – xylose medium containing yeast nitrogen base as nitrogen source,Peptone + YE– xylose medium containing peptone and yeast extract as nitrogen sources

In the above figure (Fig7), it can be observed that in presence of peptone as nitrogen source, the soil isolate did not completely utilize xylose within 48h. Also it can be observed that in presence of peptone+ YE as nitrogen sources, the soil isolate slowly utilized 5% xylose.

Whereas, when YNB was used as the nitrogen source there was almost complete 5% xylose utilization within 48h. Thus, YNB proved to be a better nitrogen source than peptone, yeast extract and it clearly enhanced xylose utilization of this isolate.

CONCLUSION

The maximum xylitol yield was $0.58gg^{-1}$ of xylose, by the soil isolate in medium A with yeast nitrogen base, which also showed a slight increase in final xylitol concentration as compared to medium B containing glycerol and biotin. Thus this study clearly indicates yeast nitrogen base to be efficient nitrogen source for xylitol production as compared to peptone and yeast extract.

The highest percentage efficiency of xylose to xylitol conversion in this study is 64% of the soil isolate This study was carried out under lab scale conditions, shake flask method was used for fermentation rather than a fermentor which restricted the exact conditions required for the fermentation process i.e. baffles, sparger, impeller etc for maintaining homogeneity, equal distribution and maintaining dissolved oxygen content. Thus the percentage efficiency of xylose to xylitol conversion and the yield for this isolate could be enhanced by improvising necessary parameters. Also the yield of xylitol could be worked upon by optimizing several parameters including various concentrations of yeast nitrogen base, glycerol and biotin.

The maximum yield given by the isolates on immobilization is $0.157gg^{-1}$ with 16% xylose to xylitol conversion efficiency. The calcium-alginate concentrations could be optimized for more excretion of xylitol into the medium. Also the matrix used for immobilization can be altered with sugarcane baggase etc. Five percent xylose was rapidly and efficiently utilized under aerobic conditions than the static conditions by this isolate. Also this isolate grew well under aerobic conditions. Thus it indicates that these isolates appear to be aerobic natural xylose utilizers.

For improving the xylitol yield, the enzymes responsible for xylose to xylitol conversion can be extracted and this enzyme solution can be employed for direct xylose to xylitol conversion.

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