Assessing the Potential of Newly Isolated Pichia Fermentans for Xylitol Production Using Non-Detoxified Xylose Rich Pre-Hydrolysate Derived from Sugarcane Bagasse

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Research

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Abstract

Background: Integrated management of hemicellulosic fraction and its economical transformation to value-added products is the key driver towards sustainable second-generation biorefineries. In this aspect microbial cell factories are harnessed for sustainable production of biochemicals by valorising C5 and C6 sugars generated from agro-industrial waste. However, most of the strains can effectively consume C6 sugars but lacks pentose metabolism pathway. The effective utilization of both pentose and hexose sugars is key for economical biorefinery.

Results: In the current study, the ability of a newly isolated xylose assimilating *Pichia fermentans* was explored for xylitol production. The wild type strain robustly grew on xylose and produced xylitol with >40% conversion yield. Mutagenesis with ethyl methanesulphonate (EMS) yielded seven mutants. The mutant obtained after 15 min exposure, exhibited best xylose bioconversion efficiency. This mutant under shake flask conditions produced maximum xylitol titre and yield of 34.0 g/L and 0.68 g/g, respectively. oweverHoHHHoHowever, under same conditions, the control wild type strain accumulated 27.0 g/L xylitol with a conversion yield of 0.45 g/g. Improved performance of the mutant was attributed to 34.6% activity enhancement in xylose reductase with simultaneous reduction of xylitol dehydrogenase activity by 22.9%. Later, the culture medium was optimized using statistical design and validated at shake flask and bioreactor level. Bioreactor studies affirmed the competence of mutant in xylitol accumulation. The xylitol titre and yield obtained with pure xylose were 98.9 g/L and 0.67 g/g, respectively while xylitol produced using non-detoxified xylose rich pre-hydrolysate from sugarcane bagasse was 79.0 g/L with an overall yield of 0.54 g/g.

Conclusion: This study established the potential of *P. fermentans* in successfully valorising the hemicellulosic fraction for sustainable xylitol production.

Background

In the present global scenario, tremendous efforts are being made in the search of alternative source to overcome the reliance on petroleum products. The economy of a biorefineries largely thrives on successful extraction of renewable carbon present in the biomass in terrestrial biosphere and its transformation to economically viable products such as chemicals, fuels, energy, polymer etc [1]. Lignocellulosic biomass (LCB) is the significant source of fixed organic carbon which is not only renewable but cheap and abundant in nature. LCB principally consist of three components; cellulose, hemicellulose and lignin. As opposed to lignin which is a complex heterogeneous polymer, cellulosic and hemicellulosic fractions are more lucrative options for valorization. These structural polysaccharides not only account for large of the total biomass but provide a sugar platform upon depolymerization which can be transformed into a number of commercial bio-based products. However, state of the art reveals that presently most of the research is focused towards depolymerisation of the cellulosic fraction of LCB and its commercial exploitation. LCB biorefineries can simultaneously achieve the target of enhanced

carbon efficiency, techno-commercial success and waste minimisation if the potential of hemicellulose is also unleashed [2, 3].

Hemicellulose is a branched hetero-polysaccharide largely composed of pentose monomeric sugars specifically xylose which makes up to 30–40% of LCB[4]. Xylose can be valorised into an array of industrially important chemicals such as furfural, xylitol, ethanol, lactic acid, propionic acid, butyric acid, biopolymers etc either through chemical or biotechnological route [1]. However, the xylose valorization through biochemical route is largely ignored, as most of the industrial microbes lack efficient metabolic pathway for its assimilation. More eminently, xylose utilization in bacteria, fungi and yeast suffers a major setback due to preference of microbes for glucose as carbon substrate over xylose commonly known as carbon catabolite repression [5].

Among all the xylose derived value-added chemicals, xylitol production through biotechnological intervention has been one of the most studied product. Unlike conventional chemical route, xylitol production via microbial pathway is less energy-intensive, environmentally benign and offers numerous advantages such as reduced purification cost of xylose and direct use of biomass-derived mixed sugars [6, 7]. Xylitol is recognised as one of the twelve most promising platform chemicals, owing to its transformation potential into value-added products such as xylaric acid, glycerol, lactic acid, ethylene glycol, propylene glycol and derivatives of hydroxyfurans. Further, the low glycemic index of xylitol and its antimicrobial activity against gram-positive bacteria, makes it an essential ingredient of many food products as a natural sweetener and odontological formulations. Thus, the microbial fermentation of xylose to xylitol should preferably make use of generally regarded as safe (GRAS) organism as xylitol finds major applications in food and pharmaceuticals[6, 8, 9].

In this context, the present study aimed towards exploring the xylitol producing potential of the newly isolated xylose assimilating yeast obtained from food waste. Molecular characterisation confirmed that the said isolate belonged to the genus "*Pichia*" which is known to have a "GRAS" status [10]. Strain improvement was attempted by chemical mutagenesis followed by statistical optimisation of culture medium using the best xylitol producing mutant strain. After optimisation, the xylitol production studies were conducted both at shake flask and bioreactor. The capability of the said strain was evaluated with non-detoxified xylose rich pre-hydrolysate obtained after hydrothermal pretreatment of sugarcane bagasse (SCB) using pure xylose as the benchmark. To the best of our knowledge, this is the first report on xylose assimilation and xylitol production by *P. fermentans*.

Results And Discussion

Shake flask studies for assessing xylitol production ability of *P. fermentans*

The isolated yeast strain was examined for its ability to assimilate xylose and further fermenting it to xylitol. When *P. fermentans* was grown on pure xylose, there was rapid consumption of xylose and nearly entire supplied xylose was depleted in 48 h with concomitant rise in cell growth (Fig.1C). The cell growth

was rapid and an OD_{600} of 14 was recorded in 24 h which increased continuously till it reached more than 30. The starting pH was 7.0 and dropped below 5.5 after 48 h. The xylitol accumulation of 9.0 g/L was noticed at 24 h and highest concentration of 17 g/L was recorded at 48 h with a yield of 0.57 (g/g). These results shows strong ability of isolated *P. fermentans* to grow on xylose and valorize/ferment it to xylitol. The co-fermentation of glucose and xylose by *P. fermentans* was carried out and the impact of presence of glucose on growth pattern, substrate utilization and xylitol formation was investigated (Fig. 1D). Glucose was preferred over xylose which led to rapid depletion of glucose and the initial glucose (10.0 g/L) was consumed within 24 h of fermentation. The xylose consumption was slowed down in the presence of glucose and xylose was rapidly consumed after glucose exhaustion in next 48 h. The addition of glucose boosted up cell growth and significantly, higher OD_{600} was obtained. As a result of it, more xylose was diverted towards xylitol formation in co-fermentation than fermentation with only xylose. By 72 h, ~95% of the xylose was consumed and the maximum of 22 g/L xylitol was produced with conversion yield being 0.77 g/g. Small amount of glucose is helpful for overall xylitol accumulation and higher xylitol yield in mixed sugar fermentation could be attributed to rapid build-up of biomass from fast glucose depletion [11]. The suppression of other carbon sources in presence of glucose is quite common and could be attributed either to carbon repression enzymes, which supress the xylose uptake or competition between the transport carriers as most of the yeast transport system shows high affinity toward hexose sugars and low affinity for pentose sugars [1]. The results obtained can be correlated to similar studies of *Pichia stipites* and *Candida tropicalis* which unveiled fast glucose consumption in mixture sugar fermentation and high xylitol production [12].

The biological production of xylitol has been investigated from a long time. Though, variety of organisms inculding bacteria, yeasts and fungi have been explored for xylitol accumulation, yeasts such as *Candida, Hansunela, Debaromyces, Spathaspora, Pachysolen, Kluyveromyces, Pichia* etc are the most promising xylitol producers. Among yeasts, *Candidasp* such as *C. boidinii*, *C. guillermondii*, *C. tropicalis* etc are the most extensively studied organism [6,8,9]. However, this genus is a known opportunistic pathogen and associated with a number of skin disorders. Xylitol finds major applications in food-based industries and pathogenicity of *Candidasp* impedes the commerical applications of the yeast. On the other hand, *P. fermentans* is a non-conventional, non-pathogenic, safe, dimorphic and fast growing yeast even under microaerophilic conditions. The yeast produce flavor and aroma compounds which find wide applications in food and beverage industries. *P. fermentans* has antagonistic properties which inhibit the growth of fungi and bacteria, therefore, enhance the shelf life of food and beverage products [13,14], making it an attractive candidate for the xylitol production.

Chemical-based mutagenesis of *P. fermentans*

The random mutagenesis is unpredictable compared to rational design and can significantly alter the genome of the microbes creating a strain having superior phenotypic characteristics. The application of inverse metabolic engineering by creating a random mutation in the genome result in microbial systems with desired traits [15]. In the present study, when *P. fermentans* was subjected to EMS mutagenesis by exposing the growing cells to 20 mM EMS solution for different time intervals, in total seven mutants

were obtained. When all the mutants were screened for their ability to produce xylitol with parent strain as the yardstick, xylitol accumulated was equal or more than the parent strain for an exposure time up to 90 min and declined thereafter as shown in Fig. 2A. The best results were obtained with mutant strain which was exposed to EMS for 15 min (E015). Fig. 2 (C & D) shows batch fermentations of wild type (E000) and mutant (E015) strains in shake flask. The batch cultivations were carried out with initial xylose level of 60.0 g/L. Nearly all the xylose was consumed by both the strains within 72 h time duration and exhibited similar cell growth patterns (OD_{600}: 52-58). The major difference was in terms of xylitol production. The mutant strain produced the maximum xylitol titer and yield of 34 g/L and 0.68 g/g, respectively, 26% higher in comparison to wild type strain (designated as E000) which accumulated 27 g/L xylitol with conversion yield of 0.45 g/g. Our results are comparable to Kim et al., 2015[15], where they have subjected the *K. marxianus* ATCC 36907 to EMS based mutagenesis and reported1.79 fold increase in the xylitol titer (25 g/L) in comparison to parent strain (14 g/L).

The biosynthesis of xylitol is a single step reduction reaction and tightly linked to availability of reduced cofactors. The reaction involve reduction of xylose using electrons from redox co-factor NAD(P)H and is catalysed by xylose reductase (XR). Therefore, continuous replenishment of reduced cofactor is required for a smooth production of xylitol. In the next step, xylitol is oxidized to xylulose via NAD^+ and the reaction is mediated through xylitol dehydrogenase (XDH) [16]. The xylitol formed is partially excreted and partially metabolized to xylulose which subsequently enter central carbon metabolism for biosynthesis of precursors and eventually contributes to cellular growth. Thus, activities of XR and XDH and availability of redox co-factors play important role in determining xylitol level.

In this study, the enzymatic activities of XR and XDH were quantified to unravel the superior performance of E015 over its parent strain E000. Fig. 2B compares activities of these two enzymes in wild type and mutant (E015) strains. The enzymatic activity was carried out from the 48 h old cultures when the culture growth was in mid-exponential phase and most of the xylose was consumed. EMS treatment caused significant change in XR and XDH activity levels of wild type strain. As a result of it, the XR activity was enhanced by 34.6% while XDH activity was decreased by 22.9% in mutant strain. This changed XR/XDH ratio from 1.34 in wild type to 2.33 in mutant strain, which is clearly reflected in the xylitol production. On the other hand, the better synchronisation of XR and XDH in wild type strain due to narrow gap in their activities probably allowed more carbon flux towards central carbon metabolism and resulted in lower xylitol accumulation. It was observed that xylitol concentration was dropping after reaching the peak when xylose was also exhausted indicating xylitol was serving as carbon source in absence of xylose and re-entering the metabolism. This was also supported by increase in cell OD_{600}. However, the drop was more rapid in wild type than the mutant strain which can be correlated with low XDH activity of mutant strain. Balanced XR:XDH ratio plays a crucial role in xylitol formation. The observation made in current work is consistent with the literature information. For example, Gírio et al., 1994 [17] achieved high xylitol production when enzymatic activity of XR was double of XDH in *Debaryomyces hansenii* . Walfridsson and co-workers [18] reported that *P. stipitis* strain exhibiting XR/XDH activity ratio of 17.5 produced 0.82 g xylitol/g xylose as compared with the strain having low XR/XDH ratio of 0.06, which produced no xylitol.

Xylitol production from non-detoxified xylose rich lignocellulosic pre-hydrolysate

Agriculture wastes such as corn cobs, rice straw, SCB, wood chips etc contains considerable amount of hemicellulose content and are cheaper feed stock in comparison to pure xylose [3]. Despite all the cited drawbacks, presently microbial platforms using biomass-derived xylose are predominated by yeasts owing to their robust nature and high tolerance to inhibitory compounds associated with it. Therefore, it was important to check robustness of *P. fermentans* to manufacture using hemicellulosic feedstocks. To this end, the xylitol production ability of *P. fermentans* was investigated at shake flask level, using non-detoxified xylose rich pre-hydrolysate obtained by hydrothermal pretreatment of SCB. The toxic effect of acetic acid was extenuated by adjusting the pH of pre-hydrolysate to 7.0 [19]. The wild type (E000) and mutant (E015) *P. fermentans* strains were grown on pre-hydrolysate containing 20.0 g/L xylose (Fig. 3A & 3B). The xylose consumption was smooth as with pure xylose and a large fraction of initial xylose concentration of 20.0 g/L was consumed in 48 h. Both the strains exhibited high cell growth (OD_{600}: 35-38) concomitant with xylose assimilation. The xylitol titre and yield obtained with mutant strain were 11.8 g/L and 0.59 g/g while wild type strain was able to accumulate 9.3 g/L xylitol with conversion yield of 0.46 g/g on xylose rich carbon source. Again, mutant outperformed wild type strain for xylitol production and therefore, was used in all further experiments. Despite presence substantial amount of acetic acid and little furfural in pre-hydrolysate, the strain grew very well and was able to produce large amount of xylitol. The high cell concentration in inoculum used and cell density achieved during the course of fermentation might be favourable for mitigating toxic effects of inhibitors [20]. The results obtained in terms of xylose metabolism, cell growth and product yield were similar to pure xylose in section 3.2 and 3.3 indicating robustness of *P. fermentans* in valorising lignocellulosic feedstock.

Effect of aeration on xylitol production in shake flask

Aeration plays a game changing role in xylitol production specially in yeasts as it affects the activities of XR and XDH thereby regulating xylose metabolism [8,21]. The partition of xylose flux between xylitol synthesis and cell growth is highly dependent on oxygen levels. Under high aeration condition, the NADH formed in the second step is re-oxidized favouring oxidation of xylitol to xylulose, as a result, the xylitol formed enters the pentose phosphate metabolism which is subsequently metabolized to cell mass and other metabolites and resulting in less xylitol accumulation. Conversely, oxygen deprived/limited condition will result in increased intracellular NADH levels which leads to imbalance between XR and XDH causing accumulation of xylitol [6,16,22]. Thus, it was indispensable to study the effect of aeration on the xylitol production by mutant strain E015, as it altered the dissolved oxygen and hence affected the oxygen uptake rate as well.

There are two ways to play with the oxygen levels at shake flask level. The first method is by varying the agitation speed of the shaker and other being altering the media volume in a flask of fixed capacity. When the effect of aeration by examined by varying the agitation speed of the shaker from 100 to 300 rpm with an interval of 50 rpm, following results were obtained as shown in Fig. 4. Xylose was completely utilized within 120 h at 100 rpm and the consumption time significantly reduced with increase in shaking speed.

It was 96 h at 150 and 200 rpm while at 250 and 300 rpm, it was reduced to 72 h. The cell growth increased continuously as agitation speed was raised from 100 (OD_{600}: 35.0) to 300 rpm (OD_{600}: 57.1). The xylitol titer and yield also enhanced linearly with increase in agitation till 250 rpm and thereafter, the numbers dropped. The highest titer and yield of 36.6 g/L and 0.61 g/g were recorded at 250 rpm. On the other hand, increasing agitation speed to 300 rpm resulted in higher biomass production but reduced xylitol accumulation.

The results of the present study are in agreement with literature which suggested that higher agitation speed enhanced cell growth while moderate agitation speed was found to be beneficial for xylitol accumulation. Xylose to xylitol conversion is a biotransformation reaction and a high concentration of biocatalyst is beneficial for accumulating high product level which is xylitol in current study. However, a biomass concentration beyond a given threshold could negatively affect xylitol biosynthesis by imposing oxygen limitation [23]. Similar observation was made by Unrean and Ketsub, 2018 [24] that increment in oxygen levels up to certain level enhanced cell growth and xylitol production and too high oxygen levels reduced xylitol accumulation. Zhang et al., 2012[25] attempted both single-stage and two-stage fermentation processes to investigate impact of shaking speed on xylitol accumulation by *Candida athensensis* strain. They achieved highest xylitol concentration of 112.6 g/L with xylose to xylitol bioconversion efficiency of 82.6% in single stage fermentation. In two-stage fermentation, 200 rpm was maintained for first 36 h and thereafter, agitation speed was reduced to 100 rpm for remaining time. This arrangement resulted in maximal xylitol production (115.6 g/L) and highest bioconversion efficiency of 84.6%. In both the cases, cell growth was compromised to maximize xylitol formation. Thus, a trade-off between the biomass formation and xylitol accumulation requires a moderate shaking speed or a microaerobic condition to maximize the xylitol yield. All these results show a fine-tuning of oxygen level is necessary for high level accumulation of xylitol.

Optimization of media components for maximizing xylitol production

To analyze the effect of medium components on xylitol production, we adopted a three-level Box Behnken design. The design matrix along with the observed and predicted responses for xylitol production is shown in Table 1. Multiple regression analysis was carried out to study the model accuracy and based on the evaluation, the second-order polynomial model was fitted to equation.

Where, X_1 = xylose (g/L), X_2 = ammonium sulphate (g/L), X_3 = KH_2PO_4 (g/L) and X_4 = Yeast extract (g/L).

Table 2 shows the statistical tool ANOVA that was used to interpret the results. Fisher *F* test was highly significant, with $P<0.05$. The coefficient of determination (R^2) was used to determine the model's goodness of fit and it was found to be 0.98, which leads to the conclusion that 98.0% of the variation in the model could be explained. Another function called as the "lack of fit" analyses the failure of the model and represents data in experimental domains at points not mentioned in the model. Herein, the model lack of fit was found to be insignificant ($p>0.05$) with F value of 0.92.

Production of the xylitol was well explained by the tested variables with the help of 3D surface plots. The plot was so built against any two independent variables that the response (xylitol titer) was plotted on z-axis while maintaining other variables at their mid-levels, as shown in the Fig. 5 A-F. It is evidenced from the graph that there is a direct correlation between xylose concentration and xylitol production. There is a steep increase in xylitol production with growing amount of xylose leading to the maximum xylitol production. Likewise, the same pattern is shown by yeast extract (Fig. 5 C, E & F), where the optimum yeast extract concentration showed an improved maximum production of xylitol. From these graphs, it can be noted that maximum xylitol production was achieved with a mid-level concentration of xylose and higher concentration of yeast extract. On the contrary, at mid concentration of ammonium sulfate (Fig. 5 A, D & E) and KH_2PO_4 (Fig. 5 B, D & F) turned out to be more helpful in increasing the xylitol production. Previously Ling et al., 2011[26] adapted central composite design (CCD) for enhanced xylitol production using *Candida tropicalis* HDY-02. They found that medium components such as KH_2PO_4, yeast extract, $(NH_4)_2SO_4$ and $MgSO_4{\cdot}7H_2O$ had significant effect on xylitol production. Likewise, Yewale et al., 2017 [27] performed Plackett–Burman (PB) design followed by CCD and reported components such as KH_2PO_4, yeast extract and xylose are the key factors for xylitol production. These studies indicate the importance of statistical method to identify the important components affecting growth and product formation.

The validation experiment was performed at the value obtained by Derringer's desired function methodology shown in Table S2 (Additional File 1). The target value was set for 50 g/L and the desirable value was found to be 0.99, showing higher probability to achieve the set target value. The optimal batch experiments were performed in two different sets in shake flask: with pure xylose (Fig. 6A) and xylose rich hemicellulosic pre-hydrolysate (Fig. 6B). The high initial xylose level of 150 g/L caused a lag phase of 24 h. After 24 h, xylose uptake was significantly improved, growth was picked up and cells entered log phase. The major fraction of the xylose was depleted between 120-144 h time interval where a residual xylose concentration left was ~5.0 g/L. The cell growth enhanced linearly, nearly became steady after 120 h and reached ~80 (OD_{600}). The results show that the cell growth was unaffected by inhibitors such as acetic acid and furfural present in the pre-hydrolysate. With pure xylose as carbon source the *P. fermentans* was able to convert 48.9% of xylose to xylitol with the titer of 70.5 g/L. In case of pre-hydrolysate, the strain was able to accumulate of 62.3 g/L of xylitol with the conversion yield of 42.7%. The optimized medium composition positively affected cellular growth and xylose assimilation, which eventually boosted xylitol synthesis. The concentration of xylitol produced is far superior to the results obtained by Huang et al. (2011)[11], however, yields were lower. They used non-detoxified xylose rich rice straw and SCB hydrolysate for xylitol production by *Candida tropicalis* JH030. The yeast accumulated ~ 31.0 g/L with conversion yield of 0.71 g/g from SCB while xylitol titer and yield achieved with rice straw was 12.5 g/L and 0.51g/g, respectively. Similarly, de Freitas Branco et al. (2011)[28] obtained 36.3 g/L of xylitol with yield of 0.60 g/g by *Candida guilliermondii* FTI 20037 using SCB as crude renewable source.

Batch cultivation in bioreactor

The data was scaled up from shake flask to bioreactor and the batch experiments were performed in 2.5 L bench scale bioreactor with 1L working volume using pure xylose and pre-hydrolysate. The process parameters were same to shake flask validation studies expect the aeration maintained at 1.0 L/min and pH was not controlled. The time course profiles for the fermentation are shown in Fig. 7. Xylose uptake, xylitol titer and yield were significantly improved from shake flask to bioreactor cultivation and the xylose utilization was more than 95% in both the cases. The lag phase was largely reduced in comparison to shake flask cultivation. The xylose underwent fast depletion and the maximum cell OD_{600} of 98 was achieved at 168 h with pure xylose, which was significantly higher than obtained in shake flask studies. The highest amount of xylitol recorded was 98.9 g/L of xylitol with the yield of 0.67 g/g at 168 h time interval (Fig. 7A). The fermentation profile of *P. fermentans* with pre-hydrolysate is shown in Fig. 7B. The maximum cell OD_{600} achieved with pre-hydrolysate was 99, which is quite similar to growth obtained with pure xylose. However, the xylitol production (79 g/L) and yield (0.54 g/g) was substantially lower than with pure xylose. Such improvement in xylitol production could be due to the better mixing effects and consequently improved mass transfer in the bioreactor. More particularly, the role of controlled aeration in a bioreactor cannot be overlooked as it directly affects the oxygen transfer rate, a key parameter affecting xylitol production as established earlier [6,8,22].

For the cost effective industrial production, it is desirable to have excellent tolerance against pH fluctuations and high substrate concentration. The pH declined in both the cases as fermentation progressed and the final pH at the end of 168 h was 4.8 and 5.4 with pure xylose and pre-hydrolysate. The *P. fermentans* displayed excellent pH tolerance without compromising the xylitol production which is an added advantage that for industrial production of xylitol. After optimizing media, when the xylose concentration was increased from 60 to 150 g/L, the yeast amassed good level of xylitol from pure as well as crude xylose and the yield was unaffected. Most of the xylitol producing yeasts displayed higher production when the initial xylose concentration was maintained between 100 to 200 g/L and our results are in consistent with previous reports [22,29]. The difference in the xylitol production with pure xylose and pre-hydrolysate could be attributed to presence of known as well as unknown/undetected inhibitors, which negatively affected the performance.

Table 3 compares the xylitol production achieved in the current study with other reports using pure xylose with/without co-substrate or xylose rich hemicellulosic hydrolysates for xylitol production. The range of xylitol titer and yield obtained in these studies broadly varies from 12.5 to 172.4 g/L and 0.51 to 1.0 g/g depending on feedstock/carbon source, inhibitor composition and/or detoxification method. The results obtained in current work are comparable or even better than data available in literature, making it competitive, however, the volumetric productivities were lower. In last decade, a variety of wastes streams rich in xylose have been investigated for xylitol production. The processing of LCB-based materials is must to release fermentable carbon, which not only generate sugars but also fermentation inhibitors such as weak acids, furan derivatives, phenolics etc. Therefore, detoxification methods such as over liming, activated charcoal, ion exchange resin adsorption treatment or a combination of these methods is required to remove these inhibitors for a smooth and effective fermentation [6,8]. The process of

detoxification need additional facilities and significantly increases the capital investment. Moreover, detoxification usually leads to loss of sugars, curbing down the fermentation performance. Thus, it would be very attractive to manufacture xylitol from crude xylose without detoxification [11]. Usually, the amount of xylitol obtained with untreated non-detoxified hemicellulosic hydrolysate are much lower in comparison to synthetic medium containing pure xylose. Therefore, majority of reports using hydrolysate for xylitol production have detoxified the hydrolysate for improving fermentation performance [11]. However, in current study, the high xylitol titer (79.0 g/L) and yield (0.53 g/g) obtained with SCB pre-hydrolysate without detoxification and proves the immense potential of *P. fermentans*.

Conclusions

The work describes the xylose assimilation and xylitol production capability of the newly isolated yeast strain *P. fermentans* from food waste. The strain was able to accumulate high levels of xylitol even at high concentration of pure as well as crude xylose, competitive with previous work. About 79.0 g/L xylitol with a conversion yield of 53% was obtained from xylose rich hemicellulosic pre-hydrolysate using *P. fermentans*. The advantageous feature of the strain is that it was able to synthesize xylitol from hydrolysate without detoxification and lack of detoxification requirements can make the process more economical. Unlike other hyper-producer of xylitol, *P. fermentans* is a safe organism and well-known for producing flavour and aroma metabolites with applicatios in the food and beverage industries. The work demonstrates remarkable potential of the isolated strain to valorise xylose rich waste streams and can contribute towards establishing profitable lignocellulosic biorefineries. Low volumetric productivity and the substantial amount of complex nitrogen sources used in the current work are the bottlenecks for bulk production of xylitol and needs to be taken care. *P. fermentans* could be used as starting point and commercial feasibility for xylitol production could be achieved by application of metabolic engineering approaches and bioprocess optimization.

Further studies are underway.

Methods

Materials used in this study

All the chemicals used in this study were of analytical grade and purchased from Sigma Aldrich (USA). The molecular biology reagents were procured from New England Biolabs (USA) and NBS Biologicals (UK). The xylose rich pre-hydrolysate obtained after hydrothermal pretreatment of SCB was kindly provided by Nova Pangaea Technologies, UK. The composition of the concentrated pre-hydrolysate was as follows: xylose, 500.0 g/L; glucose, 50.0 g/L; arabinose, 37.5 g/L; acetic acid, 55.0 g/L; furfural < 1.0 g/L.

Isolation and maintenance of xylose assimilating yeast

The xylose assimilating yeast used in the present study was isolated from mixed food waste, collected from Cranfield Student Association (CSA) (52°04'20.4"N 0°37'45.0"W) at Cranfield University, UK. The morphological characteristics were analyzed with microscopic observation. The isolated yeast showed an ellipsoid structure having 3-4 µm diameter (Fig. 1A). Further, 26s rRNA and ITS (internal transcribing spacers) sequence of isolated yeast were aligned with sequences available in NCBI database using Clustal W and Mega 10.0 software. The alignment results showed that isolated yeast strain shared 99 % homology with *Pichia fermentans*. Phylogenetic trees were constructed based on 26S rRNA sequence of the isolated strain using Neighbor-Joining method, further the distance between the evolutionary species were determined according to Kimura 2-model (Fig. 1B).

It was routinely maintained on modified yeast extract-xylose-malt extract (YXM) agar containing 3.0 g/L yeast extract, 3.0 g/L malt extract, 5.0 g/L peptone, 10.0 g/L xylose, 20.0 g/L agar. Bacterial contamination was prevented by adding 10.0 µg/ml chloramphenicol to the said medium and adjusting initial pH to 3.5. The yeast was regularly sub-cultured every two weeks and plates were stored at 4°C in refrigerator.

Shake flask cultivation of *P. fermentans* for xylitol production

Shake flask studies were conducted to investigate the xylitol production potential of *P. fermentans*. Two different strategies were adopted to assess the ability of the strain for xylitol production, one being use of single substrate or pure xylose and other being use of glucose (10 g/L) as co-substrate with xylose. The medium used for preparing seed culture and submerged xylitol fermentations was according to [30] with slight modification, having the following composition: 10.0 g/L yeast extract, 20.0 g/L peptone, 30.0 g/L xylose, 0.5 g/L KH_2PO_4, 0.5 g/L $(NH_4)_2SO_4$, and 0.5 g/L $MgSO_4$. The seed culture was grown in a 100 mL Erlenmeyer flask containing 25 mL of above-mentioned medium using single colony of *P. fermentans*. The final pH of the medium prior to sterilization was adjusted to 7.0. Cultivation was carried out for 24 h at 30 °C on a rotary shaker with agitation speed of 250 rpm. The submerged cultivations were carried out in 500 mL Erlenmeyer flasks containing 100 mL working volume. The flask were inoculated with fresh inoculum at OD_{600} of 1.0 and kept at 30 °C under constant shaking at 250 rpm on a rotary shaker (Excella 24, New Brunswick).

Random mutagenesis using ethyl methanesulphonate (EMS)

The chemical mutagenesis was performed according to protocol by [31]. A single colony of *P. fermentans* was grown overnight in 5 mL of YPX medium at 30 °C on a rotary shaker with agitation speed of 250 rpm and then centrifuged. The collected cell mass was washed thrice with sterile distilled water and suspended in 10 mL of phosphate buffer (0.1M, pH 7.0). The sample concentration was adjusted to obtain the cell count of ~2×10^8 cells/mL. One mL of the cell solution was centrifuged for 30 sec at 20000 x g. The cell pellet was suspended in 1 mL of phosphate buffer (0.1 M, pH 7.0) in a 15 mL sterile centrifuge tube and 50 µL of 20 mM EMS was added to cell suspension. This was followed by vortexing and incubation for different intervals of time at 30 °C. The reaction was stopped by adding 8.0 mL of 5%

sterile sodium thiosulfate to 0.2 mL of mutated culture. Finally, 0.1 mL of the culture was plated on YPX agar medium at pH 7.0 and 28 °C until colonies were formed.

Measurement of enzymatic activities

The enzymatic activities of xylose reductase (XR) and xylitol dehydrogenase (XDH) were measured according to protocol as described earlier [32].

Media optimization to maximize xylitol production using Box Behnken Design

Media optimization to maximize xylitol production was carried out using Box Behnken Design (BBD) as described in Prabhu et al., 2017[33]. The medium components chosen for optimization were xylose, ammonium sulphate($(NH_4)_2SO_4$), potassium dihydrogen phosphate (KH_2PO_4) and yeast extract. The levels of each variables are shown in Table S1 (Additional information). The optimal levels were predicted with the aid of second order polynomial.

Batch cultivation in bioreactor

The batch experiments were performed in a 2.5 L bioreactor (Electrolab Bioreactors, UK) with 1.0 L working volume. The inoculum was prepared using optimized media, and the optimum values of media components were as follows (g/L): xylose, 150.0; KH_2PO_4, 0.30; $(NH_4)_2SO_4$, 0.46; yeast extract, 18.36. The starting pH was 7.0 and not controlled during the fermentation. The temperature and agitation speed were controlled at 30°C and 250 rpm, respectively, while the aeration rate was maintained at 1.0 L/min.

Analytical methods

The samples were withdrawn periodically and analysed for OD, pH, residual glucose, xylose and xylitol. Cell growth was quantified by measuring the optical density at 600 nm wavelength in a 1 mm-path-length cuvette using a double beam spectrophotometer (Jenway 6310, UK). The concentrations of glucose, xylose and xylitol were measured by HPLC (Agilent Technologies 1200 series, USA) as describe in [32]. All measurements were conducted in triplicates and values were averaged. The standard deviation was not more than 10 %.

Declarations

Acknowledgement

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decision to publish, or preparation of the article.

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Contributions

AAP, EB and YA carried out all the experimental work. VK analysed the data and wrote the manuscript. DA, VV, VKT and FC were involved in proof reading the manuscript and revised the manuscript critically. All authors read and approved the final manuscript.

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Ethics declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

All data generated or analyzed during this study are included in the manuscript.

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Tables

Table 1: Box-Behnken design matrix with un-coded values along with observed and predicted response for xylitol production

Xylose (g/L)	Ammonium sulphate (g/L)	KH_2PO4 (g/L)	Yeast extract (g/L)	Xylitol (g/L)	Xylitol (Pred)
50	0.1	0.55	11	14.4	14.39
150	0.1	0.55	11	32.33	31.91
50	1	0.55	11	10.17	11.59
150	1	0.55	11	21.02	22.04
100	0.55	0.1	2	20.53	20.10
100	0.55	1	2	17.81	17.28
100	0.55	0.1	20	19.05	20.58
100	0.55	1	20	12.65	14.08
50	0.55	0.55	2	32.48	32.76
150	0.55	0.55	2	25.96	27.79
50	0.55	0.55	20	14.04	12.44
150	0.55	0.55	20	45.42	45.38
100	0.1	0.1	11	11.52	13.82
100	1	0.1	11	7.93	7.64
100	0.1	1	11	8.78	9.31
100	1	1	11	4.89	2.83
50	0.55	0.1	11	11.03	10.05
150	0.55	0.1	11	44.89	42.75
50	0.55	1	11	23.21	24.10
150	0.55	1	11	19.64	19.38
100	0.1	0.55	2	18.03	16.87
100	1	0.55	2	12.33	12.33
100	0.1	0.55	20	18.55	17.31
100	1	0.55	20	9.26	9.18
100	0.55	0.55	11	34.17	35.05
100	0.55	0.55	11	36.04	35.05
100	0.55	0.55	11	37.85	35.05

100	0.55	0.55	11	34.22	35.05
100	0.55	0.55	11	32.99	35.05

Table 2: ANOVA for quadratic model*

Source	DF	Seq SS	Adj SS	Adj MS	F value	P value
Regression	14	3685.87	3685.87	263.28	77.1	0
Linear	4	778.17	778.17	194.54	56.97	0
X1	1	587.02	587.02	587.02	171.9	0
X2	1	120.4	120.4	120.4	35.26	0
X3	1	65.19	65.19	65.19	19.09	0.001
X4	1	5.56	5.56	5.56	1.63	0.223
Square	4	2179.18	2179.18	544.79	159.54	0
X1*X1	1	158.66	0.58	0.58	0.17	0.685
X2*X2	1	1106.41	1532.83	1532.83	448.87	0
X3*X3	1	698.9	825.68	825.68	241.79	0
X4*X4	1	215.2	215.2	215.2	63.02	0
Interaction	6	728.52	728.52	121.42	35.56	0
X1*X2	1	12.53	12.53	12.53	3.67	0.076
X1*X3	1	350.25	350.25	350.25	102.57	0
X1*X4	1	359.1	359.1	359.1	105.16	0
X2*X3	1	0.02	0.02	0.02	0.01	0.936
X2*X4	1	3.22	3.22	3.22	0.94	0.348
X3*X4	1	3.39	3.39	3.39	0.99	0.336
Residual Error	14	47.81	47.81	3.41		
Lack-of-Fit	10	33.28	33.28	3.33	0.92	0.588
Pure Error	4	14.53	14.53	3.63		
Total	28	3733.67				

*DF=Degree of freedom; Seq SS= Sequential sum of square; Adj SS= Adjusted sum of square; Adj MS= Adjusted mean square; F= Variance ratio (Fisher F-value); P=Probability value

Table 3: Comparison of xylitol production by different microorganisms

Microorganism	Feedstock	Detoxification	Xylitol titer (g/L)	Xylitol yield (g/g)	Reference
Candida tropicalis BSXDH-3	Pure xylose, glycerol & glucose	-	48.6	0.98	[34]
Debaryomyceshansenii	Pure xylose	-	68.6	0.76	[35]
Debaryomyces hansenii	Pure xylose & glycerol	-	53.7	0.95	[16]
Kluyveromycesmarxianus YZJ015	Pure xylose	-	71.5	0.83	[21]
E. coli	Pure xylose & glucose	-	172.4	-	[36]
Yarrowia lipolytica	Pure xylose & glycerol	-	53.2	0.97	[32]
P. fermentans	Pure xylose	-	**98.9**	**0.66**	This study
Candida guilliermondii FTI 20037	Sugarcane bagasse	Detoxified	36.3	0.64	[37]
Candida tropicalis W103	Corncob	Detoxified	68.4	0.70	[19]
Candida tropicalis JH030	Sugarcane bagasse	Non-detoxified	12.5	0.51	[11]
Candida tropicalis JH030	Rice straw	Non-detoxified	~32	0.71	[11]
Debaryomyceshansenii	Sugarcane bagasse	Detoxified	13.8	0.69	[35]
Pichia stipitis	Corn stover	Detoxified	12.5	0.61	[38]
Candida athensensis SB18	Horticultural waste	Detoxified	100.1	0.81	[25]
E. coli	Corncob & glucose	Detoxified	150.0	>0.95	[36]
P. fermentans	Sugarcane bagasse	Non-detoxified	**79.0**	**0.53**	This study

Figures

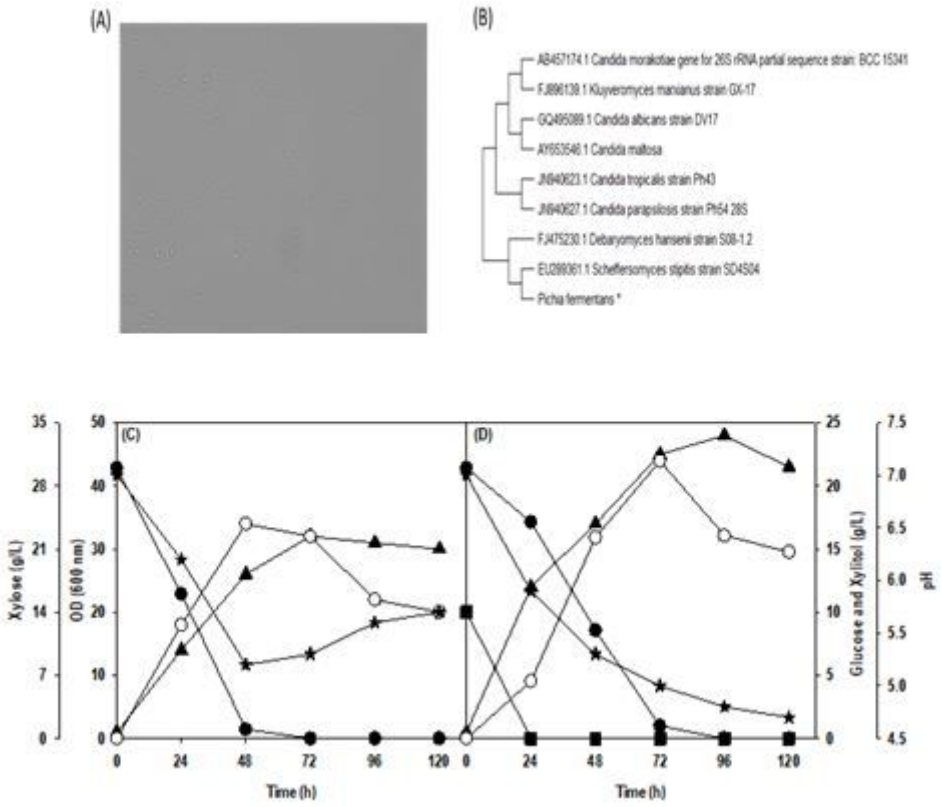

Figure 1

(a) Morphological identification of Pichia fermentans by microscopy. (b) Phylogenetic tree based on 26s rRNA sequence, using neighbor joining method clustered with bootstrap test with 1000 replicates. The isolated strain reported in the present study is marked with a star. Time course profiles for shake flask cultivation of P. fermentans on; (C) xylose and (D) xylose + glucose. Symbols: filled square (glucose), filled circle (xylose), filled triangle (OD600), empty circle (xylitol) and filled star (pH).

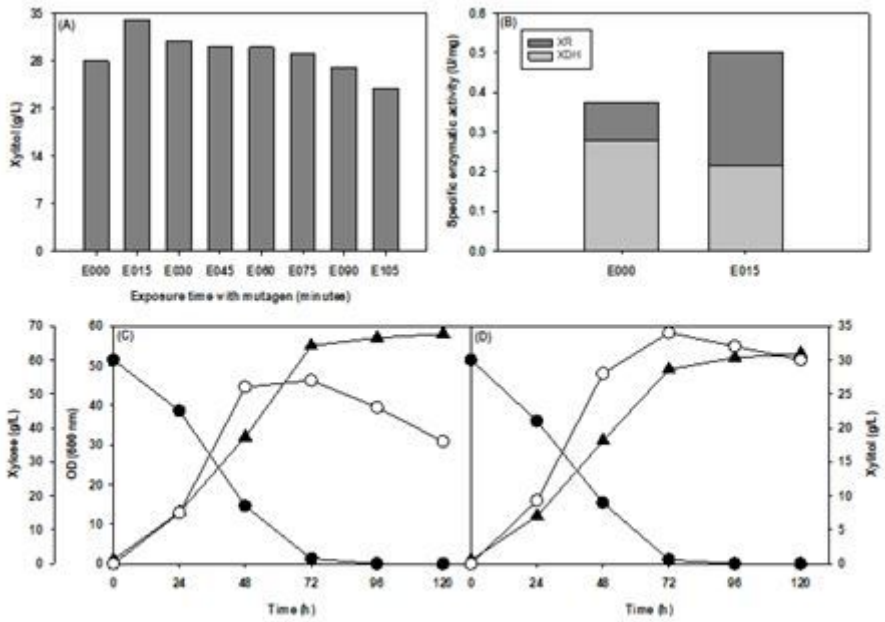

Figure 2

(A) Exposure of wild type P. fermentans with chemical mutagen EMS for different times; (B) Specific Enzymatic activity of xylose reductase and xylitol dehydrogenase during fermentation; Batch fermentation for xylitol production by (C) wild type and (D) mutant (E015) strain of P. fermentans. Symbols: filled circle (xylose), filled triangle (OD600) and empty circle (xylitol).

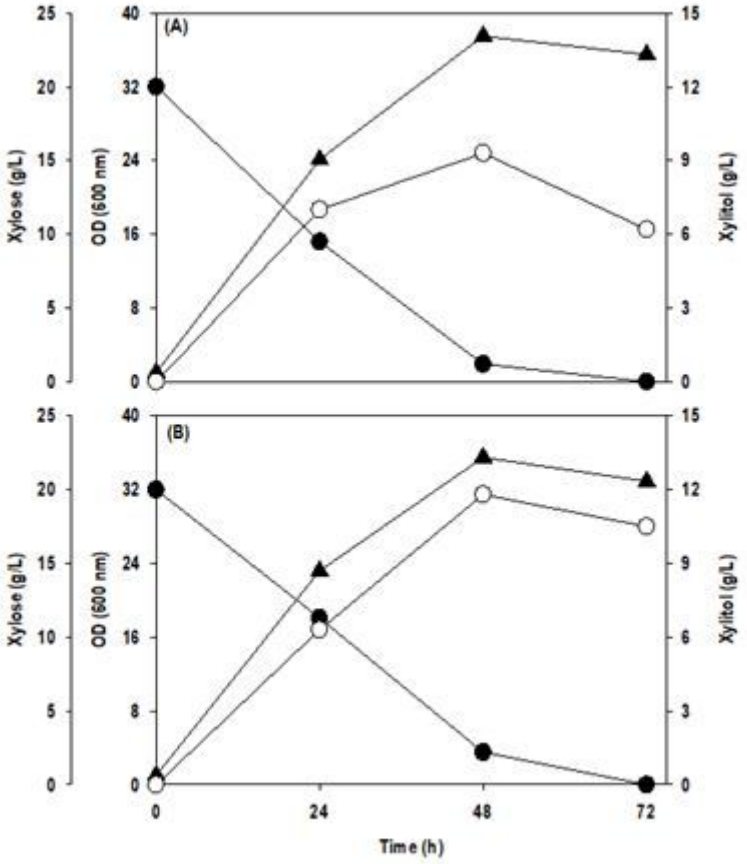

Figure 3

Xylitol production using xylose rich hemicellulosic pre-hydrolysate from sugarcane bagasse by (A) wild type and (B) mutant strain of P. fermentans. Symbols: filled circle (xylose), filled triangle (OD600) and empty circle (xylitol).

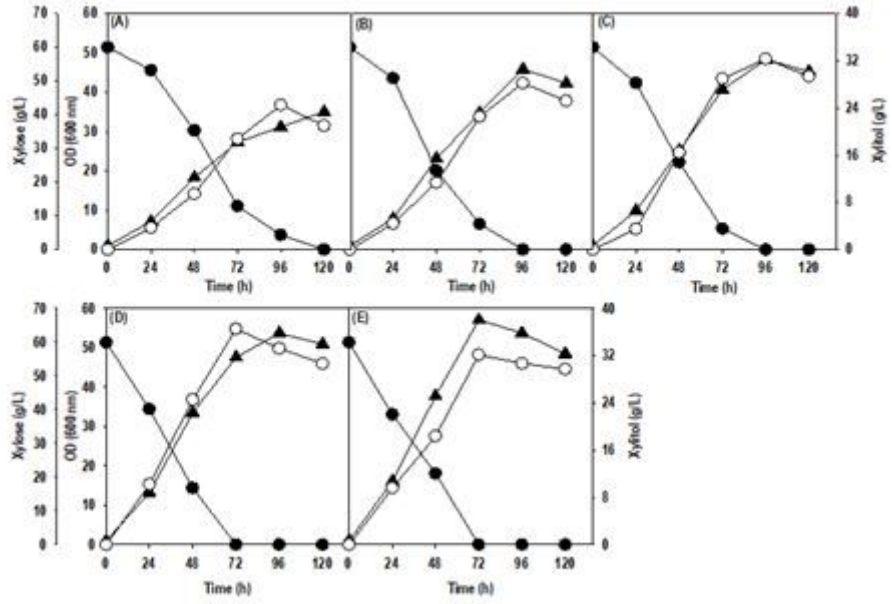

Figure 4

Impact of agitation speed on xylose uptake, cell growth and xylitol formation by P. fermentans in shake flask (A) 100 rpm, (B) 150 rpm, (C) 200 rpm, (D) 250 rpm and (E) 300 rpm. Symbols: filled circle (xylose), filled triangle (OD600) and empty circle (xylitol).

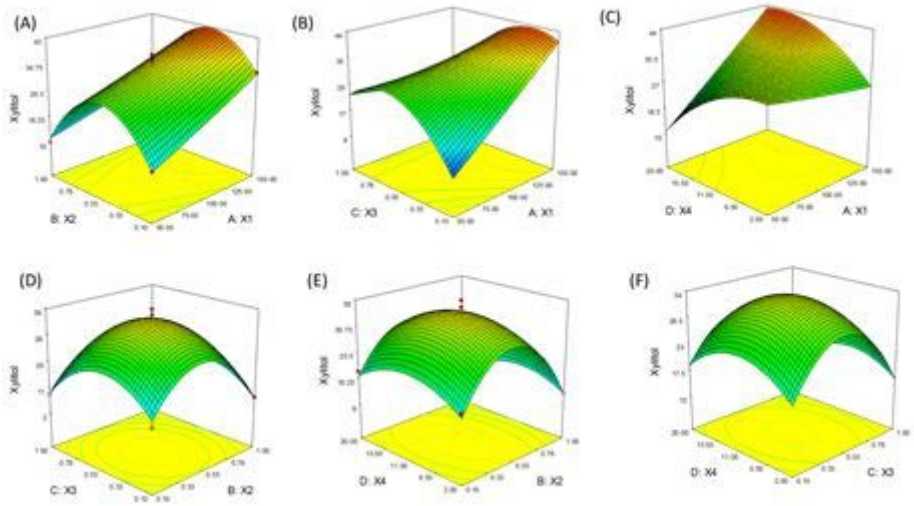

Figure 5

Three-dimensional response surface plot for xylitol production showing the interactive effects of (A) xylose (X1) and ammonium sulphate (X2) (B) xylose (X1) and KH2PO4 (X3) (C) xylose (X1) and yeast extract (X4), (D) ammonium sulphate (X2) and KH2PO4 (X3) , (E) ammonium sulphate (X2) and yeast extract (X4), (F) KH2PO4 (X3) and yeast extract (X4) with the remaining factors kept constant at the middle level of the central composite experimental design.

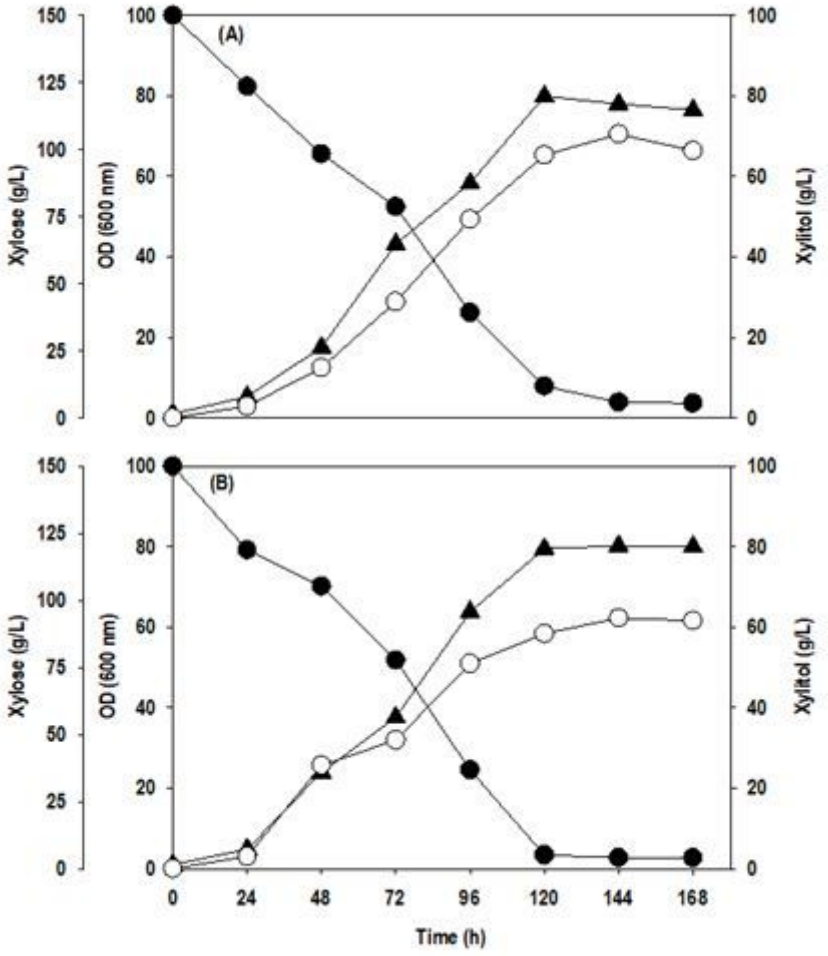

Figure 6

Shake flask cultivation of P. fermentans using optimized media on; (A) pure xylose and (B) xylose rich hemicellulosic pre-hydrolysate from sugarcane bagasse. Symbols: filled circle (xylose), filled triangle (OD600) and empty circle (xylitol).

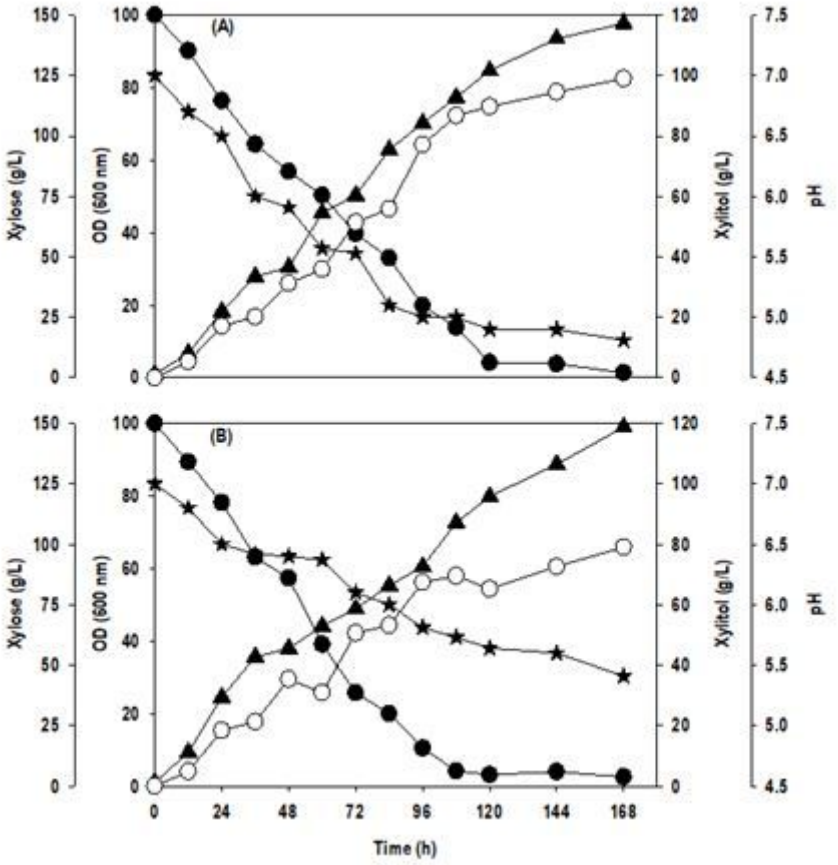

Figure 7

Batch kinetics of substrate assimilation, cell growth, pH and xylitol formation by P. fermentans in bioreactor on; (A) pure xylose and (B) xylose rich hemicellulosic pre-hydrolysate from sugarcane bagasse. Symbols: filled circle (xylose), filled triangle (OD600), empty circle (xylitol) and filled star (pH).

Supplementary Files

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