Summary

Xylitol, 5-carbon polyol, commercially used in food and pharmaceutical industries due to its some of health benefits like anticariogenicity, tooth rehardening, and capability of preventing otitis, ear and upper respiratory infections etc. This sugar has also been used as food preservative due to its high chemical and biological properties which helps in increasing shelf life of the food products and moreover, it does not undergoes detrimental maillard reaction causing loss of nutritional food value due to reaction with amino acid during thermal processing (Soleimani et al., 2006). Xylitol has also been used to promote cooling effect in chewing gums, chocolates and other confectionary products because of its high endothermic heat in solution (34.8 cal/g) (Mussatto and Roberto, 2002). It is present naturally in fruits and vegetables but in small quantity, that makes its extraction uneconomical in large scale. Therefore, for large scale xylitol production, pure xylose is chemically reduced to xylitol at high temperature (80-140°C) and pressure (5000 KPa) using nickel as metal catalyst. Requirement of high temperature, pressure and pure xylose, makes chemical process cost and energy intensive, which forced researcher to explore other alternative routes for production of xylitol. Biotechnological production of xylitol emerged as a promising inexpensive method due to involvement of microorganism (bacteria, yeast, fungus, and mold) which requires mild environmental conditions, moreover availability of biomass as a source for xylose makes this process more economical as compared to chemical process. Among microorganisms, yeasts are considered to be best xylitol producers with high yields. Many potential yeast strains have been isolated from nature and efforts have been made for improvement of strain for increasing xylitol yield. *D. hansenii* is counted among best natural xylitol producer with 54 % total xylose conversion to xylitol. Xylose metabolism pathway in this yeast was characterized by Biswas et al., 2013, where xylose is first reduced to xylitol by NADPH dependent xylose reductase followed by xylitol conversion to xylulose by NAD^+ dependent xylitol dehydrogenase. Difference in cofactor requirement by both enzymes results in cofactor imbalance and thus acts as a dominant reason for natural xylitol accumulation in yeast cell. However, some of the xylitol produced get oxidized to xylulose which is further phosphorylated to xylulose-5-phosphate by xylulose kinase which is an intermediate of pentose phosphate pathway and this results in

lowering the xylitol yield i.e. g of xylitol/g of xylose produced. An attempt was made to increase xylitol yield by disrupting *XDH* gene of *D. hansenii* CBS 767 and xylitol production studies were performed. Xylitol production was initially tested in YPD and YPG media. It was observed that glycerol grown cells of disrupted mutant DBX 11 and DBX 12 accumulated 2.5 fold higher xylitol i.e. ~15 g/L and ~13 g/L xylitol respectively, as compared to glucose grown cells which produced ~6 g/L and ~5.5 g/L xylitol respectively. Whereas, wild type *D. hansenii* CBS 767 produced ~5 g/L in glycerol media and ~2 g/L xylitol in glucose containing media. This showed increased xylitol accumulation after disruption of *XDH* which blocked further oxidation of xylitol to xylulose. Moreover, in control experiment with YNB medium containing xylose as the only carbon substrate, negligible xylitol accumulation was found which confirmed *XDH* disruption in DBX11 and also strain was unable to grow due to blocked pathway at xylitol in comparison with *D. hansenii* 767. Optimizing xylose concentration (25 g/L to 100 g/L), pH (4-6.8), and xylose addition time (0-48 h) in shake flask studies resulted in best xylitol yield (0.93 g xylitol/g xylose) and productivity (0.71 g/L/h) in shake flask with 50g/L of initial xylose added after 24 h post inoculation in YPG with no pH control. Effect of aeration was also studied in terms of volumetric oxygen transfer coefficient (k_La) at fermenter scale (7 L fermenter with 4 L working capacity). Fermentation batches performed with cells grown for initial 24 h at constant agitation and aeration and then varying k_La k_La from 23.87-35.34 h^{-1} with change in aeration rate from 1.5 to 2.5 LPM while keeping agitation constant at 250 rpm resulted in increase in xylitol volumetric productivity from 0.36 g/L/h to 0.43 g/L/h whereas xylitol yield remained in the range of 0.85 – 0.99 g of xylitol /g of xylose. However, a significant increase in xylitol volumetric productivity from 0.76 to 2.33 g/L/h was observed when k_La was increased from 50.24 to 87.96 h^{-1} by increasing agitation rate from 300 to 450 rpm and keeping aeration rate constant at 3 LPM with average xylitol yield of 0.92 g of xylitol / g of xylose consumed. Next, efforts were made to further increase xylitol volumetric productivity by increasing OSR (mmoles of oxygen/L) by supplementing 10 % (v/v) pure oxygen keeping air flow rate at 3 LPM. However, this did not help in increasing xylitol volumetric productivity. To investigate xylose as a limiting factor affecting xylitol volumetric productivity, fermentation broth was supplemented with additional xylose (50g/L) at 36 h and 48 h post inoculation. It was observed that low substrate concentration was

not limiting the xylitol volumetric productivity. It was thought that this could be due to unavailability of cofactor (NADPH) for xylose reduction as glycerol (source of NADPH) was completely metabolized in initial 36 h of fermentation.

As *D. hansenii* has been reported to cause bone infection (Wong et al., 1982) and is not regarded as GRAS for industrial production of xylitol. However, *S. cerevisiae* is regarded as safe (GRAS) by FDA for industrial production of value added products like ethanol, xylitol etc. But, as this yeast lacks efficient xylose metabolism pathway, *Dh*XR was successfully expressed in *S. cerevisiae* and xylitol production was studied in both shake flask and in large scale bioreactor. For stable expression of heterologous genes in *S. cerevisiae*, GPD promoter was cloned in yeast integrative vector pRS405. Then, *Dh*WT was cloned in pRS405-GPD vector followed by successful stable transformation in *S. cerevisiae* 131 by homologous recombination. Correct orientation of integrated genes were checked by two set of diagnostic PCR using primers corresponding to *S. cerevisiae* genomic DNA and pRS405-GPD-XR. Positive clones were screened in terms of XR enzymatic activity and xylitol production. It was observed that among positive transformant, Y-*Dh*WT, # 16 produced highest amount of xylitol with yield of 0.96 g/g of xylose with 73 mU/mg of XR activity (in protein crude extract) using NADPH as a cofactor. Comparison of xylitol productions by simple batch fermentations using a glucose-xylose mixture or a glycerol-xylose mixture was done where glucose (2% w/v) or glycerol (2% w/v) was used as a co-substrate for NAD (P) H/NADH regeneration and cell growth. It was observed that, though the growth rate of recombinant strains were higher in glucose media as compared to glycerol media till glucose depletion (12 h of fermentation) but final xylitol concentration was found to be higher in glycerol media. It was also observed that both xylitol production and growth rate were higher in glycerol media after 16 h fermentation, where, glycerol grown N16 cells produced 27.31±0.6 g/L xylitol with volumetric productivity of 0.49±0.002 g/L/h. Xylitol production from D-xylose has been reported to be limited by two factors such as: low intracellular D-xylose level and unavailability of NADPH for xylose reduction. The intracellular xylose level was previously seen to be have restricted by repression of xylose transport when glucose was used as cosubstrate (Peng et al., 2012). Xylose uptake was seen to be interfered by glucose due to sequential consumption of glucose and D-xylose before glucose depletion. Whereas, in case of the glycerol-xylose

mixture, co-utilization of glycerol and xylose was observed as glycerol did not interfere in xylose uptake. In present study, problem related to preference of glucose over xylose was bypassed using glycerol as cosubstrate for cofactor regeneration and cell maintenance. Effect of various other process conditions like inoculum size (% age of fermentation medium (5-15%)), xylose initial concentration (25 g/L to 100 g/L), xylose addition time (0-48 h), initial pH of fermentation media (4-6.8) and rate of aeration were studied in shake flask. Xylitol yield of 0.89 g/g xylose was obtained by recombinant N16 *S. cerevisiae* when 50 g/L initial xylose concentration was added to 24 h YPG grown cells without pH control. Effect of aeration was also studied in batch culture at fermenter scale (7 L bioreactor using 4 L fermentation (YPG) media). A set of three batch fermentations was carried out using N16 *S. cerevisiae* strain in different k_La condition i.e. 18.37 h^{-1}, 74.40 h^{-1} and 97.20 h^{-1}. In this, cells were allowed to grow for first 24 h post inoculation at constant k_La of 50.24 h^{-1} and then 50 g/L xylose was added followed by change in k_La values (by varying agitation/ aeration). It was observed that, increase in k_La resulted in increase in xylitol volumetric productivity from 0.52 g/L/h with xylitol concentration of 26.26 g/L (k_La - 18.37 h^{-1}) to 0.86 g/L/h with 41.26 g xylitol/L (k_La - 74.40 h^{-1}). However, further increase in to k_La – 97.20 h^{-1} resulted in decrease in volumetric productivity to 0.74 g xylitol/L/h with xylitol concentration of 35.31 g/L after 48 h post xylose addition. However xylitol yield (g xylitol/g xylose) ranged between 0.99-1.03 g xylitol/ g xylose in above three cases. Decrease in xylitol productivity inspite of increased k_La (i.e. oxygen availability) was assumed to be due NADPH limitation, as G6PDH gene production and its enzyme activity both were reported to be decrease with increase in k_La above 60 h^{-1} (Silva et al., 2002). Moreover, recombinant *S. cerevisiae* (expressing *S. stipitis* XR) was also shown to have repressed activity of G6PDH when grown on xylose as compared to natural xylose assimilating yeats like *S. tipitis*, *K. lactis* etc. (reported to have induced activity of G6PDH when grown on xylose) (Hector et al.,2011).

It has been suggested that XR from most of the yeasts and fungi are NADPH dependent including that of XR of *D. hansenii*. Since the NADPH cofactor is regenerated by a few metabolic steps, insufficient supply of NADPH may limit the rapid assimilation of xylose. Cofactor regeneration is one of the main concerned area for increasing xylitol production and it has been observed that cofactor imbalance results in surplus NADH in cell. Moreover, NADH is found to be more stable and

frequently available in cell as compared to NADPH. Considering these facts, it was hypothesized that utilization of both NADPH and NADH as cofactors would ameliorates the xylose conversion process and thus xylitol biosynthesis. Accordingly, *Dh*XR specificity was modified by mutating residues in cofactor binding site so that *Dh*XR could use both NADPH and surplus NADH for reduction of xylose. Multiple sequence alignment using Clustal W was done to find out conserved IPKS cofactor binding site and based on previously reported literature, NADH-preferring *Dh*XR mutants were designed by site directed mutations in cofactor binding pocket. Four single mutants (R275H, N271D, K269R, and K269G), five double mutants (N271D/R275H, K269R/R275H, K269R/N271D, K269G/R275H, K269G/N271D) and two triple mutants (K269R/N271D/R275H, K269G/N271D/R275H) were designed. All *Dh* XR mutant clones were successfully expressed at 16° C after 0.2 mM IPTG induction for 18 h and were purified to homogeneity by Ni-NTA affinity chromatography. Recombinant *Dh*XR WT enzyme showed specific activity of 7.49 and 0.12 U mg^{-1} with NADPH and NADH respectively. *Dh*XR $K_m^{(xylose)}$ with NADPH and $K_m^{(NADPH)}$ (using xylose as a primary substrate) was observed to be 36.71 mM and 0.84 µM respectively which showed strong preference of WT *Dh*XR towards NADPH. Among single mutants, R275H showed significant activity with both cofactors i.e. 3.75 and 0.96 U mg^{-1} with NADPH and NADH respectively. ~8 fold increase in activity with NADH as compared to WT *Dh*XR was seen in compensation with~2 fold loss of NADPH associated XR activity upon introduction of R275H mutation. Similar decline in XR activity has been observed with NADPH when R276H mutation was introduced in *Ps*XR, however activity with NADH remained same (Watanabe et al., 2007). Introduction of R275H mutation also resulted in ~21 fold increase in affinity towards NADPH ($K_m^{(NADPH)}$=0.04 µM) and $K_m^{(NADH)}$ was observed to be 0.23 µM. Affinity towards xylose was also found to be enhanced due to ~1.3 fold decrease in $K_m^{(xylose)}$ (29.35 mM) with NADPH cofactor, whereas, it was found to be 104.60 mM with cofactor NADH. It has been reported that lysine270 plays important role in both NADPH and xylose binding (Kostrzynska et al., 1998) and loss of 80-90% XR activity using xylose as substrate was revealed when this conserved amino acid was substituted. Similar effect of loss of 2 fold activity was observed with NADPH as a cofactor and xylose as substrate whereas no increment in activity with NADH was observed when Lysine269 was replaced with Arginine in

*Dh*XR. Reduced affinity with NADPH due ~2 fold increase in K_m^{xylose} along with ~ 6 fold decreases in K_{cat}^{NADPH} was observed in K269R *Dh*XR mutant, clearly indicating importance of Lysine269 in both NADPH and xylose binding. Complete loss of XR activity with both cofactors was observed when Lys269 was replaced with a simplest amino acid Glycine. Among double mutants R275H/N271D showed 2.72 and 0.98 U mg^{-1} XR activity, whereas loss of ~2.8 fold activity with NADPH was observed in comparison to WT. Other *Dh*XR mutants did not show any impressive activity with any of the two cofactors. All recombinant proteins were found to be structurally stable as CD profile of all mutant enzymes was overlapping with *Dh* XR. Melting temperature of WT *Dh*XR was computed to be 52°C whereas +3°C and +1°C increase in melting temperature was observed in case of R275H and R275H /N271D respectively.

R275H *Dh*XR and R275H /N271D *Dh*XR were successfully expressed in *S. cerevisiae* 131 and xylitol production studies were performed. Xylitol yield of 0.93 g/g xylose was observed in case of #5 Y-*Dh*R275H showing XR activity of 11.25 mU/mg NADPH as cofactor. However, #6 Y-*Dh*R275H/N271D produced comparatively less xylitol with yield of 0.78 g xylitol/g xylose due to less XR activity of 8.42 mU/mg with NADPH cofactor. Xylitol production was carried out in two set of media (YPG and YPD), where, higher xylitol accumulation of 25.05±0.65 g/L with volumetric productivity of 0.45±0.01 g/L/h was observed in glycerol media, whereas, glucose grown cells produced 24.52 g/L±0.82 xylitol with 0.44±0.02 g/L/h of xylitol volumetric productivity. Batch fermentation in shake flask performed with RH5 cell using optimized condition (i.e. conditions optimized for N16 cells), produced xylitol yield of 0.91±0.21 g xylitol/g xylose with volumetric productivity of 0.61 ±0.04 g xylitol/L/h.

In present study, effect of disruption of XDH, cloning of heterologous gene and modification of cofactor specificity was examined on xylitol production. It was observed that disruption of *XDH* gene, blocked the pathway at xylitol, thus helped in increasing xylitol productivity and yield in *D. hansenii* DBX11. Majority of xylitol fermentation studies have been done expressing *XR*s from *P. stipitis* and *XR* analoges from *C. shehatae* (Govinden et al., 2001), *C. bondii* (Kang et al., 2003), and *C. tropicalis* (Li et al., 2013). Even though remarkable yield and volumetric productivity

of upto 1 g xylitol/g xylose and 12 g/L/h has been obtained using *Candida* species using cell recycling fermentation (Rafiqul and Sakinah, 2013), however, opportunistic pathogenic nature of *Candida* species, limits their application on commercial scale specially in food manufacturing industries. *XR* from *D. hansenii* (*Dh*XR) has been functionally and structurally well characterized (Biswas et al., 2013). The *Dh*XR wild-type enzyme or its variant has so far not studied for whole-cell xylitol production by recombinant *S. cerevisiae.* In present case, recombinant *S. cerevisiae* expressing *Dh*XR WT resulted in good xylitol yield using glycerol as cosubstrate for cofactor (NADPH and NADH) regeneration and cell growth in batch fermentation and results were comparable with other recombinant strains *S. cerevisiae* reported in literature, carried out under glucose limited fed-batch fermentation. Further, attempts were made to increase xylitol yield by modifying *Dh*XR coenzyme specificity, so that it can utilize both cofactors (i.e. NADPH and NADH). Insertion of R275H mutation resulted in increased *Dh*XR activity with NADH cofactor but at the expense of NADPH associated XR activity and as a result slightly lower xylitol yield by recombinant *S. cerevisiae* expressing *Dh*XR R275H than *S. cerevisiae* expressing *Dh*XR WT was observed. This study could be beneficial for further improvement of organisms own metabolism for increasing xylitol yield.