Biovalorisation of crude glycerol and xylose into xylitol by oleaginous yeast *Yarrowia lipolytica*

Ashish Prabhu
Cranfield University
Dominic J Thomas
Cranfield University
Rodrigo Ledesma- Amaro
Imperial College London
Gary A Leeke
University of Birmingham
Angel Medina Vaya
Cranfield University
Carol Verheecke-Vaessen
Cranfield University
Frederic Coulon
Cranfield University
Vinod Kumar (vinod.kumar@cranfield.ac.uk)
Cranfield University https://orcid.org/0000-0001-8967-6119

Research

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Abstract

Background: Xylitol is a commercially important chemical with multiple applications in food and pharmaceutical industries. According to the US Department of Energy, xylitol is among the twelve platform chemicals that can be produced from biomass. The chemical method for xylitol synthesis is however expensive and energy intensive. In contrast, the biological route involving microbial cell factories offers a potential cost-effective alternative process. The bioprocess occurs under ambient conditions and makes use of biocatalysts which can be sourced from renewable carbon coming from a variety of cheap feedstocks classified as wastes.

Result: In this study, biotransformation of xylose to xylitol was investigated using *Yarrowia lipolytica* an oleaginous yeast grown on glycerol/glucose screening of primary carbon source, media optimisation in shake flask, scale up in bioreactor and downstream processing of xylitol were carried out. With the two step medium optimization involving central composite design and artificial neural network coupled with genetic algorithm, the yeast amassed 53.2 g/L of xylitol using glycerol and xylose with a bioconversion yield of 0.97 g/g. Similar results were obtained when pure glycerol was substituted with crude glycerol from biodiesel industry (titer: 50.5 g/L; yield: 0.92 g/g). Even when xylose from lignocellulosic hydrolysate was used as opposed to pure xylose, a xylitol yield of 0.54 g/g was achieved. Xylitol was successfully crystallized from fermented broth using pure glycerol/xylose and crude glycerol /xylose with a recovery of 35 and 39.45%, respectively.

Conclusion: To the best of the author's knowledge, this is the first study demonstrating the potential of *Y. lipolytica* as microbial cell factory for xylitol synthesis from inexpensive feedstocks. The results obtained are competitive with other xylitol producing organisms.

Background

The development of green technologies is highly desired as the world is facing problems of progressive depletion of non-renewable fossil fuels coupled with exponentially growing human population [1]. Biorefineries, processing biomass for integrated chemicals, fuels and energy production, are promising alternative options and good examples of green and sustainable technologies. First generation biorefineries making use of edible feedstocks are well established. The food versus feed debate has necessitated the use of non-edible feedstocks, especially the wastes containing renewable carbon [2, 3]. Waste biorefineries are receiving significant attention because they not only generate high value products but also contribute to sustainable waste management. The utilisation of such cheap substrates is necessary to create economical bioproduction. Thus, over the last two decades, there has been a great deal of research dedicated to the development of bioprocesses based on agro-industrial wastes[4].

Lignocellulosic biomass (LCB) is the most abundant biomass on earth. The three major components of LCB are cellulose (34–50%), hemicellulose (19–34%) and lignin (11–30%). Hemicellulose is the second most abundant polymer in LCB after cellulose. It is a heterogeneous biopolymer, majorly consist of xylose

(~ 90%), contributing 15–35% of the total dry cell weight of LCB [5, 6]. Despite this, the application of xylose as a potential feedstock is overlooked for biorefineries and discarded as waste. This is due to a lack of efficient fermentation systems, as many of the microorganisms do not have native pathway for metabolizing xylose. In addition, uptake of xylose is suppressed in presence of glucose due to carbon catabolite repression. However, utilisation of xylose is necessary for commercial viability of lignocellulosic biorefineries [7, 8]. Like xylose/LCB, crude glycerol (CG) is another carbon source waste product of interest for microbial conversion. CG is an inevitable and major by-product of industries such as bioethanol, oleochemical, chemical and biodiesel. After the advent of rapidly growing biodiesel industries, the increment in CG production has been exponential [9]. It has been estimated that the production of every 100 kg of biodiesel generates about 10 kg of CG. The biodiesel production ascended exponentially from 2005 to 2015 and is forecasted to enhance by another 35% by 2025[10]. It is imperative to manage these wastes otherwise; they are going to contribute to environmental pollution. Therefore, the design of sustainable processes for transforming biowastes into higher value products has become an urgent requirement.

Xylitol is a commercially important chemical. According to the US Department of Energy, xylitol is among the top twelve platform chemicals, which can be produced from biomass [11]. Chemically, it is a polyol (sugar alcohol) containing five carbon atoms where an hydroxyl group is attached to each carbon atom. Xylitol is produced chemically/biochemically by reduction of xylose. The molecule is equivalent to sucrose in sweetness but has less calories and lower glycaemic index [5, 12]. Due to its high endothermicity, low glycaemic rates, cariostatic properties, lack of carcinogenicity, non-involvement in insulin metabolic pathway and non-interference with food nutritional value, xylitol finds applications in food and pharmaceutical industries[13]. Xylitol has a huge market and 12% share of the total polyols market, which is expanding rapidly. The global market for xylitol in 2016 was 190.9 thousand metric tons and is anticipated to reach 266.5 thousand metric tons in 2022 with an amount above US$1 billion[6].

Yarrowia lipolytica is a non-conventional, oleaginous, safe and robust yeast with multiple biotechnological applications. It displays a versatile characteristic such as high cell density cultivation, metabolic flexibility and tends to accumulate a wide array of industrially important metabolites. In addition, the yeast is non-pathogenic and has a GRAS (generally regarded as safe) status [14]. The unique features of Y. lipolytica makes it a promising cell factory for the production of value added chemical. Glycerol is the most preferred carbon source for Y. lipolytica and can metabolize it with great efficiency. As per the literature, majority of the Yarrowia strain cannot grow on xylose as it possess strong xylose reductase activity but have low xylitol dehydrogenase activity, however, some can bio transform xylose into xylitol. The current study was undertaken to investigate xylitol accumulating ability of Y. lipolytica Po1t (Ura^+, Leu^+) [15]. The biotransformation of xylose into xylitol was carried out by cell biocatalysts grown on pure/crude glycerol or glucose. The work included screening of primary carbon source, media optimization in shake flask, scale up in bioreactor and downstream processing of xylitol.

Results

Xylitol production in shake flasks

The biochemical production of xylitol takes place through reduction of xylose to xylitol mediated by xylose reductase (XR) and the electron transfer for this reaction is facilitated through participation of the redox cofactor NAD(P)H (Fig. 1). The produced xylitol is further metabolized and xylitol derivatives enter the central carbon metabolism for cell maintenance and growth [13]. The *Y. lipolytica* Po1t (Ura^+ Leu^+) strain used in this study has the ability to transform xylose into xylitol but it cannot grow on xylose as sole carbon source (data not shown). Therefore, the biotransformation medium was supplemented with a co-substrate which can promote substantial cell growth of the yeast. For this reason, glucose and glycerol were employed to accumulate *Y. lipolytica* cells to biotransform xylose into xylitol. The results obtained with glucose and glycerol are shown in Fig. 2. The assimilation of glycerol was faster than glucose. All the supplied glycerol was completely exhausted within 48h whereas more than 15% of glucose was left unconsumed in the same time. The highest OD_{600} values obtained with glycerol and glucose were quite similar, 29.2 and 28.0, respectively. Nearly at that time, when a major amount glucose/glycerol was almost exhausted (~48h), biotransformation began and in next 24-48h, the maximum production of xylitol was recorded. The biotransformation rate was significantly faster with glycerol than glucose with maximum amount of xylitol produced in 72h with glycerol and 96h with glucose. The xylitol titre was 16.0 g/L with the yield of 0.80 g/g when glycerol was a co-substrate while 12.7 g/L of xylitol was achieved with a conversion yield of 0.64 g/g using glucose. The pH dropped during growth phase, reduced below 4.0 after 48h and then remained almost constant during xylitol production phase. The biotransformation yield obtained was higher with glycerol and therefore, selected as co-substrate for further experiments.

Media optimization in shake flask to maximize biotransformation of xylose to xylitol

Central composite design (CCD)

Statistical methods measure the effects of change in operating variables and their mutual interactions on the process through experimental design techniques. In the present study, the central composite design (CCD) was employed to determine the optimum value of media components (xylose, YNB, NH_4Cl and phosphate buffer) to maximize xylitol production in shake flask. The design matrix and the corresponding results of observed and predicted responses (xylitol titre) are shown in Table 2. The results were analysed using the Analysis of Variance (ANOVA) shown in Table 3. The error term, which indicates that the amount of variation in the response data is very low. According to the ANOVA, the regression model for the xylitol production showed high significance with the F value of 22.41. The model with high Fisher's F value clearly explains most of the variations present in the experimental design [16]. Further the F-value for lack of fit was 0.92 and implies that the response (xylitol) is not significant relative to the pure error. The correlation coefficient (R^2) between the between experimental and model-predicted values of response variables showed high statistical significance of 94.16 %, which implies that only 5.84 % of the total variation was not explained by the model. The Student's t distribution and the corresponding p values depicts that most of the interaction terms are statistically significant($P<0.05$), except the

interaction terms involving xylose with NH_4Cl and phosphate showed insignificance. The second-order polynomial equation for xylitol production by CCD is given in equation 6.

$$Y_{xylitol} = 25.90 + 4.45X_1 + 1.46X_2 - 0.75X_3 - 0.68X_4 + 0.008X_1^2 - 3.64X_2^2 + 0.27X_3^2 - 1.35X_4^2 + 0.36X_1X_2 + 1.42X_1X_3 - 0.33X_1X_4 + 3.7X_2X_3 - 0.15X_2X_4 - 2.8X_3X_4 \quad (6)$$

The response surface plot for the interaction between the media components is depicted in Fig 3. The 3D surface plot generated gives the overview of the interaction effect between the two components on xylitol production by keeping other parameters as central value. The interaction between xylose and NH_4Cl showed a positive effect, with the increase in the concentration of xylose and NH_4Cl, there is a progressive increase in the xylitol production but at higher concentration of xylose there was reduced production of xylitol due to the substrate inhibition effect. Similarly, the interaction between xylose, YNB (Fig 3B) and NH_4Cl, YNB (Fig 3D) showed positive effect ($P<0.05$), which indicates that higher the concentration of the YNB and NH_4Cl will enhances the production of xylitol. On the other hand, the interactive term between xylose, phosphate buffer (Figure 3C), phosphate buffer, NH_4Cl (Figure 3E) and YNB, phosphate buffer (Figure 3F) showed statistical insignificant values, which indicates one of the components have to be kept at minimal level to enhance the xylitol production.

Optimization of Process parameters using artificial neural network linked genetic algorithm (ANN-GA)

The experimental design generated by the CCD is used as input feed for ANN algorithm. The overall data set were divided into three subsets- training (20 data points), validation (5 data points) and test sets (5 data points). The network training was carried out by adapting Levenberg-Marquardt (LM) backpropagation algorithm, which is a mere approximation of Newtons method. The training was carried for 1000 epochs and the mean square error (MSE) and R^2 value of training, validation and test points for xylitol production is shown in Table S1 (supplementary data). The data points apart from the training used to examine the validation. During training the data over fits and substantial error will be accumulated on the validation. When the error on the validation reaches the threshold point the weights and biases are adjusted to minimize the error[17,18]. Network topology have a crucial role in predicting results, the input-output neuron of ANN is the resemblance of input and output data used in this study. The number of neurons in the hidden layer was determined by trial and error method to minimize MSE. The MSE of xylitol production was found to be 7.425. The predicted value of ANN for xylitol is shown in Table 2. The optimum value was achieved with 4 inputs, 8 hidden layers and 1 output layer. The simulation of ANN resulted in R^2 of 0.938 between the actual experimental production values (Fig 4a). In order to further optimize the solution space for global optimum, GA was adapted to train ANN values. The values of GA specific parameters used in the optimization technique were as follows: population size = 20, cross over probability = 0.8, mutation probability=0.01, No. of generation = 100. The maximum of 47.72 g/L of xylitol production was observed with 160 iterations. The best fitness plot for the GA of xylitol production (Fig 4b) maps the gradual convergence of the best fitness values of successive generations

towards the final optimum value. The optimum values found to be xylose: 55 g/L, NH_4Cl: 3.94 g/L, YNB: 5 g/L and phosphate buffer: 132.5mM.

Validation of optimisation results

The validation experiments were performed in shake flasks based on the global optimum values obtained on the basis of ANN-GA training. Three different sets of experiments were conducted: pure glycerol + xylose (Fig. 5A); crude glycerol + xylose (Fig. 5B); pure glycerol + crude xylose (Fig. 5C). The crude carbon sources were included to test the ability of the *Y. lipolytica* strain to tolerate, utilize and valorise crude renewable sources. The glycerol uptake rate was similar for the case of pure and crude source and the major fraction of glycerol carbon was depleted in first 48 h indicating no effect of impurities in crude glycerol on *Y. lipolytica*. The cell concentration obtained with pure glycerol (OD_{600}: 34.2) was higher than crude glycerol (OD_{600}: 24.9). The low cell OD_{600} in comparison to pure glycerol might be attributed due to the presence of some of the inhibitory components such as methanol present in the crude glycerol [19]. The results showed significant improvements in xylitol titer and yield in comparison to unoptimzed composition. In case of pure glycerol and xylose, 98% of xylose was transformed into xylitol and a xylitol concentration of 54 g/L was achieved. On the other hand, for the co-fermentation of crude glycerol and xylose, the xylitol titre of 48.2 g/L was obtained with conversion yield of 0.88 g/g. This difference could be attributed to the contents of crude glycerol. The higher buffer concentration of optimized medium suppressed the reduction in pH and therefore, after some initial drop, pH was stable around 5.5. The pH plays a crucial role in the transportation of xylose across the membrane [20].

The ability of *Y. lipolytica* to produce xylitol from crude xylose was also tested. For this, crude xylose obtained from sugarcane bagasse was used. Lignocellulosic material often contains compounds such as phenols, furan derivatives and aliphatic acid in a large amount which tends to inhibit microbial growth [21]. However, the cell growth (OD_{600}: 29.1) was almost unaffected by the presence of impurities/inhibitory molecules in crude glycerol and crude xylose. The higher cell growth could also be contributed by other sugars such as glucose in the hydrolysate[22]. The conversion of crude xylose was 54 % with the xylitol titre of 5.4 g/L. The low biotransformation yield in comparison to pure xylose could be due to the negative impact of impurities on enzyme xylose reductase as the hydrolysate was used without any detoxification as it contains phenolic compounds such as furfural and hydroxymethyl furfural. All these strongly indicates potential of *Y. lipolytica* as microbial cell factory for xylitol production.

Submerged batch cultivations in bioreactor

In order to scale up the fermentation and validate the optimized medium composition, batch cultivations were carried out in a 2.5 L scale bench bioreactor with 1L working volume. The process condition was mimicked exactly as to that of the shake flask studies except the aeration. The aeration rate was maintained at 2.0 L/min for first 48h and then reduced to 1.0 L/min for the rest of period. Two separated batch fermentation were run with pure glycerol/xylose and crude glycerol/xylose. The time course for

both the fermentations were similar to shake flask cultivations (Fig. 6). Glycerol is the most preferred carbon source for *Y. lipolytica* and the presence of glycerol repressed the uptake of xylose as evident in Fig. 6. The gradual uptake of xylose concomitant with xylitol production was noticed when glycerol was almost completely exhausted. The maximum cell OD_{600} of 48.6 was observed at 120 h with co-fermentation of pure glycerol and xylose, which is higher than achieved during shake flask cultivation. The complete consumption of xylose was observed at 168 h and a maximum of 53.2 g/L of xylitol was produced with a yield of 0.97 g/g (Fig 6A). The fermentation profile of *Y. lipolytica* with crude glycerol and pure xylose is shown in Fig 6B. The maximum cell OD_{600} recorded was 31.8, not far from value obtained in shake flask studies. The yield of the xylitol was about 0.92 g/g with the titre of 50.5 g/L. Further, the pH in fermentation using pure carbon source fluctuated between the range of 6.5-5.55, whereas in fermentation with crude renewable glycerol the pH dropped to 4.3. Low aeration is maintained during xylitol production phase in the bioreactor because excess aeration causes re-oxidation of NADH, a co-factor necessary for xylitol production from xylose and NAD^+ produced can facilitate further metabolism of xylitol for cell growth [23].

Xylitol production by resting cells

Resting cells are metabolically active non-growing cells [24]. Resting cells show an advantage over active cells such as simple operation, no requirement of nutrient medium and convenient downstream processing [25,26]. *Y. lipolytica* used in the current study is carrying out single step biotransformation of xylose to xylitol. Therefore, it is worth to explore the potential of the yeast as a biocatalyst that can be employed for continuous production of xylitol through reusability of the cells. In other words, the growth phase was split from the xylitol production phase. The *Y. lipolytica* cells were grown using the pure and crude glycerol in shake flask using the culture medium described in section 2.4 without xylose. The fermentation was terminated once the cell OD_{600} reached 20-25, nearly after 10h and the cells were collected through filtration. The obtained cell pellet was suspended in buffer containing only xylose. The experiments were carried out using different concentrations of xylose (30, 70 and 100 g/L). It is evident from Fig. 7 that the conversion of xylose to xylitol was not satisfactory. The xylitol obtained for the case of cells accumulated on crude glycerol was ~10 g/L regardless of xylose concentration. The results obtained with pure glycerol were different. The highest amount of xylitol recorded with pure glycerol grown cell was ~28 g/L from 30 g/L xylose. However, the xylitol biotransformation yield significantly reduced with further increase in xylose levels. The probable cause for the cessation of the biocatalytic activity, may be due to the lack of ability of the already built biomass of *Y. lipolytica* cells to produce enough cofactors for continuous and smooth biotransformation as there was no nutrient in the bioconversion stage to support cofactor supply. The presence of xylose alone in the media would not be sufficient to produce the cofactors required for the conversion.

Purification of xylitol from the fermentation broth

Downstream processing costs are usually one of the major obstacles for the economical production of chemicals. Crystallization is the more commonly adopted method in the purification of polyols as it

allows recovery of xylitol in a purified form in a single step. In terms of energy consumption, crystallization is less energy intensive when compared to a distillation process. Crystallization can be performed using various methods such as solvent evaporation, cooling, salting out etc [27]. Xylitol is a solid at room temperature with melting point >90 ^{0}C. In the initial step, the coloured substance was clarified using charcoal treatment, with 5% activated charcoal. The fermentation broth almost became translucent and the impurities such as residual xylose were removed. The recovery of xylitol after charcoal treatment step was 76.2 and 77.1% with crude glycerol/xylose and pure glycerol/xylose, respectively, as shown in Table 5. A subsequent alcohol precipitation step further reduced the recovery of xylitol. The final crystallization step carried out at -20°C for 72 h resulted in 35.3 and 39.5% recovery for crude glycerol/xylose and pure glycerol/xylose, respectively.

Discussion

Xylitol is a polyol of commercial interest due to its high sweetening power and anticariogenic properties. Due to its multiple benefits, the market is growing with an increase of 6% per year. The industrial route requires pure xylose and the process is operated at high temperature and pressure. The chemical route is advantageous in terms of yield but has a number of hurdles to be overcome such as extensive purification, product recovery, catalyst deactivation, and energy intensity. All these factors make the process expensive and non-sustainable [13]. The biotechnological production of xylitol is environmentally safe and does not use toxic catalysts. The approach would be sustainable and create economic benefits if integrated with waste feedstocks rich in renewable carbon [12, 28]. The review of literature shows that there has been two strategies for bioproduction of xylitol; use of xylose as sole carbon source for cell growth as well as xylitol accumulation; co-feeding another carbon source along with xylose for biomass formation. The co-fermentation of xylose and another substrate is preferred over using only xylose for microbial xylitol production [29–31]. In the former approach, low yield and productivities are obtained due to a number of reasons; slow metabolism on xylose, partitioning of xylose flux between cell growth and xylitol formation, and re-consumption of produced xylitol. Therefore, it is more desirable to grow cell biocatalysts on a preferred carbon source so that a high cell density could be generated in a short time leading to higher productivities and yield.

The current study examined the potential of non-conventional yeast Y. lipolytica for xylitol production which is known to accumulate high levels of lipids, organic acids and polyols [14]. The xylose metabolism in yeast starts with reduction of xylose to xylitol (XR) followed by oxidation of xylitol to xylulose (XDH) which is then phosphorylated to xylulose-5-phosphate (XK) to be part of the pentose phosphate pathway (Fig. 1). The absence of XDH enzymes, its weak activity and/or imbalance in activities of XR/XDH result in accumulation of xylitol in the broth [13, 30, 32]. Majority of Y. lipolytica strains cannot grow on xylose as documented by many literature reports [15]. According to Rodriguez et al. (2016), the complete xylose pathway exists in Y. lipolytica but inability to grow robustly on xylose arises due to poor expression of key enzymes (XDH and XKS) controlling the pathway (Fig. 1). The Y. lipolytica Po1t (Ura$^+$ Leu$^+$) strain used in the current study and in a previous work, has been shown to

accumulate xylitol when cultured on xylose [15]. The high yield obtained was a stimulus to carry out a detailed study to investigate the potential of Y. lipolytica for xylitol production.

Xylitol is an extracellular metabolite and its production is affected by many factors including medium composition, cell density and growth rate. Besides high xylose concentrations, an optimal balance of other nutrients is necessary to achieve industrial levels of xylitol [32, 33]. We started growing Y. lipolytica on a mixture of glucose or glycerol and xylose. We obtained similar results in terms of cell growth, however, biotransformation yield on glycerol was significantly higher than glucose. Glycerol being a more reduced carbon source than traditional carbohydrates (glucose/sucrose/xylose) can provide better supply of reducing equivalents (NAD(P)H which is beneficial for xylitol formation [30, 34]. Zhang et al., (2014) [35] also found glycerol as a better co-substrate than glucose for xylitol accumulation. In addition, being an oleaginous yeast, Y. lipolytica, can eat even the crude glycerol, a major industrial byproduct with same the efficiency as pure glycerol [36]. The media optimization using CCD coupled with ANN-GA resulted in higher biotransformation efficiency, attaining a yield more than 90% using pure and crude glycerol with xylose. Previously Pappu and Gummadi, (2017)[37] adapted the ANN-GA model for optimizing process parameters for maximizing xylitol production in Debaryomyces nepalensis. With hybrid ANN-GA optimization they reported an optimum predicted error of 3.5% and maximized xylitol and biomass production. Nitrogen source (NH_4Cl) and YNB showed a momentous effect on the xylitol bioconversion. NH_4Cl plays a crucial role in enhancing the protein/enzyme expression level as the transcription of carbon metabolising gene is relied upon as the nitrogen source [38]. On the other hand, YNB comprises of essential components such as amino acids, vitamins, salts and trace elements required for yeast growth. Xia et al., 1995[39], reported excellent fermentation capability of xylose by C. shehatae in medium supplemented with YNB, whereas impaired growth was witnessed when the medium was devoid of YNB supplementation. The results of shake flask were replicated when the experiments were scaled up in bioreactor. The fermentation profiles can be divided into two phases; growth and biotransformation phase. The biotransformation of xylose started only after when a large fraction of co-substrate was consumed, and it may be due to carbon catabolite repression. The interesting observation was that cell growth was continuously increasing even after complete consumption of glycerol as probably, some xylose was contributing to cell growth after exhaustion of glycerol. More work is required to decode this. However, results are in agreement with Ledesma-Amaro et al. (2016)[15]. Table 4 shows the comparison of results obtained in the current work with existing literature. The competitive titer and yield near to the theoretical demonstrates the remarkable potential of Y. lipolytica when compared with other xylitol accumulating yeasts. The idea of the resting cell experiment was to investigate the reusability of the cell biocatalysts repeatedly, which will improve the bioprocess economics. Somehow, the results were not very encouraging. We suspect that it could be due to disrupted supply of reduced pyridine nucleotides and in future studies the biotransformation medium could be supplemented with co-substrate at regular interval for uninterrupted supply of a redox cofactor to carry out the reduction reaction [13, 40].

The final step of recovery and purification of a product in a bioprocess is challenging and determines the feasibility of the process, especially in terms of products synthesized from crude renewable sources. Its

complexity depends on the nature of product and composition of the fermentation broth. The information on xylitol recovery from fermented broths is scarce in literature [41]. We employed the method of Rivas et al., (2006)[42] based on crystallization and obtained a final recovery of 35–40%, similar to what they achieved (43.8%). Misra et al., (2011)[43] reported a 44% yield of xylitol using 15 g/L activated charcoal and crystallizing the solution at -20 °C. Wei et al., (2010)[44] included an additional step for purification using two ionic exchange columns and were able to enhance the recovery to 60%. The results of xylitol recovery are still encouraging as crude glycerol contains numerous impurities such as fatty acids, methanol etc.

In the current study, the bioproduction of xylitol was coupled with two waste streams, crude glycerol, a major industrial by-product and xylose, the second major sugar present in the hemicellulosic fraction of biomass. The impurities present in crude glycerol limits its application in chemical industries as the refining costs offset the profit and because it has limited applications. For example, the large amount of crude glycerol generated in UK from biodiesel industries (Greenergy, Croda, Oil Works) has no proper use and is exported to Germany, Holland, East Europe and sold at very low price (£40-£150/ton). Some small manufacturers are even paying for collection of CG. However, if the CG could be used locally, then environmental and economic benefits may be gained. To the best of our knowledge, this is the first study where crude glycerol was used towards xylitol production. The results obtained with pure as well as crude glycerol were consistent indicating a high level of tolerance by the yeast to the impurities in crude glycerol as no inhibition was observed during course of fermentation. These results are in agreement with many other studies where Y. lipolytica has been cultured on crude glycerol for the production of organic acids such citric acid, succinic acid etc [14, 45, 46].

Biological production of xylitol has been studied for decades using a number of organisms including bacteria, yeasts and fungi. The biological production of xylitol by bacteria and fungi have lower performance in comparison to yeasts. Among yeasts, Candida species are the most researched and best organisms for bioproduction of xylitol, yielding high conversion rates and productivities [47, 48]. The main application of xylitol is in food and pharmaceutical industries, but most of the xylitol producing organisms are pathogenic and suffer from their pathogenic behaviour, occluding commercialisation of a bioprocess. For example, Candida sp are prolific pathogens and cause globally almost 90% of fungal infections. As a result, the use of Candida sp are not allowed in food industries [32, 33, 49]. The limited ability of Y. lipolytica to cause only mild, self-limiting infections confer the GRAS status of the yeast which is an advantageous feature [50]. The high yield of xylitol achieved with Y. lipolytica along with safe behaviour keep it in superior position.

Conclusion

Xylitol is a platform chemical with vast commercial potential. This is the first detailed report of bioproduction of xylitol by Y. lipolytica. The current work demonstrates enormous potential of Y. lipolytica to convert xylose to xylitol with a yield near to the theoretical (> 90%). It produces similar concentrations of xylitol to some of the best xylitol producing organisms such as Candida strains. Moreover, it is a safe

organism to use with GRAS status and exhibited high tolerance to crude glycerol and xylose. Employment of unconventional feedstocks as carbon sources is highly desirable for the economic viability of biorefineries and becomes a good destination for renewable carbon-rich wastes. The study also demonstrated the feasibility of simultaneous valorisation of two major wastes, crude glycerol and xylose, which can be utilized as cheaper feedstocks. The strategy can be conducive towards development of a bioprocess as an alternative to the commercial chemical route and could support the sustainability of biodiesel industries/lignocellulosic biorefineries. More work is required to optimise the metabolic engineering and process scale-up to improve the economics of the bioprocess.

Material And Methods

Materials

All chemicals used in this study were of analytical grade and purchased from Sigma-Aldrich and Fisher scientific, unless stated otherwise.

Microorganism, culture maintenance and inoculum preparation

The current study made use of *Y. lipolytica* Po1t (Ura^+, Leu^+) derived from wild-type strain W29 (ATCC20460). The *Y. lipolytica* strain was preserved in 20 % glycerol (v/v) at - 80 °C and maintained on a petri dish containing YPD agar medium (1% yeast extract, 2% Peptone, 2% Dextrose and 2% Agar) at pH 7.0 and 30 °C. The seed culture was grown in a 250 mL Erlenmeyer flask containing 50 mL YPD broth. 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 at an agitation speed of 250 RPM.

Submerged cultivations in shake flask

The fermentation medium had the following composition: (g/L) pure or crude glycerol/glucose, 20 (2% w/v); xylose, 20 (2% w/v); yeast nitrogen base (YNB), 1.7 (0.17% w/v); NH_4Cl, 1.5 (0.15% w/v).The medium was prepared in 50 mM phosphate buffer. The initial pH was adjusted to 6.8 before inoculation by using 5N NaOH. The submerged cultivations were carried out in 500 mL shake flasks containing 100 mL working volume. The flasks were inoculated with fresh inoculum at OD_{600} (optical density) of 0.1 and kept at 30 °C under constant shaking at 250 RPM on a rotary shaker (Excella 24, New Brunswick). Crude glycerol used was kindly provided by Greenergy, UK. The crude glycerol contained glycerol (72.8%), non-glycerine material (soaps, fatty acids, esters, salts, other organic byproducts) (5.7%), methanol (2.0%), water (12.2%) and ash (9.6%). The xylose (26.4 g/L) rich lignocellulosic hydrolysate from sugarcane bagasse was obtained from Nova Pangea Technologies, UK.

Central composite design (CCD) and artificial neural network linked genetic algorithm (ANN-GA) for media optimization

The CCD was carried out, with the view of optimizing the variables and to give insight over the combined effect of four variables (xylose, YNB, NH_4Cl and phosphate buffer) at constant glycerol concentration on maximizing the production of xylitol concentration. Design-Expert software (version 7.0) was used to develop CCD for four independent variables and five levels (Table 1). The total number of experiments (*N*) was based on Equation (1)

$$N = 2^k + 2k + 6 \qquad (1)$$

where *k* is the number of independent variables. The experiment comprised 2 axial points and 6 replicates for centre points for the evaluation of pure error. The second-order polynomial for predicting the optimal levels was expressed according to the Equation (2).

$$Y_i = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} {X_i}^2 + \sum_{i<j} \sum \beta_{ij} X_i X_j + \varepsilon \qquad (2)$$

where, Y_i is the Predicted response; β_0 β_i, β_{ij}, β_{ii} are constant & regression coefficients of the model, X_i, X_j represent the independent variables in coded values and ε represents the error.

To further optimise the media components, the artificial neural network (ANN) methodology was adapted. ANN is biological inspired model, which mimics neural system and tends to optimize non-linear systems. Multi-layer perceptron method was incorporated, and training of the network was based on feed-forward back propagation method. The network architecture consisted of four input layers (xylose, YNB, NH_4Cl, phosphate buffer), eight hidden layers and one output layer representing xylitol concentration. In the feed-forward training system, the data was channelized from input to output via., hidden layer, which was connected by parameters such as weights (w) and biases (b). Transfer functions such as tan sigmoid (*f1*: tansig) and Pure linear (*f2*: purelin) were situated between hidden and output layer, respectively. Tansig sums up weighted input including the biases, and the purelin carried out the linearization function for the output. The predicted output function is represented by the Equation (3)

$$Y_p = f2[w^0 \times f1 \times (w^H \times input\ vector + b^H) + b^0] \qquad (3)$$

where Y_p is the predicted response, w^o, b^o & w^H, b^H are weights and biases of the output and hidden layer, respectively. The network training was done using Levenberg–Marquardt algorithm, which calculates error function based on the difference between actual output and predicted output. The algorithm was trained repeatedly until subsequent minimisation in the error between the input and output layer is met [51]. Mean squared error (MSE) was used to calculate error function using Equation (4).

$$MSE = \frac{1}{N} \sum_{i=1}^{N} (Y_a - Y_p)^2 \qquad (4)$$

where, Y_a is the actual output, Y_p is the predicted output and N is the number of data points. The simulation of the network was carried out by in built neural network toolbox of MATLAB (version 2010a).

Genetic algorithm (GA) is a heuristic method used to determine the global optimal solution for a non-linear problem and are independent of initial values; GA is often coupled with ANN to achieve precise optimization values. GA follows four steps to find a global solution. In the first step, initialization of the solution for the population takes place followed by fitness computation. The selected individual based on the fitness computation then undergoes crossing over and mutation, creating a new set of individuals (Yasin et al. 2014; Sushma et al. 2017). This process is repeated until a global optimum value is achieved.

The trained neural network model was used as a fitness function to further optimise the input space. The schematic representation of ANN-GA algorithm for optimisation of medium components to maximize xylitol production was shown in Fig S1 (supplementary data). The objective function of GA is given by Equation 5:

$$\text{Maximize } Y = f(x, w), x_i^L \leq x_i \leq x_i^u, i = 1,2,3 \cdots P \quad (5)$$

where f is the objective function (ANN model), x denotes input vector, w denotes corresponding weight vector, Y refers to the xylitol experimental yield, X denotes operating conditions, P denotes number of input variables, x_i^L & x_i^U are lower and upper bounds of x_i fitness of each candidate solution.

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 optimised media and the optimum values of media components were as follows (g/L): pure/crude glycerol, 20; xylose, 55; YNB, 5.0; NH_4Cl, 3.94; phosphate buffer, 132.5 mM. The starting pH was 6.8 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 2.0 L/min for initial 48 h and then changed to 1.0 L/min for the rest of fermentation period.

Biotransformation by resting cells

For active cells, *Y. lipolytica* was grown on optimised medium with pure glycerol in 250 mL flasks containing with 20% working volume. The temperature, pH and agitation speed were the same as mentioned in section 2.3. For the second stage (biotransformation), the cells were harvested in the late exponential period (after 48 h) when the OD_{600} was somewhere between 20-25. Immediately after, the culture was centrifuged at 2800xg for 10 min, and the resulting pellet was washed with ice-cold 100 mM phosphate buffer (pH 7.0). The cells were resuspended in a bioconversion medium containing xylose (30, 70 and 100 g/L) in phosphate buffer (100 mM). The bioconversion experiments were carried out at 30 °C with freshly prepared biomass.

Downstream processing of xylitol

The purification protocol for xylitol was performed according to Rivas et al., (2006). The 100 ml of spent fermentation broth was subjected to centrifugation at 20000 *g* to separate the cells and the clarified broth was treated with 5% activated charcoal. The charcoal treated broth was precipitated by adding four volume of absolute ethanol and incubated at 4°C for 1h. After 1h, the precipitates were removed by centrifuging the mixture at 4000xg for 10 min. The supernatant was vacuum concentrated at 40 °C. The concentrated sample and ethanol were mixed at a ratio of 1:4 and incubated at -20 °C with slight agitation (50 RPM) until crystals were observed. To improve the crystallization about 1 g/L of xylitol was mixed with the concentrated sample.

Analytical methods

The samples were withdrawn periodically and analysed for OD, pH, residual glycerol/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). One unit of absorbance at 600 nm corresponded to a cell dry weight (CDW) of 0.21 g/L. The concentrations of glycerol, glucose, xylose and xylitol were measured by high performance liquid chromatography (Agilent Technologies 1200 series, USA). The supernatants, obtained by centrifugation of the culture samples at 10,000×g for 10 min, were filtered through a 0.22 µm PVDF membrane (Sartorious, Germany)) and eluted using Rezex ROA-Organic Acid H+ (Phenomenex, USA) column at 60 °C attached with refractive index detector (RID). The mobile phase and flow rate were 0.5 mM H_2SO_4 and 0.4 mL/min, respectively. All measurements were conducted in triplicates and the values were averaged. The standard deviation was not more than 10 %.

Declarations

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Author information

Ashish A Prabhu[a*], Dominic J Thomas[a*], Rodrigo Ledesma- Amaro[b], Gary A Leeke[c], Angel Medina Vaya[a], Carol Verheecke-Vaessen[a], Frederic Coulon[a], Vinod Kumar[a**]

Affiliations

[a] School of Water, Energy and Environment, Cranfield University, Cranfield MK43 0AL, UK

[b] Department of Bioengineering and Imperial College Centre for Synthetic Biology, Imperial College London, London SW7 2AZ, UK

[c] School of Chemical Engineering, University of Birmingham, B15 2TT, UK

Contributions

Ashish Prabhu and Dominic J Thomas carried out all the experimental work, analyzed the data. Vinod Kumar analyzed the data and wrote the manuscript. Gary A Leeke, Angel Medina Vaya, Carol Verheecke-Vaessen, Frederic Coulon was involved in proof reading the manuscript. Rodrigo Ledesma- Amaro provided useful suggestions for experimental design and revised the manuscript critically. All authors read and approved the final manuscript.

Corresponding author

Correspondence to Vinod Kumar.

Availability of data and materials

All data generated and analysed during this study are included in this published article and its additional files.

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.

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Tables

Table 1: Experimental codes, range and levels of the independent variables used for central composite design (CCD) experiments

Independent Variables	Units	Symbol code	Coded Value				
			+α	-1	0	1	-α
Xylose	g/L	X_1	5	20	35	50	65
YNB	% (w/v)	X_2	0.05	0.2	0.35	0.5	0.65
NH_4Cl	% (w/v)	X_3	0.05	0.2	0.35	0.5	0.65
Phosphate buffer	mM	X_4	2.5	35	67.5	100	132.5

Table 2: CCD and ANN design matrix of variables, experimental responses and predicted values of xylitol concentration by *Y. lipoliytica*.

Experiment	Xylose (g/L)	NH_4Cl (%)	YNB (%)	Phosphate buffer (mM)	Xylitol (g/L)	CCD Predicted	ANN Predicted
1	20	0.2	0.2	35	17.47	18.04	18.79
2	50	0.2	0.2	35	22.20	22.80	22.48
3	20	0.5	0.2	35	13.11	11.81	14.99
4	50	0.5	0.2	35	20.05	19.51	20.45
5	20	0.2	0.5	35	10.20	10.52	4.83
6	50	0.2	0.5	35	21.77	22.48	19.70
7	20	0.5	0.5	35	18.70	20.81	18.92
8	50	0.5	0.5	35	37.22	35.73	33.50
9	20	0.2	0.2	100	18.99	26.02	23.06
10	50	0.2	0.2	100	29.53	27.94	30.42
11	20	0.5	0.2	100	17.85	17.65	19.66
12	50	0.5	0.2	100	23.30	22.52	23.14
13	20	0.2	0.5	100	4.74	5.79	5.90
14	50	0.2	0.5	100	14.07	14.91	13.92
15	20	0.5	0.5	100	15.00	13.95	14.27
16	50	0.5	0.5	100	26.08	26.03	31.47
17	35	0.35	0.35	67.5	20.71	24.25	24.96
18	35	0.35	0.35	67.5	27.18	24.25	24.96
19	35	0.35	0.35	67.5	24.53	24.25	24.96
20	35	0.35	0.35	67.5	24.84	24.25	24.96
21	5	0.35	0.35	67.5	20.57	19.33	21.37
22	65	0.35	0.35	67.5	34.98	36.16	37.22
23	35	0.05	0.35	67.5	12.41	10.67	11.56
24	35	0.65	0.35	67.5	13.88	15.55	15.66
25	35	0.35	0.05	67.5	29.69	30.82	29.96
26	35	0.35	0.65	67.5	28.00	26.80	23.25
27	35	0.35	0.35	2.5	23.62	23.15	23.75
28	35	0.35	0.35	132.5	21.03	21.43	21.08
29	35	0.35	0.35	67.5	24.87	27.46	24.96
30	35	0.35	0.35	67.5	29.78	27.46	24.96

Table 3: Analysis of Variance for Xylitol Production*

Source	DF	Seq SS	Adj SS	Adj MS	F	p
Blocks	1	68.61	68.61	68.61	15.35	0.002
Regression	14	1402.25	1402.25	100.16	22.41	<0.001
Linear	4	489.28	489.28	122.32	27.37	<0.001
Xylose	1	424.91	424.91	424.91	95.08	<0.001
NH_4Cl	1	35.71	35.71	35.71	7.99	0.01
YNB	1	24.22	24.22	24.22	5.42	0.04
Phosphate	1	4.44	4.44	4.44	0.99	0.37
Square	4	404.82	404.82	101.21	22.65	<0.001
Xylose*Xylose	1	9.54	0.14	0.14	0.03	0.86
NH_4Cl*NH_4Cl	1	341.93	352.75	352.75	78.93	<0.001
YNB*YNB	1	7.65	3.14	3.14	0.7	0.42
Phosphate*Phosphate	1	45.7	45.7	45.70	10.23	0.01
Interaction	6	508.15	508.15	84.69	18.95	<0.001
Xylose*NH_4Cl	1	8.73	8.73	8.73	1.95	0.18
Xylose*YNB	1	51.99	51.99	51.99	11.63	0.004
Xylose*Phosphate	1	8.09	8.09	8.09	1.81	0.2
NH_4Cl*YNB	1	273.24	273.24	273.24	61.14	<0.001
NH_4Cl*Phosphate	1	4.56	4.56	4.56	1.02	0.33
YNB*Phosphate	1	161.56	161.56	161.56	36.15	<0.001
Residual Error	14	62.57	62.57	4.47		
Lack-of-Fit	10	29.03	29.03	2.90	0.35	0.92
Pure Error	4	33.54	33.54	8.38		
Total	29	1533.43				

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

Table 4: Comparison of xylitol production by various yeast strains

Strain	Initial Xylose (g/L)	Xylitol (g/L)	Yield (g/g)	Reference
Candida guilliermondii FTI-20037	104	77.2	0.74	(Barbosa *et al.*, 1988)
Candida tropicalis	150	131	0.87	(Oh and Kim, 1998)
Candida boidinii NRRL Y-17213	150	53.1	0.35	(Vandeska *et al.*, 1995)
Candida sp.559-9	200	173	0.87	(Ikeuchi *et al.*, 2000)
Pichia sp.	40	25	0.63	(Rao *et al.*, 2007)
Debaryomyces hansenii UFV-170	100	76.6	0.73	(Sampaio *et al.*, 2008)
Hansenula polymorpha	125	58	0.62	(Suryadi *et al.*, 2000)
Y. lipolytica	55	53.2	0.97	This study

Table 5: Xylitol titer and percent recovery obtained during different steps of downstream processing of fermented broth from batch cultivation of *Y. lipolytica* in shake flask on crude glycerol/xylose and pure glycerol/xylose.

Purification steps	Crude Glycerol + Xylose		Pure Glycerol + Xylose	
	Xylitol (g/L)	Recovery (%)	Xylitol (g/L)	Recovery (%)
Crude fermentation broth	48.72	100	51.53	100
Activated charcoal treatment	37.13	76.21	39.73	77.10
Alcohol precipitation	24.22	49.71	27.11	52.60
Crystallization	17.20	35.30	20.33	39.45

Figures

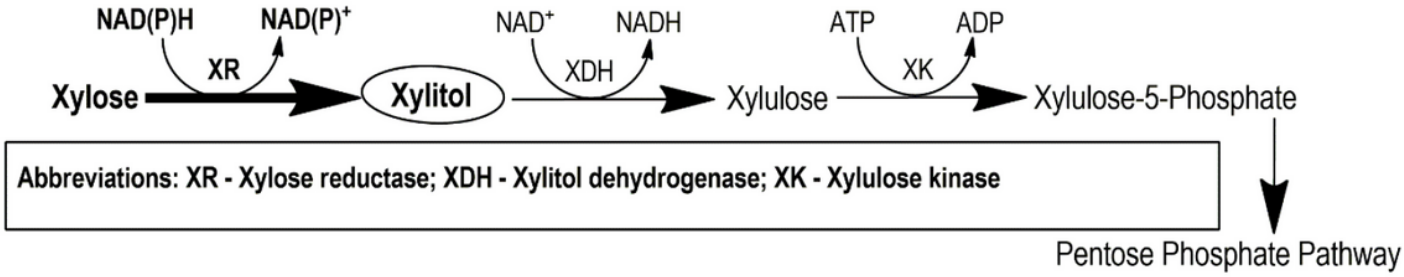

Figure 1

Xylose metabolism in yeast (Pal et al. 2013; Ur-Rehman et al. 2015).

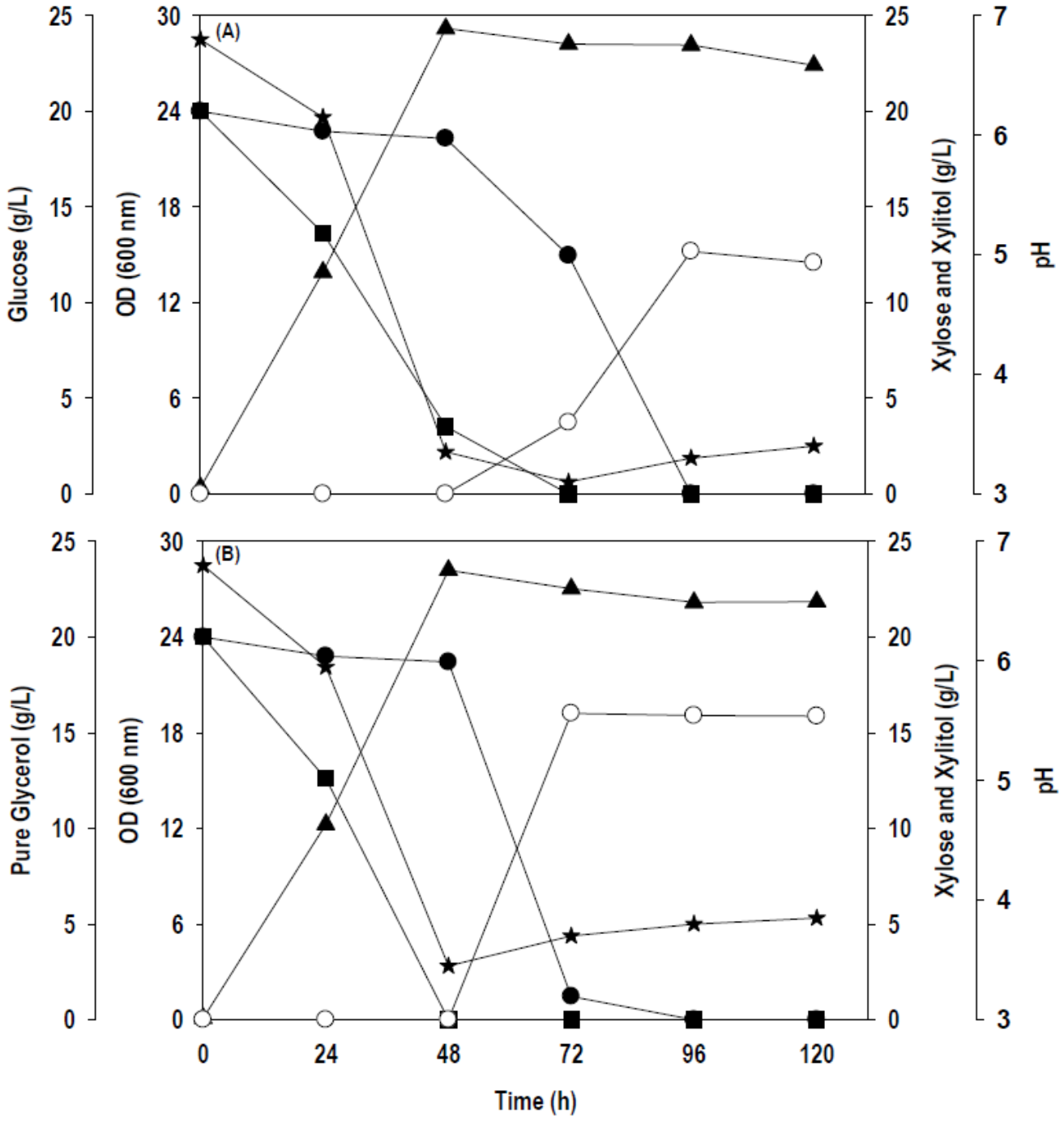

Figure 2

Time course profiles of Y. lipolytica on; (A) glucose and xylose, (B) pure glycerol and xylose. Symbols: filled square (glucose or pure glycerol), filled circle (xylose), filled triangle (OD600), empty circle (xylitol) and filled star (pH).

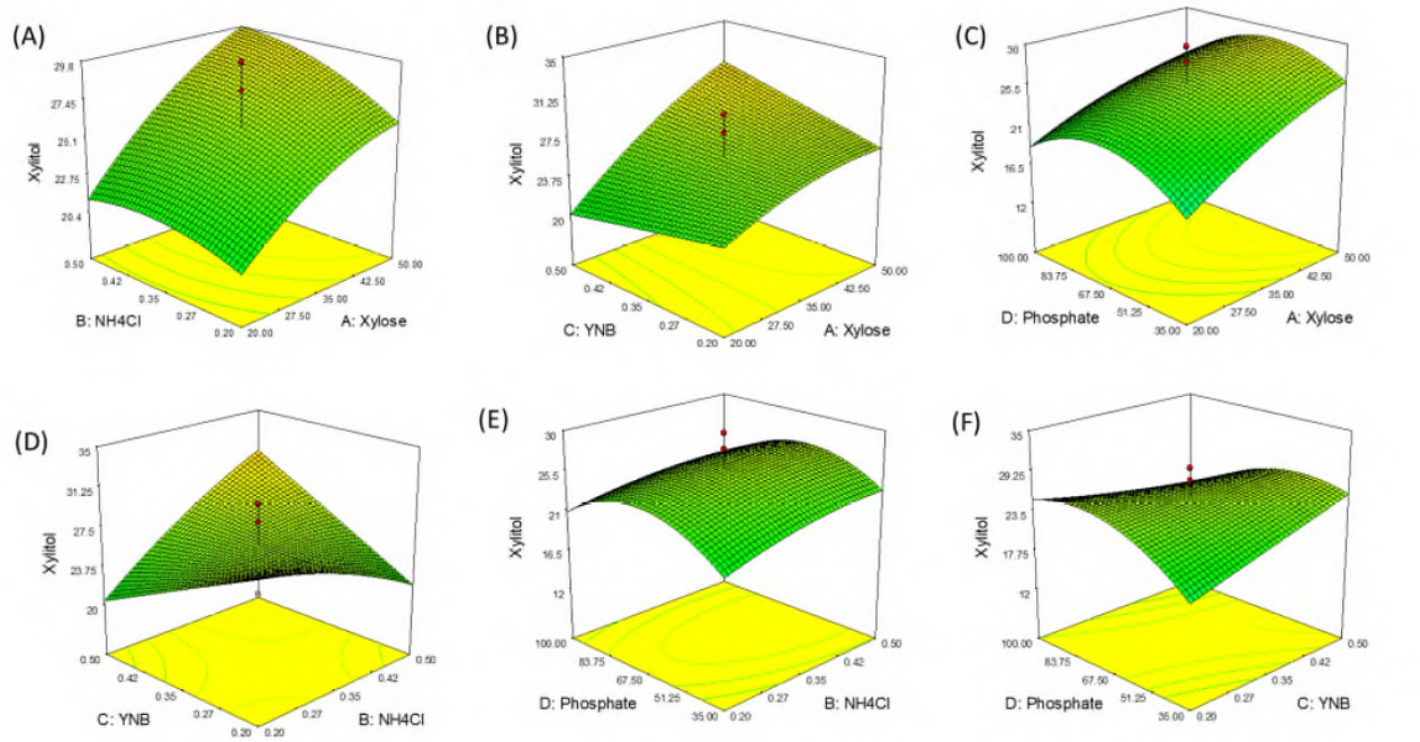

Figure 3

Three-dimensional response surface plot for xylitol production showing the interactive effects of (a) xylose and NH4Cl (b) xylose and YNB (c) xylose and phosphate buffer, (d) YNB and NH4Cl, (e) phosphate buffer and NH4Cl, (f) YNB and phosphate buffer with the remaining factors kept constant at the middle level of the central composite experimental design.

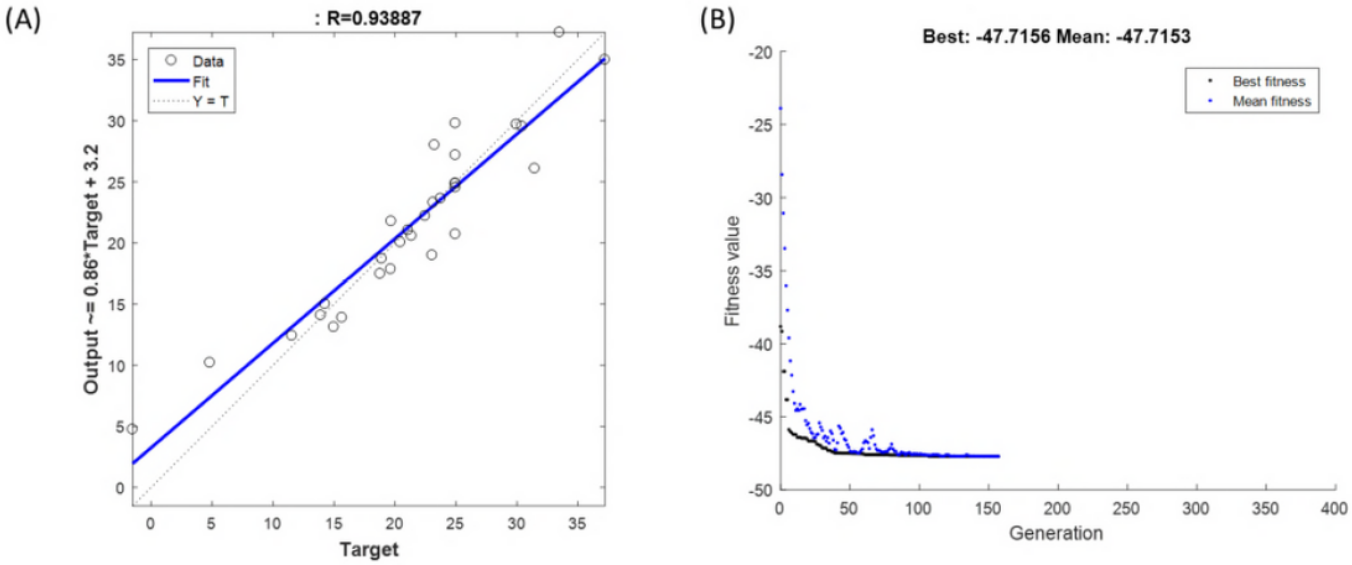

Figure 4

The prediction performance of ANN models for xylitol Production. (a) Overall regression value of simulated ANN (b) Best and average fitness values with successive generations showed gradual convergence to the optimum value for xylitol production.

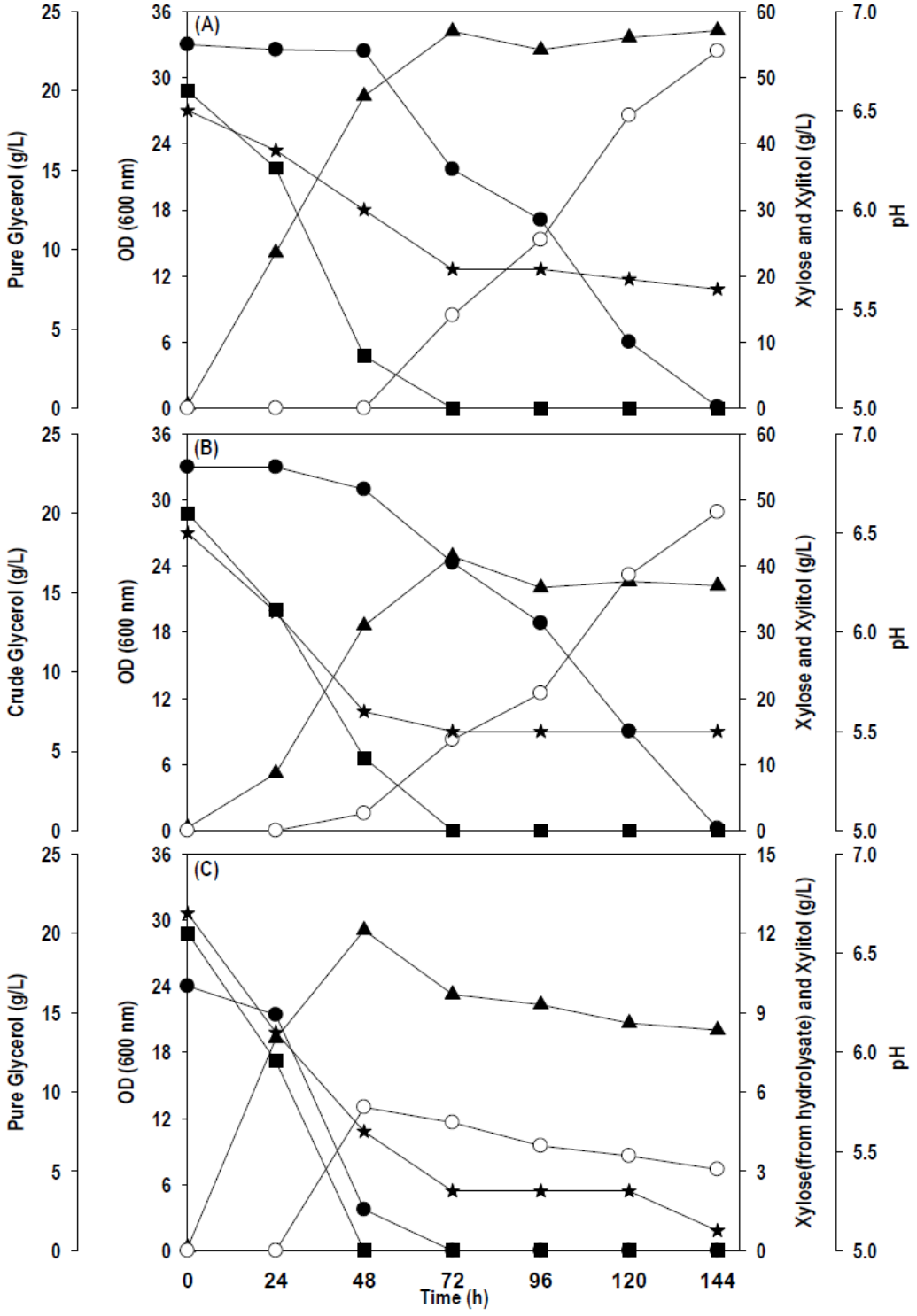

Figure 5

Shake flask cultivation of Y. lipolytica using optimized media on; (A) pure glycerol and xylose, (B) crude glycerol and xylose, (C) pure glycerol and xylose from hydrolysate. Symbols: filled square (pure or crude

glycerol), filled circle (xylose), filled triangle (OD600), empty circle (xylitol) and filled star (pH).

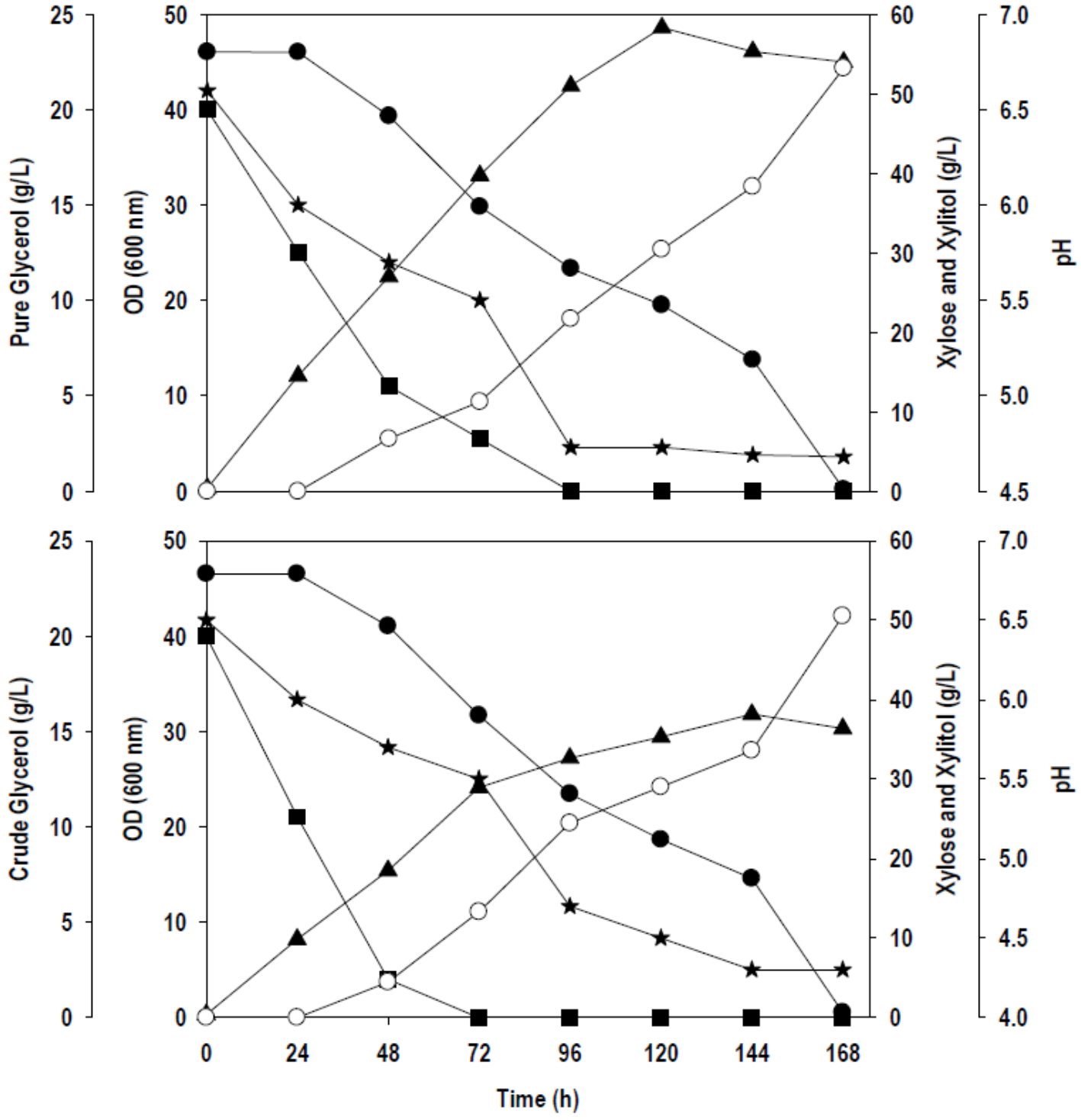

Figure 6

Batch kinetics of substrate assimilation, cell growth, pH and xylitol formation by Y. lipolytica in bioreactor during co-fermentation on; (A) pure glycerol and xylose, (B) crude glycerol and xylose. Symbols: filled square (pure or crude glycerol), filled circle (xylose), filled triangle (OD600), empty circle (xylitol) and filled start (pH).

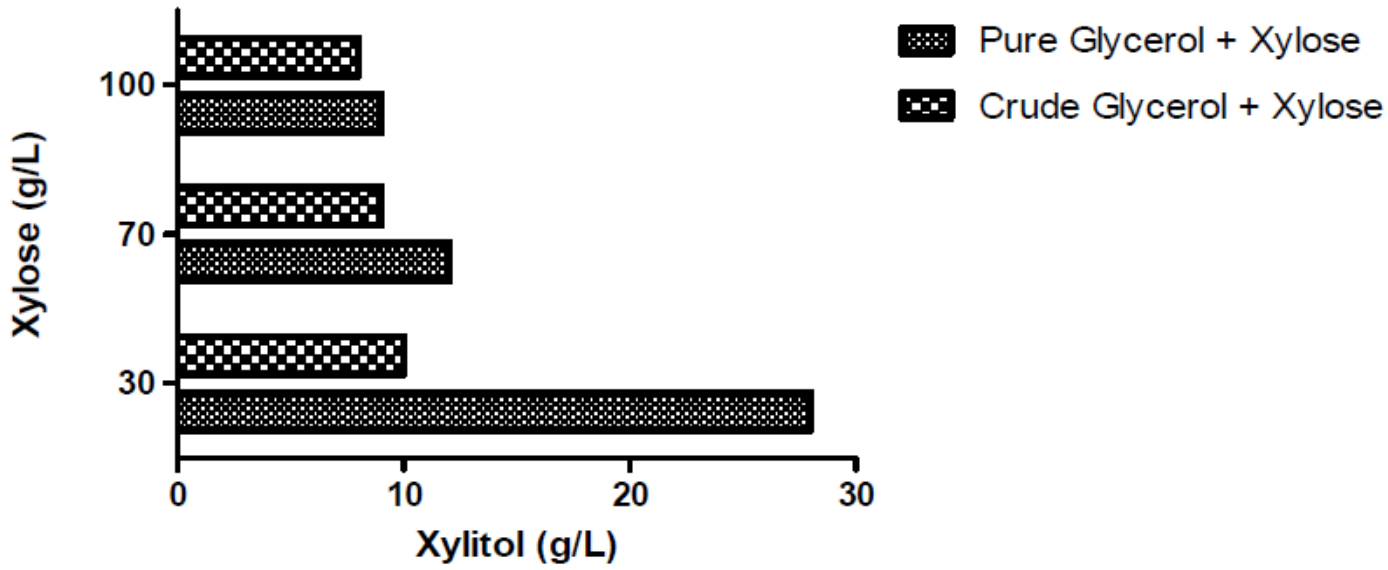

Figure 7

Biotransformation of xylose to xylitol by resting cells of Y. lipolytica grown using pure and crude glycerol.

Supplementary Files

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- Graphicalabstract.pptx
- Supplementary.docx