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OVAT ANALYSIS FOR XYLOSE REDUCTASE PRODUCTION FROM *CANDIDA* SP. XLT-01, *ASPERGILLUS* SP. XLT-11 AND *PSEUDOMONAS GESSARDI* HPUVXLT-16 (GENBANK ACCESSION NO: MG770460)

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ABSTRACT: Xylitol is an important commercial bio-product having immense biological potential in preventing tooth decay, strengthening of bones and teeth besides a safer sweetener alternative for diabetic patients. Due to diverse pharmacological applications, xylitol ranks among top twelve commercial pharmaceutical bioproducts available in market. In the present work three hyper xylose reductase (XR) producer microbial isolates i.e. *Candida sp.* (Xlt-01), *Emericella nidulans* (Xlt-11) and *Pseudomonas gessardi* (Xlt-16) were selected out of 228 microbial isolates obtained from soil samples collected from several locations from Shimla in Himachal Pradesh and Jhansi in Uttar Pradesh. Comparative evaluation for XR production by OVAT (one factor/ variable-at-a-time) method resulted in 2.40, 2.11 and 3.03-fold increase in XR activity respectively by the three isolates selected. *Candida* sp. Xlt-01 resulted in highest yield of xylitol (25.65±0.13 U/mg) followed by *Pseudomonas gessardi* HPUVXLT-16. Higher yield of both enzyme (xylose reductase) and the product i.e. xylitol from bacterial isolate is one of the major outcome of the present work which is also comparable to the other yeast strains reported earlier. Moreover, this is probably the first report of OVAT analysis for production of enzyme xylose reductase (XR) from yeast, bacteria and filamentous fungus. The initial results suggest the potential utility of all three hyper producer isolates for xylitol production at higher scale after further R&D efforts.

KEYWORDS: Xylitol, xylose reductase, *Candida* sp Xlt-01, *Emericella nidulans* Xlt-11, *Pseudomonas gessardi* HPUVXlt-16.

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1. INTRODUCTION

Xylitol is a natural pentose sugar alcohol with equivalent relative sweetness to sucrose but less calorific value. Additionally, xylitol is resistant to mallard's reaction due to absence of a reducing carbonyl group in comparison to respective aldoses/ketoses sugar molecules which contributed to its high application in food industries [1, 2]. Xylitol is useful in preventing tooth decay and ear infection since most of the bacteria responsible for tooth decay and ear infection are unable to utilize xylitol [3]. Earlier it was considered that xylitol metabolism is insulin independent but later it has been found that inhibition of carbohydrate hydrolyzing enzymes, delayed absorption of glucose in intestine and enhanced uptake of glucose by skeletal muscle contributed to anti-diabetic effect of xylitol [4, 5, 6]. Xylitol as an important commodity has been widely researched due to its low glycemic index [7, 8], high cooling power [9], anti-cariogenic influence, non-neoplasticity and increased nutritional value [10, 11]. As a result, over the years the market demand of xylitol has increased specially due to its immense health benefits besides natural origin and low calorie content. Global annual demand of xylitol recorded 161.5 million metric tons in 2013 (equivalent to US$ 670 million) is expected to touch US$1 billion (250 MMT) by 2020 [12]. Major fraction (upto 70%) of consumption is shared by chewing gums and confectionery products [13]. Among global competitors, DuPont Danisco from Finland, China and USA, are the leading xylitol manufacturer [14], followed by Xylitol Canada Inc. followed by DFI Corp. (USA) and Novagreen Inc. (Canada) [15] and the Chinese player Shandong Futaste [16]. Commercial production of xylitol is very expensive and energy consuming since it involves catalytic hydrogenation of pure d-xylose in the presence of Ni under high temperature and pressure. To counter the drawbacks and reduce the production cost, biotechnological routes have been explored by the researchers. Recent R&D efforts have suggested lignocellulosic residues (LCR) as an alternate and cheap source of xylose for its biocatalytic conversion into xylitol. LCR has also been utilized earlier for production of 2nd generation bioethanol but, it has also emerged as more promising source for xylitol production which can compensate ethanol production cost [17, 18]. The work reported in the present manuscript details the efforts of screening microbes from varied habitats for their ability to produce xylose reductase and further efforts to optimize xylose reductase and xylitol production by following one variable-a-a-time (OVAT) approach using yeast, fungal and bacterial isolates.

2. MATERIALAND METHODS

Sample collection: For the isolation of xylose utilizing and xylose reductase producing microbes, soil samples were collected from waste disposing area of Mandi, Palampur, Solan and Shimla in Himachal Pradesh and Jhansi and Lalitpur (Uttar Pradesh) during the month of July-August. Samples collected in polyethylene bags were pooled together, brought to laboratory and processed further in RL-V, Department of Biotechnology, Himachal Pradesh University Shimla.

Enrichment & Screening: Before isolation, soil samples were enriched with 1% xylose and

incubated at 30^0C for one to two weeks in order to increase the microbial population with enhanced xylose utilization ability. Isolates were screened by three stages of screening as discussed below:

Xylose assimilation: Isolates were cultured on synthetic medium supplemented with xylose as sole carbon source. Isolates were incubated at 27°C (yeast and fungi) and 35°C (bacteria) for 48-72 hours.

Screening for XR-XDH: Isolates able to assimilate xylose were further screened by estimating the activity of XR [19, 20], XDH [21] and XI [22] enzymes.

OVAT analysis for XR production: OVAT analysis refers to optimization by considering one variable at a time. Yeast, fungal and bacterial isolates were selected for XR production and comparative analysis.

Application for xylitol production: Xylitol yield from all three isolates were estimated by Megazyme-KSorb02/15 kit [23] and high performance liquid chromatography with refractive index detector (HPLC-RID). For HPLC analysis acetonitrile: water was used as mobile phase.

3. RESULTS AND DISCUSSION

Total 228 microbial isolates including 160 fungal and 68 bacterial were obtained. After primary screening, total 186 isolates were screened further for determining XR, XDH and XI activities. Finally, 98 isolates including 23 bacteria and 75 fungal and yeast emerged active for XR-XDH pathway. On the basis of highest XR activity, three isolates one each of yeast (XYLFV-01), fungus (XYLFV-11) and bacteria (XYLBV-16) were selected and used for further studies. The isolates were characterized and identified as *candida* sp. Xlt-01, *Emericella nidulans* Xlt-11 and *Pseudomonas gessardi* HPUVXlt-16 with Genbank accession no: MG770460.

OVAT analysis for XR production

OVAT analysis, was done with first selection of production medium for XR production depending upon type of isolate viz. yeast, fungi and bacteria. The medium comprised of (g/l); Xylose-30, yeast extract-10, ammonium sulfate-3, potassium dihydrogen phosphate-15, magnesium sulfate-1 (for *Emericella nidulans* Xlt-11) [24], xylose-10, malt extract-20, yeast extract-5 (for *Candida* sp. Xlt-01) [25] and Yeast extract-1.00, $(NH4)_2SO_4$-0.20, $MgSO_4$x7 H_2O-0.50, $CaCl_2$ x $2H_2O$-0.25, KH_2PO_4-0.60, xylose-1.00 (for *Pseudomonas gessardi* HPUVXlt-16) [26]. Further production parameters were optimized for comparative analysis (Table 1).

Table 1: OVAT analysis of XR production from selected microbes.

Parameters	***Candida sp.* Xlt-01**	***Emericella nidulans* Xlt-11**	***Pseudomonas gessardi* HPUVXlt-16**
	(Activity units : U/mg)		
Seed age	24 (9.88)	24 (15.67)	22 hours (5.01)
Seed size (%)	5 (9.67)	8 (16.93)	6 (6.27)
Carbon source	Xylose (9.75)	Xylose (16.45)	Xylose (6.21)
C-source (%)	3 (19.67)	3.5 (17.86)	1.5 (7.49)
Nitrogen source	Yeast extract (15.67)	Egg albumin (19.27)	Amm. sulfate (8.89)
N-source (%)	0.4 (16.98)	1 (21.23)	0.2 (10.36)
Co-substrate (Cs)	Xylan (18.17)	Dextrose (22.65)	Xylan (12.94)
Cs- conc. (%)	0.05 (21.71)	0.07 (25.12)	0.1 (12.79)
Production profile (hr)	104 (22.47)	98 (29.98)	54 (14.99)
pH	6 (23.87)	7 (30.07)	7 (15.13)
Temperature (^oC)	27^oC (23.75)	30^oC (33.17)	30^oC (15.04)
RPM (/min)	150 (23.75)	150 (33.07)	150 (15.19)
Fold increase	2.40	2.11	3.03

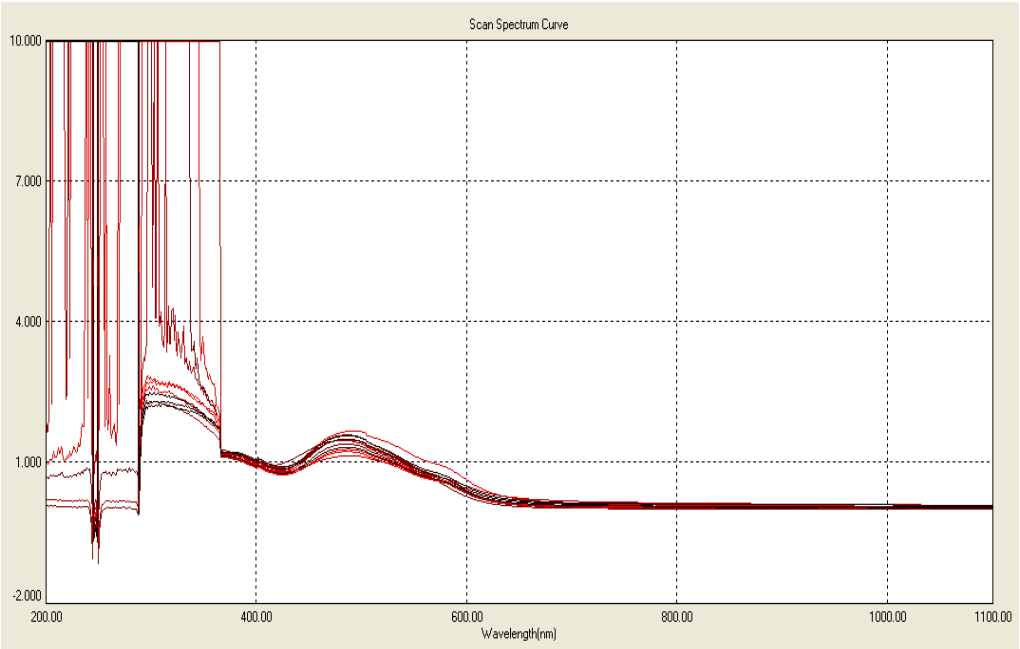

Figure 1: Spectrum analysis of isolates for INT-formazan formation from xylitol

Xylitol production: After optimization through OVAT approach, all the three isolates were compared for their xylitol production potential with optimized parameters and the results have been

summarized in Table 2.

Table 2: Comparative xylitol yield from the selected hyper producers

Isolate	**Assay Kit** (%)	**HPLC-RID** (%)
Candida sp. Xlt-01	26.23±0.65	25.65±0.13
Emericella nidulans Xlt-11	23.54±0.49	22.98±0.89
Pseudomonas gessardi HPUVXlt-16	24.67±0.12	23.12±0.56

Results indicated that although all the three isolates could produce xylitol production but in comparison to bacteria, yeast were more prominent and the number of isolates obtained was maximum in case of yeasts. Among selected isolates, highest XR activity was recorded in case of bacteria followed by yeast while highest xylitol yield was from yeast. Recently lignocellulosic biomass recovered from different sources have been targeted for generation of commercial byproducts. Although xylose reductase (XR) is the key enzyme for xylitol production but it is not sufficient for xylitol production hence multiple enzyme systems are required for conversion of xylose to xylitol. Due to its low lignin content, *Agave americana* has been reported as an ideal feedstock for the production of commercial bioproducts. Among 3 microbial isolates tested by the earlier researchers, *Cellumonas xylanilytica* XIL11 displayed better hydrolysis ability while higher bioethanol (0.92g/g) and xylitol (0.98g/g) yield was reported from *Bacillus* strain (65S3) and *Pseudomonas* strain (CDS3) respectively [27]. *Candida tropicalis* MTCC 25057 from soil sample, expressing cellulases and xylanases over a wide range of temperatures helped in releasing sugars from feedstocks and their biotransformation into xylitol and ethanol [28].

The wild strain of *Saccharomyces cerevisiae* doesn't have ability to produce xylitol hence xylose reductase (XR) genes from *Candida tropicalis, Pichia stipitis, Neurospora* crassa, and an endogenous gene GRE3 have been inserted and successfully overexpressed in *Saccharomyces cerevisiae.* Another gene SUT1 encoding for xylose specific transporter from *Pichia stipitis* was cloned to improve xylose transportation with highest volumetric (0.28 gL-1 h-1) and specific (34 mgg-1 h-1) xylitol yield found in strain overexpressing GRE3 gene. Glucose and xylose present in production medium were reported to be consumed and transformed into ethanol and xylitol respectively [29]. In another work corn biomass residue (corn stover, husk, and cob) and corn bran were evaluated as alternative by chemical and thermochemical routes. Chemical pretreatment has been found superior for processing and xylitol production from LCR [30]. Presence of salt has synergic effect on biotransformation of D-xylose to xylitol by *Debaryomyces hansenii* NRRL Y-7426 using detoxified corncob hydrolyzates [31]. Earlier it was thought that after pretreatment, LCR has to be detoxified before fermentation but some of the recent work suggested that pretreated hydrolysate can be used without detoxification. Among different LCRs, sisal fibers having higher availability as low cost raw material have been evaluated for simultaneous production of xylitol and

ethanol from yeast *Candida tropicalis* CCT 1516 with maximum xylitol yield of 0.32 $g \cdot g^{-1}$and 0.27 $g \cdot g^{-1}$ of ethanol was recorded in 60 h [32]. Rapeseed straw hemicellulosic hydrolysate was also evaluated for xylitol production by *Debaryomyces hansenii* and *Candida guilliermondii*, using different hydrolysate detoxification strategies. It was reported that *C. guilliermondii* have high tolerance to toxic compounds than *D. hansenii* and higher yield was reported as 0.55 g/g and 0.45 g/g respectively without any detoxification [33].

4. CONCLUSION

Yeast like *candida* sp have active XR-XDH pathway more frequently than fungi. As prokaryotic cells, bacteria have fewer chances to have active XR-XDH pathway. In present work *Candida* sp. *Emericella nidulans* Xlt-11, & *Pseudomonas gessardi* HPUVXlt-16 were obtained from soil samples. OVAT analysis showed that all three isolates have potential for xylitol production which can further be replicated at large scale. However active XR-XDH pathway and comparable xylitol yield from *Pseudomonas gessardi* HPUVXlt-16 was one of the most significant findings of the present work.

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