Elucidation of New Xylose Metabolizing Pathway in *Pseudomonas Gessardii* VXIt-16 and its Correlation with Xylitol Production from Sugarcane Bagasse Hydrolysate

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Title:

Elucidation of new xylose metabolizing pathway in *Pseudomonas gessardii* VXlt-16 and its correlation with xylitol production from sugarcane bagasse hydrolysate

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Abstract

Scientific interventions have identified lignocellulosic biomass as potential raw material for various industrial processes. However toxic byproducts released during the process result in deterioration of environment to a greater extent. Microbes can utilize these wastes for production of products of commercial value like bio-fuels, protein, organic acids and xylitol. However, high production cost and astringent operating conditions have been the major bottlenecks for its commercial production. In microbes, xylose is metabolized by xylose isomerase (XI) and xylose reductase-xylitol dehydrogenase (XR-XDH) pathways, with later having ability to transform pure xylose as well as xylose rich lignocelluloses. Efforts to find hyper producer isolates for xylitol production resulted in identification of one such isolate *Pseudomonas gessardii* VXlt-16 (MG770460) by 16s rDNA sequencing. Statistical optimization resulted in 7.28 folds' increase in xylitol yield with 64.76% xylose bioconversion. Conversion of xylose to xylitol even at large scale suggests the possible application of bacterial isolate for the production of this useful product at industrial scale.

Keywords: Bacterial xylose reductase; *Pseudomonas gessardii*; Xylitol

Abbreviations: XR: xylose reductase; XDH: xylitol dehydrogenase; XI: xylose isomerase; LCR: lignocellulosic residues.

Introduction

Unmanaged waste majorly comprises of organic matter as most of industries utilize some of the fraction of raw material and discarded rest Organic matter from agricultural practices and relevant industries such as paper-pulp is rich in xylan and xylose can be a potential substrate for many commercial by products including nutraceuticals, biofuels, phenols, organic acids, biopolymers, enzyme, animal feed, bio-fertilizer and even xylitol if handled scientifically for the production of commercial byproducts [1–4]. Xylitol is a 5-carbon sugar alcohol having equal sweetness to table sugar but with lesser calories besides other pharmacological benefits such as anti-cariogenicity, antimicrobial and oral hygiene. It doesn't add to blood glucose pool which makes it a suitable sweetener for diabetic patients. Moreover, its antimicrobial potential adds its application in maintaining oral hygiene and preventing demineralization of tooth by limiting the growth of oral bacteria. Its unique metabolism and pharmacological potential makes it one of the top twelve bioproducts, having high market demands [4]. Extraction from plants was the only source for xylitol initially which was replaced by more efficient processes such as chemical reduction of xylose for xylitol. Chemical catalysis operates at high temperature and pressure. However the process is efficient but requirement of high energy and extensive purification enhance the production cost [5, 6]. Bio-production of xylitol may be helpful in reducing the production cost along with higher yield and efficient production. Cells can metabolize xylose by two metabolic pathways *i.e.* xylose isomerase (XI) pathway and xylose reductase-xylitol dehydrogenase (XR-XDH) pathway. XR-XDH pathways are common in eukaryotes such as yeast while prokaryotes including bacteria follows XI pathway mostly. However earlier researches have also enlisted some of the bacteria such as *Cellulomonas*, *Serratia*, *Corynebacterium* [7] *Bacillus* and *Pseudomonas* [8] for XR-XDH pathway and xylitol production. Xylose reductase is the key enzyme for xylitol production therefore purified enzyme can also be used

for xylose bioconversion specifically but it requires purified substrate which also adds cost to pure substrate. Moreover, cells are able to produce number of enzymes which can degrade polymeric biomolecules such as carbohydrates into monomers and further used for energy generation. To overcome the drawbacks and limitation of chemical catalysis and enzymatic conversion, continuous efforts have been made to find some robust hyperproducer microorganism for the bio-transformation of xylose into xylitol [9–11]. *Pseudomonas* is one of the most prominent strains among bacterial kingdom. Some of the species of *Pseudomonas* have been reported for xylitol production [8] but no literature is available for xylose reductase pathway and xylitol production from fluorescent *Pseudomonas* and it is possibly the first report for describing the XR-XDH pathway and xylitol production from *Pseudomonas gessardii*. To enhance the product yield, growth conditions can be optimized either by one variable at a time (OVAT) approach or by statistical modeling like response surface methodology. Statistical modeling is preferred as it comprises the interactions of all the factors to maximize the production of desired products [12, 13]. Therefore, the current investigation was to enhance the production of xylose reductase from *Pseudomonas gessardii* VXlt-16 and establish the relation between XR activity and xylitol field.

Experimental

Enrichment and isolation

Soil samples were collected from the waste disposing area and agricultural fields of Shimla, Solan, Palampur, Mandi (H.P.) and Jhansi, Lalitpur (U.P.). Collected samples were enriched with xylose syrup at 35°C for 72 h and inoculated on nutrient agar. Bacterial isolates were further grown on xylose selective medium comprised of 4.0% xylose; 0.4% (NH₄)₂SO₄ and 0.4% KH₂PO₄, and 1.5% Agar [13]. Only xylose assimilating colonies were able to flourish and thus evaluated for 'xylitol consumption potential' by growing in 0.2% (NH₄)₂SO₄; 0.05% MgSO₄; 0.025% CaCl₂; 0.06% K₂HPO₄, and 3% xylitol. To determine the xylitol

producing potential, bacterial isolates were cultured in production medium comprised of: Xylose: 5; tryptone: 5; peptone: 5; yeast extract: 5 [6, 14] at 35°C. After 72 h cells were harvested, sonicated and centrifuged at 10000 rpm for 10 min. XR and XDH activities were determined with cell free culture extract. Protein concentrations in each sample was measured by Bradford method [15].

Identification and characterization of bacterial isolate

Selected bacterial isolate was cultured onto nutrient agar and Kings A & B media (Himedia, India) to determine the morphological characteristics. Biochemical characteristics *i.e.* catalase test, nitrate broth, starch hydrolysis test, oxidase test, methyl red/voges-proskauer (MR/VP), citrate utilization, urease test, and carbohydrate utilization potential was determined with 24h old inoculum of 0.1 OD. 16s rRNA gene sequence (provided by Xcelris Genomics Ahmedabad) was analyzed for confirmatory identification.

Hydrolysate preparation for xylitol production

Sugarcane bagasse hydrolysate was generated by combining dilute acid and steam blast treatment using 0.3% HCl, at 121°C for 4 cycles of 20 min each. After this, sample was cooled and settled down to separate liquid fraction. Liquid extract was analyzed for the sugar content, which was further used for fermentation.

Statistical modeling for batch fermentation of sugarcane hydrolysates

Statistical model was designed with six production parameters including hydrolysate concentration, inoculum size, pH, production time, temperature and rpm using Taguchi Orthogonal Array (TOA) approach.

Scale-up: Batch fermentation

For higher xylitol yield, 1500 mL of sugarcane bagasse hydrolysate was used for fermentation in 2L fermenter (New Brunswick Scientific, USA). For xylitol production kinetics, XR, XDH activities and xylitol yield were quantified after every 4 h till 96 h, as discussed in earlier sections.

X-ray Diffraction (XRD)

Every material has its own X-ray diffraction pattern due to interaction between X-ray and atoms/crystals. It covers almost all kind of interactions like elastic, inelastic scattering, photoelectric absorption and pair production [16]. X-ray diffraction pattern composed of peaks at different angles which aid in elucidation of crystal lattice structures. XRD pattern of xylitol crystals were prepared by using X-ray diffraction instrument at Rigaku MiniFlex 600; Central Instrumentation Facility 'CIF'-Jiwaji University, Gwalior. A thin film of purified and dried xylitol crystals was prepared and scan at the rate of 10 min^{-1} in the wavelength range of $2\text{-}90^\circ$. $\text{CuK}\alpha$ radiation source of 40 kV was used as source of X-ray.

Fourier-Transform Infrared Spectroscopy (FTIR)

Functional groups and chemical bonds in a compound own a distinct transmission/adsorption behavior for infrared radiation. Spectrum profiling can be very helpful in qualitative estimation of samples' purity [17]. In addition, the FTIR profile may also help in estimating the damages and changes associated with heating and chemical exposure. To confirm the functional moieties and bonds in sample, purified xylitol crystals were analyzed between wavenumber of 450-4000 cm^{-1} in transmission mode. The spectrum was prepared with FTIR spectrometer at IIT Roorkee Uttarakhand.

Product recovery

Xylitol was crystallized from the fermentation broth after removal of solid particulate matter and other unwanted microbial byproducts. To remove the particulate matter, fermented broth was centrifuged at 10000 rpm for 10 minutes at 4°C. Supernatant was detoxified with activated charcoal followed by ion exchange resins [18]. The decolorizing efficiency was determined as follows (1):

$$\eta (\%) = \frac{A_0 - A}{A_0} \cdot 100 \quad (1)$$

where η is decolorization ratio; A_0 is absorbance of fermentation broth before decolorization and A is absorbance after decolorization

Detoxified broth was concentrated at $40 \pm 5^\circ\text{C}$ under vacuum till supersaturation of xylitol solution. Concentrated broth was seeded with 1% xylitol crystals and kept at low temperature for up to 24-48 h to crystallize the xylitol by nucleation. Recovered crystals were washed with petroleum ether and acetone to remove residual impurities.

Analytical methods

Xylitol concentration in fermentation broth was determined by HPLC (Shimadzu HPLC 20 A unit) with refractive index detector using acetonitrile: water (70:30) as eluent at 40°C. Xylose and xylitol (Alfa Assar US) of high purity was used as standard.

Reuse of activated carbon

Xylitol production from hydrolysate and its purification holds relies on activated carbon for detoxification and clarification but its one-time use imparts substantial burden on product cost as well as on environment. Physical and chemical methods were employed for carbon regeneration and each sample was evaluated for restoration of detoxification strategy. Along with that selected method was used for successive cycles of regeneration to determine the regeneration potential of spent carbon. Spent carbon was regenerated by washing with acetic acid [19], chromic acid and distilled water. Spent carbon was mixed with acetic acid/chromic acid/distilled water (1:4) at 30°C for 30 min and filtered. The process was followed by washing with distilled water for 4-5 time to attain neutral pH. Virgin, spent and regenerated carbon were characterized for their iodine number (D28 Committee, n.d.), broth decolorizing power and removal efficiency for dye (methylene blue), furfurals and phenols.

Characterization of fresh, spent and regenerated carbon

Effectiveness of regeneration treatment represented as regaining of adsorption power which relies on the availability of pores and binding sites. Regeneration of functional/active sites was determined with ‘iodine number’ and ‘methylene blue number’. After every cycle of treatment and regeneration, carbon samples were dried to equivalent moisture content *i.e.* below 10%. Moisture and ash content of each sample was determined as defined in standard

procedure ASTM-D3173; ASTM D-3174 respectively (D05 Committee 2021b; D05 Committee 2021c). Porous structure of carbon facilitates the adsorption of molecules of different sizes. Porosity of carbon can be determined with ‘Iodine number (X/M)’ which is the milligram of iodine adsorbed per gram (g) of carbon. Iodine number of virgin, spent and regenerated carbon was determined by iodine-sodium thiosulfate titration as defined in ASTM D4607-94R11. X/M (2) and residual iodine ‘C’ (3) were determined by the following equations:

$$X/M = \frac{((N \cdot 126.93 \cdot V_1) - \left[\frac{(V_1 + V_{HCl})}{V_f} \right] \cdot (N_{Na_2S_2O_3} - 126.93) \cdot V_{Na_2S_2O_3})}{M} \quad (2)$$

$$C = N_{Na_2S_2O_3} \cdot V_{Na_2S_2O_3} \quad (3)$$

where X/M iodine number; N1 is the normality of iodine solution; V1 is the volume of iodine solution used; V_{HCl} is the volume of 5% HCl used; V_F is the filtrate volume used for titration; N_{Na₂S₂O₃} is the normality of sodium thiosulfate solution; V_{Na₂S₂O₃} is the volume of sodium thiosulfate solution consumed; M_C is the mass of activated carbon and C is residual iodine.

However, iodine number does not predict the adsorption potential exactly for which methylene blue number (MBN) and inhibitor removal efficiency (IRE) was determined. MBN is associated with the adsorption of large sized molecules in mesopores on adsorbents’ surface. The methylene blue number of activated carbon was determined as defined in ASTM-D2330-20. Briefly 10.0 mg of activated carbon mixed with 10.0 mL of methylene blue solution at different concentrations (10-1000 mg/L) at room temp for 24 h. The absorbance of solution was recorded at 645 nm to determine the residual dyes and MBN was calculated by the equation. To determine the IRCs of activated carbon for furfurals, phenolics

and dyes, respective standard solutions (1-100 mg/L) were treated with 3% carbon samples for 15 min and IRC can be calculated as follows (4 and 5):

$$q_{eq} \left(\frac{mg}{g} \right) = \frac{(C_0 - C_e) \cdot V}{M} \quad (4)$$

$$\eta(\%) = \frac{C_0 V_0 - C_1 V_1}{C_0 V_0} \cdot 100 \quad (5)$$

where C_0 (mg/L) and C_e (mg/L) is the concentration of dye/furfurals/phenolics before and after treatment, V_0 (L) and V_1 (L) are the volume of the solution before and after treatment and M (g) is the mass of the adsorbent/activated carbon. MBN is maximum amount of dye adsorbed on 1.0 g of carbon. η is the removal efficiency of activated carbon.

Results and Discussion

Isolation and strain identification

Total 68 microbial isolates were obtained from 6 soil samples; among them 22 bacterial isolates were producing xylitol. Among them, Xlt-16 was selected based on maximum XR activity of 4.94 U/mg and conversion rate of 8.82% for further xylitol production from residual biomass. Bacterial isolate was gram negative, light colored/transparent on nutrient agar, showing bluish-green fluorescence on King's B medium when exposed to UV light. It was oxidase, catalase and urease positive. It was able to utilize xylitol, xylose, glucose and sucrose. 16s rRNA gene sequence analysis aligned Xlt-16 near *Pseudomonas gessardii* PBCUUn101 considering highest identity score, query coverage and minimum e-value (Fig. 1). Based on both biochemical and molecular characteristics, Xlt-16 was identified as *Pseudomonas gessardii* VXlt-16 (Genbank accession No: MG770460).

Statistical modeling for xylitol production at pre-bench scale

Statistical model, designed with Taguchi Orthogonal Array (TOA) approach considering six production parameters offered twenty-five combinations. XR activity and xylitol yield from provided combinations were calculated by the following equation (6):

$$R_{av} = 67.44 - 9.96 \cdot A[1] - 5.50 \cdot A[2] + 3.45 \cdot A[3] + 10.67 \cdot A[4] + 1.59 \cdot C[1] - 0.97 \cdot C[2] + 10.15 \cdot C[3] + 5.49 \cdot C[4] \quad (6)$$

Among given experimental runs, maximum XR activity (271.93 U/mL; 94.75 U/mg protein) was recorded with 80% hydrolysate after 60 h while xylitol yield was maximum (58.36%) with 60% hydrolysate. This may be because, growth conditions presented by 24th promoted the rapid consumption of available substrate while 9th run XR/XDH ratio was optimum. Design expert 10.0.3 provides the numerical data to predict the model suitability and reliability after statistical analysis of responses from the combinations offered. Model will be significant only if α is 0.05 and p-value is lower than α . p-value of the designed model was 0.0026 and Adj precision for the model was 9.02. The p-value of the model was much lower than 0.05 thus model was significant.

The purpose of the correlation study was to identify the inherent factor responsible for maximum xylitol yield. The key findings were determined by measuring enzyme activities of both the enzyme (XR and XDH) for XR-XDH pathway (Fig. 2). Results suggested that the ratio between XR to XDH tend to favor higher xylitol yield instead of XR alone. This fact also supports the benefit of using whole cell catalysis over enzymatic transformation.

Batch fermentation for xylitol production

Once the production conditions for xylitol were optimized using TOA scale of xylitol production was increased to 1.5L working volume in 2L fermenter (New Brunswick

Scientific, USA). 1.5L of sugarcane bagasse hydrolysate was prepared by acidified steam blast and fermentation was carried under optimized conditions for xylitol production. The samples were withdrawn at the intervals of 4 h till 96 h and analyzed for XR, XDH activity, XR/XDH ratio and xylitol yield in each case. Maximum conversion (71.98%) of the hydrolysate with productivity of 0.76 g/L.h xylitol was recorded after 56h in comparison to lab scale. The production time reduced by 4h which was 60h under lab scale although maximum XR activity was recorded after 52h but xylitol yield was only 68.93% while maximum xylitol yield recorded only after 56h at maximum XR/XDH ratio (2.39). Fermentation broth contains unwanted microbial products and acid hydrolysis side products like furfurals, and hydroxyfurfurals. Presence of these contaminants was recorded as broth turbidity, conductivity and salt concentration. For the removal of these impurities, broth was treated with 2% activated charcoal as maximum decolorization (94.03 %) was achieved with minimum loss of xylitol (5.35 ± 0.56 g). The AC-treatment is followed by ion exchange resins which further reduced the presence of various contaminants with 94.91% decolorization efficiency. The clarified broth was concentrated at 40°C and crystallized at -20°C which was followed by lyophilization. Light-pale coloured crystals were washed with acetone and diethyl ether to remove the undesired contaminants without affecting crystals (Fig. 3). Purification profile showed that purification strategies have helped in removing the contaminants, which resulted in lowering the salt concentration and conductivity (Table 1). Purification on one hand resulted in 99.68 ± 0.67 % decolorization of fermentation broth, and removal of 79.88% ions but it also resulted in loss of 12.70% of xylitol. Finally, 48.49 g of xylitol crystals was recovered with up to 94.56% purity.

X-ray diffraction pattern of the xylitol crystal showed 14 major peaks at (2θ) 14.116, 14.306, 17.925, 19.995, 22.464, 22.817, 24.974, 28.232, 28.461, 31.705, 31.829, 38.0278, 41.796, and 52.3504. The detailed analysis of peaks revealed that both standard and test

sample were packed as orthorhombic crystals and have average crystallite size of 112.36 nm (Fig. 4).

Purified xylitol was scanned in the wavenumber range of 400-4000 cm^{-1} and transmittance recorded as adsorption bands of different intensity. FTIR spectrum have broad and intense band at 3910.06 and 3756.38 cm^{-1} for water stretch. Test xylitol samples showed broad and intense peaks around 3395.00 cm^{-1} and 2940.95 cm^{-1} for intermolecular and intramolecular hydrogen bonded hydroxyl groups (-OH). Peak at 1642.75 is attributed to C-H stretching vibration. Furthermore, the peaks at 1405.52, and 1017.22 cm^{-1} are fingerprinting bands for alcohol [20]. Bands between 1200-1000 cm^{-1} are the fingerprinting region for each polysaccharide. Band at 1125.96 cm^{-1} , 1095.25 and 1061.17 are attributed to tertiary, secondary and primary alcohol respectively [21]. Peak at 661.81 cm^{-1} represents -OH groups that are out of plane (Fig. 5).

Among screened physical and chemical methods, acetic acid was selected due to higher regeneration efficiency and thus used for successive cycle to regenerate the spent carbon. Carbon wash from the first regeneration cycle was also evaluated for the presence of organic molecules absorbed from the broth by activated carbon. Xylose reductase is the key enzyme for xylitol production. Yeast may be efficient for xylitol production. Most of the available literature also reported Yeasts like *Candida* as an efficient organism for bioproduction of xylitol [4,22,23]. Bacteria are able to utilize lignocellulosic biomass and can survive under harsh conditions. Bacterial cells mostly followed XI pathway for xylose metabolism except few including *Enterobacter*, *Corynebacterium* and *Serratia* have been reported for xylitol production [16,24] *Pseudomonas gessardii* first time reported for xylitol production. Statistical optimization of XR and xylitol production from *Pseudomonas gessardii* VXlt-16 (Genbank accession no: MG770460) resulted in maximum enzyme activity of 84.05 U/mg with conversion rate of 64.19%. Xylitol and xylose reductase was purified after fermentation.

Bacterial xylose reductase was of 67KDa composed of two monomeric units of 33.5 KDa. LCB is a complex structure which can't be utilized completely by XR alone hence whole cell or multiple enzyme system is suitable for xylitol production from low cost complex substrates like lignocellulosic residues [25]. Recently *Candida tropicalis* CCT 1516 was used for simultaneous production of xylitol and ethanol from sisal fiber hydrolysate and 0.32 g/g xylose and 0.27 g/g consumed sugar was recorded [21]. Similar efforts have been made by using *Pseudomonas* CDS3, *Bacillus* 65S3, and *Duganella* 55S2, for simultaneous production of xylitol and ethanol. Out of these three strains, *Pseudomonas* CDS3, and *Bacillus* 65S3 was found superior for xylitol production with the xylitol yield of 0.98g/g and 0.82g/g respectively [8]. Earlier *Candida tropicalis* was used for xylose reductase production using meranti wood sawdust (MWS) as substrate and highest activity of NADPH-dependent crude XR measured was 11.16 U/mL [27]. OVAT analysis between yeast, fungus and bacteria showed that xylitol yield from *Pseudomonas gessardii* HPUVXlt-16 was comparable to *Candida* and *Emericella nidulans* Xlt-11 [27]. OVAT analysis is a time-consuming approach hence to analyzed interactions of multiple factors together, statistical modeling was preferred. Obtained results from CCD showed 19.82 folds rise in enzyme activity with up to 68% xylose conversion. Moreover, in obtained results from repetitive validation run and scale up showed the variation was within admissible range. Tolerance to extreme conditions is one of the major advantages of using bacterial isolate such as *Pseudomonas gessardii* VXlt-16. It may be a good alternative to yeast in dealing with industrial waste with less treatment.

Conclusion

Xylitol is a natural sweetener with high commercial value high has high market demand. Bio-production of xylitol has immense future potential but this requires hyperproducer microbes and economically viable production process. *Pseudomonas gessardii* VXlt-16 (Genbank

accession no: MG770460) was selected on the basis of highest XR activity 4.94 U/mg and better conversion (8.82%) of xylose to xylitol. Isolate was used in the present investigation. Statistical approach used to enhance the XR and xylitol production resulted in 17.01-fold increase in enzyme production with 7.28 folds more xylitol yield. The potential of *Pseudomonas* for bioremediation is well known fact which can be explored for the utilization of LCB for commercial interest. Xylitol production from *Pseudomonas gessardii* using LCB has not been studied earlier. The results obtained indicate future possibility of xylitol production on large scale using LCB.

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Availability of Data and Materials

The data sets supporting the results of this article are included within the article and its additional files.

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Ethical standards

This study following Compliance with Ethical Standards; this study does not involve human participants, animals, and potential conflicts of interest.

Declarations

Ethical Approval

Not applicable.

Consent to Participate

All authors agreed to participate in the publication of this manuscript.

Consent to Publish

All authors read and agreed to the publication of this manuscript.

Competing Interests

The authors declare no competing interests.

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Fig. 1 Phylogenetic analysis of 16s rRNA gene sequence form Xlt-16

Fig. 2 Relation between xylose reductase and xylitol yield (a) and XR/XDH and xylitol (b)

Fig. 3 Xylitol yield at bench scale (a) and purified xylitol crystals (b)

Fig. 4 X-ray diffraction pattern of the xylitol crystal

Fig. 5 FTIR spectrum of various functional groups xylitol standard (a) and xylitol sample (b)

Table 1 Regeneration profile of carbon

Figures

Figure 1

Phylogenetic analysis of 16s rRNA gene sequence form XIt-16

Figure 2

Relation between xylose reductase and xylitol yield (a) and XR/XDH and xylitol (b)

Figure 3

Xylitol yield at bench scale (a) and purified xylitol crystals (b)

Figure 4

X-ray diffraction pattern of the xylitol crystal

Figure 5

FTIR spectrum of various functional groups xylitol standard (a) and xylitol sample (b)