Bioproduction of succinic acid from xylose by engineered Yarrowia lipolytica without pH control

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Abstract

Background

Xylose is a most prevalent sugar available in hemicellulose fraction of lignocellulosic biomass (LCB) and of great interest for the green economy. Unfortunately, most of the cell factories cannot inherently metabolize xylose as sole carbon source. Yarrowia lipolytica is a non-conventional yeast to produce industrially important metabolites, and it is able to metabolize a large variety of substrates including both hydrophilic and hydrophobic carbon sources. However, Y. lipolytica lacks effective metabolic pathway for xylose uptake and only scarce information is available on utilization of xylose. For the economically feasible of LCB-based biorefineries, effective utilization of both pentose and hexose sugars is obligatory.

Results

In the present study, succinic acid (SA) production from xylose by Y. lipolytica was examined. To this end, Y. lipolytica PSA02004 strain was engineered by overexpressing pentose pathway cassette comprising of xylose reductase (XR), xylitol dehydrogenase (XDH) and xylulose kinase (XK) gene. The recombinant strain exhibited a robust growth on xylose as sole carbon source and accumulated SA (3.8 g/L) with a yield of 0.19 g/g in shake flask studies. Substrate inhibition studies revealed a marked negative impact on cell growth and product formation above 60 g/L xylose concentration. The modelling based on inhibition kinetics revealed that Aiba model showed better fit with experimental data, which resulted the correlation coefficient ($R^2$) of 0.82 and inhibition constant ($K_I$) 88.9 g/L. The batch cultivation of recombinant strain in bioreactor resulted in a maximum biomass concentration of 7.3 g/L and SA titer of 11.2 g/L with the yield of 0.18 g/g. Similar results in term of cell growth and SA production were obtained with xylose-rich hydrolysate derived from sugarcane bagasse. The fed-batch fermentation yielded biomass concentration of 11.8 g/L (OD 600 : 56.1) and SA titer of 22.3 g/L with a gradual decrease in pH below 4.0. Acetic acid was obtained as a main byproduct in all the fermentations.

Conclusion

The recombinant strain displayed potential bioconversion of xylose to succinic acid. Further this study
provided a new insight on conversion of LCB into value-added products. To the best of our knowledge, this is the first study on SA production by Y. lipolytica using xylose as a sole carbon source.

**Background**

Currently, production of chemicals relies almost exclusively on fossil fuels which are non-renewable resources and negatively impact the environment. Growing demands on clean and sustainable processes to produce fuels and chemicals raise significant challenges for the scientific community [1]. Microbial conversion of renewable biomass such as lignocellulosic feedstock into value-added products is getting a humongous response as it can replace the dependency on petroleum based refineries, majorly responsible for rapid climate change and greenhouse gas emission [2].

Lignocellulosic biomass (LCB) from agro-industrial waste is quite abundant and considered as a prominent feedstock to produce chemical commodities as it comprises of considerable amount of carbon source. LCB is a three-dimensional polymeric material composed of lignin (15–20%), hemicellulose (25–30%) and cellulose (40–50%) (Fig. 1a). Cellulose is a linear homo-polymer of D-glucose while hemicellulose is a hetero-polymer containing D-xylose, L-arabinose, D-glucose, L-galactose, D-mannose, D-glucuronic acid and D-galacturonic acid (Fig. 1b) [3, 4]. Most of the studies are however focused on the utilization of cellulose for manufacturing value-added products, while the hemicellulosic portion is usually discarded as most microbes lack an efficient pathway for utilization of pentose sugars [5]. In addition, carbon catabolite repression suppresses the assimilation of pentose sugars. Xylose is the predominant sugar in a hemicellulose and can constitute up to 30–40% of the LCB [6, 7]. Therefore, efficient bioconversion is a prerequisite for economic feasibility of lignocellulosic biorefineries. Hence, more attention is paid to the rewiring of metabolic networks of microbial strains to utilize multiple carbon sources simultaneously, especially glucose and xylose, from the feedstock which will be essential for de-risking the commercial viability of the bioprocesses [8, 9].

Succinic acid (SA) (C₄H₆O₄), is one of the twelve high-value bio-based chemicals listed by the U.S Department of Energy [10]. It provides a chemical platform for a wide range of industrial applications in the pharmaceutical, food, polymers, plasticizers and green solvent sectors [11]. Specifically, the presence of two carboxyl groups makes SA a precursor molecule for the synthesis of a variety of
chemical compounds such as tetrahydrofuran, 1,4-butanediol, \( \gamma \)-butyrolactone, adipic acid, aliphatic esters. Due to its versatility, the global market value of SA was USD 191 million in 2013, and it is expected to grow up to USD 992 million by 2020 [12]. Also the volumetric productivity was reported to be 50,000 metric ton in 2016, and it is expected to be doubled by 2025 [13]. The chemical routes for SA synthesis are oxidation of paraffin, reduction of maleic acid or anhydride. These chemical methods result in mixture of products reducing the yield and purity of main product. Nevertheless, the yield and purity may be improved by proper selection of catalyst, the operation of process is not sustainable. As a result, there is a growing interest towards creating a cleaner and greener technology for SA production and in recent years, there is a paradigm shift from petrochemical synthesis towards bio-based production of SA [14]. The bio-based production of SA constituted 49% of the total market in 2013-2014 (around 38,000 ton) and the fraction will enhance significantly in coming years. It is envisaged that bioproduction will soon replace the conventional petroleum-based process, and the use of low-cost agricultural feedstock can contribute towards this goal [15].

The fermentative production of SA occurs through the reductive and/or oxidative TCA cycle, utilization of CO\(_2\) as co-substrate which leads to high CO\(_2\) sequestration potential [16]. The biological production of SA has been investigated using several bacterial strains such as Mannheimia succiniciproducens, Actinobacillus succinogenes and recombinant Escherichia coli as a potential host [17]. Bacteria are very sensitive towards low pH and require moderate pH for its growth resulting in large consumption of neutralizing agent and affects the productivity cost [16]. Further at neutral pH, SA is obtained in the form of succinate salts and needs an additional step of acidification to bring it back to acid form leading to accumulation of by-products such as gypsum in-turn making the downstream process more critical. This problem can be resolved by carrying out the fermentation at low pH value [18]. Yeasts are the potential host to produce organic acid because of their high tolerance, and they are naturally adapted to grow under low pH, below 4.0 [19]. The two pKa value of the SA are 4.2 (pK\(_{a1}\)) and 5.6 (pK\(_{a2}\)), and at pH 3.5 about 80% succinic acid will exist in its protonated form. Obtaining SA in unionized form will simplify the downstream processing and lead to an economical process, as it will
not require neutralization during fermentation and acidification on product recovery [13].

Over the past decade, research on a non-conventional yeast Y. lipolytica has been focused due to its versatile characteristic such as well annotated genomic model, metabolic tools, high cell density growth, highlights broad spectrum substrate uptake capability and withstand adverse conditions such as low pH and high salt concentration [20, 21]. Furthermore, the yeast has been conferred Generally Regarded as Safe (GRAS) status from the US Food and Drug Administration. Being an aerobic yeast, the flux of TCA cycle is very active in Y. lipolytica and plethora of reports on Y. lipolytica are based on the production of TCA intermediates such as citric acid, isocitric acid, α-ketoglutaric acid [22-24]. This renders this yeast species as an attractive host to produce another TCA intermediate which is known as succinic acid. Previously, Y. lipolytica has been engineered for production succinic acid using glucose and glycerol as carbon sources [25, 26]. Y. lipolytica strains can utilize a wide range of carbon source including glucose, glycerol, alkanes and lipids [27, 28]. However, to date, little information is available on the effective utilization of xylose, which is the second most abundant carbon source available in LCB. Attempts were made to overexpress pentose pathway genes in Y. lipolytica in order to enhance the uptake of xylose and produce value added products such as citric acid and lipids [29].

In the present study, SA production from xylose by Y. lipolytica was examined. To this end, Y. lipolytica PSA02004 strain was engineered by overexpression of XR, XDH and XK genes for efficient utilization of xylose and simultaneous production of SA. To further visualize the maximum carbon uptake capability, the strain was subjected to different concentration of xylose and unstructured models were used to predict the better substrate inhibition concentration and other kinetic parameters. The study was scaled up from shake flask to bioreactor with batch and fed batch cultivations to further improve SA production. The engineered strain was also evaluated for SA production from xylose-rich hydrolysate derived from sugarcane bagasse. The pH was uncontrolled in all the experiments in this work in order to understand the robustness of the strain to withstand low pH conditions, without compromising the production. To our knowledge, this is the first report of SA production in Y. lipolytica with xylose as the sole carbon source.

Results
**Shake flask cultivation of Y. lipolytica PSA02004**

Succinic acid is an intermediate of TCA cycle and produced through oxidative/reductive TCA cycle [30]. The reductive pathway is not favorable thermodynamically and responsible for glucose repression. *Y. lipolytica* prefers to use oxidative TCA cycle for SA production [11,31]. Succinate dehydrogenase is one of the enzymes of oxidative TCA cycle, which catalyzes the oxidation of succinate to fumarate and it has five subunits. Gao et al. inactivated *sdh5* encoding succinate dehydrogenase assembly factor2 (*YALI0F11957g*) in Po1f strain (derived from W29 strain) and obtained a mutant PGC01003 [32], and this strain showed impaired growth on glucose. The PGC01003 strain was subjected to adaptive evolution using glucose-based medium for 21 days and the evolved strain was designated as *Y. lipolytica* PSA02004 [26]. This strain was cultured on glucose, glycerol, xylose, glucose/xylose and glycerol/xylose (Figure 2). Glucose and glycerol are the preferred carbon sources for *Y. lipolytica*, and these carbon sources were completely depleted within 48-72 h concomitant with the cell growth, which also coincided with SA production. However, the strain was unable to grow on xylose as the sole carbon source (data not shown). The co-fermentation of xylose with glucose or glycerol resulted in xylitol accumulation along with SA synthesis indicating that *Y. lipolytica* cannot metabolize xylose to grow on it, but it can transform into xylitol with a high conversion yield (~70%). This was also supported by similar cell growth (OD$_{600}$: 20-22) observed on glucose/glycerol, as well as during co-fermentation with xylose, where xylose is mainly utilized for xylitol synthesis and not contributing for biomass/product manufacturing. The xylose was subjected to carbon catabolite repression in the presence of glucose/glycerol, and rapid consumption of xylose along with xylitol accumulation started after 48 h when large fraction of these co-substrates was utilized. Acetic acid (AA) was obtained as a main byproduct, which was evident in the late log phase of the cell growth, it can be correlated with subsequent drop in pH below 4.5 in all the fermentations. Another important observation was that the amount of SA and AA achieved during co-fermentation was marginally higher in comparison to fermentation on a single carbon source at cryptic xylose metabolism in *Y. lipolytica*.

**Introduction of xylose metabolic pathway in Y. lipolytica PSA02004**
As shown in previous section that *Y. lipolytica* PSA02004 strain showed no growth on minimal medium supplemented with 20 g/L xylose as a sole carbon source. The inability of *Y. lipolytica* to assimilate xylose for cellular growth impedes its application for lignocellulosic biorefineries. To enable growth on xylose, xylose metabolic pathway was introduced in *Y. lipolytica* PSA02004. In this study, the engineered strain was constructed by overexpressing the homologous gene of xylose reductase (XR), xylitol dehydrogenase (XDH) and of xylulose kinase (XK) from *Y. lipolytica* (Po1d strain) cloned under transcription elongation factor (TEF) promoter (Figure 3(A)). The resulting strain was designated as *Y. lipolytica* PSA02004PP. With the overexpression of XR, XDH and XK, the strain was able to grow in the medium containing xylose as a sole carbon source. The time course profiles of substrate assimilation, cell growth, product formation and pH were similar to those obtained on glucose or glycerol. There was no xylitol accumulation and probably, all the formed xylitol was funneled towards central carbon metabolism. The maximum OD$_{600}$ obtained was 14.1 at 48-72 h. The recombinant strain PSA02004PP was able to produce 3.8 g/L SA from xylose with 0.19 g/g yield. Interestingly, substantial amount of acetic acid (4.1 g/L) was accumulated. The combined production of two organic acids resulted in drop in pH with time (Figure 3b). In addition to cultivation on xylose, the activity of two key enzymes, XR and XDH, involved in xylose metabolism by *Y. lipolytica* PSA02004PP strain was monitored during the course of fermentation (Figure 3c). The activity profiles revealed that high activities of XR and XDH were maintained during exponential growth and stationary phase. The maximum XR and XDH activity of 0.85 and 0.98 U/mg, respectively, were obtained at 72 h. The slightly high XDH activity than XR allows better synchronization between two enzymes, and results in efficient conversion xylose to xylulose without accumulation of xylitol as by-product.

**Co-fermentation of xylose with glucose/glycerol by *Y. lipolytica* PSA02004PP in shake flasks**

The recombinant *Y. lipolytica* strain carrying a copy of XR, XDH and XK gene showed a superior growth characteristic along with SA synthesis in xylose containing medium under shake flask cultivation. Furthermore, the phenotypic profile in different carbon sources such as glycerol and glucose, and the effect of these substrates on the uptake of xylose were investigated. The
recombinant strain produced SA titer of 5.7 and 5.0 g/L with glycerol and glucose as carbon source, respectively (Figure 5). While in case of co-fermentation with glucose or glycerol, xylose was consumed only after the depletion of glucose or glycerol, indicating clear signs of catabolite repression effect. The co-fermentation of glucose and xylose resulted in the maximum OD$_{600}$ value of 22.7 with SA titer of 9.9 g/L, while OD$_{600}$ of 30.1 was achieved with similar resultant SA concentration (10.0 g/L) using a mixture of glycerol and xylose at 96 h. Additional accumulation of AA was observed both in individual sugars as well as co-substrates, which also reduced the pH of the fermentation broth besides reducing the SA yield. After the introduction of xylose metabolic pathway, no xylitol accumulation was observed with co-fermentations, and significant improvement in SA synthesis was noticed in comparison to control where xylose was transformed into xylitol in presence of glucose/glycerol. Thus, there was clear shift in metabolism with entry of xylose into central carbon metabolism.

**Substrate inhibition studies on recombinant Y. lipolytica PSA02004PP**

The effect of initial xylose concentration on the substrate uptake rate, cell growth, product and byproduct formation ability of Y. lipolytica PSA02004PP were investigated by growing the strain at different initial concentration of xylose ranging from 20-120 g/L. The aim of the experiment was to determine optimal level of xylose for cell growth and SA production. Figure 6 (a-e) shows the time course profiles for xylose uptake, cell growth in OD$_{600}$, SA, AA and xylitol production. Xylose was completely consumed in 72 h for fermentation media with an initial level of 20 and 40 g/L. Beyond 40 g/L, residual xylose was noticed even at 120 h. The uptake of xylose was reduced after 48 h at 60, 80, 100 and 120 g/L. The amount of unutilized xylose at 60, 80, 100 and 120 g/L was 8.0, 36.0, 52.0 and 77.0 g/L, respectively. There was a linear increase in cell growth (i.e. OD$_{600}$) from 11.7 to 17.2, as the initial xylose concentration was enhanced from 20 g/L to 60 g/L. Above 60 g/L, there was gradual decline in the biomass formation indicating the substrate inhibition. The highest value of specific growth rate (0.017 h$^{-1}$) was observed at 60 g/L initial xylose concentration and reduced significantly at higher initial xylose levels. Similar trend was obtained with SA; producing a maximum of 3.8, 6.6
and 9.9 g/L at 20, 40 and 60 g/L initial xylose concentration, respectively. Further increase in initial xylose concentration retarded the yield and productivity of SA. AA was identified as the main byproduct and accumulation enhanced at higher substrate concentration. Interestingly, xylitol formation was observed at xylose level above 40 g/L and significantly increased from 1.3 g/L to 10.5 g/L as initial xylose concentration was raised from 60 g/L to 120 g/L. The continuous increment in AA and xylitol production with increase in xylose levels can be due to overflow metabolism at higher substrate concentrations. The initial xylose concentration of 60 g/L was selected for further experiments to achieve an optimal balance between SA titer, yield and productivity.

To get the clear phenotypic relation between cell growth and substrate concentration, we have adapted unstructured inhibition kinetic models. As shown in Figure 7a, the regression model was applied on the data of the specific growth rate. The model predicted was fitted with aid of MATLAB 7.1 (curve fitting toolbox) and it is presented in Figure 7b. Most of the kinetic model showed a precise fit with the experimental data obtained under different substrate concentration regime. The parameters were estimated by applying non-linear regression on different models, and they are represented in Table 2. Among the different kinetic model used in this study, Aiba model showed a better fit with high correlation co-efficient ($R^2$) of 0.82 and lower Root mean square error (RMSE) value of 0.0012, followed by Edward model with $R^2$ of 0.79. The inhibition constant ($K_I$) of Aiba model was 88.9 g/L, which is very much near to the experimental data obtained (Table 3). The best fit 3-parameter (Aiba) and 4-parameter (Yano) models which showed improved $R^2$ values were compared for model acceptability using Akaike’s information content criteria (Table 4). In AIC model, it was seen that increment of SSE values was insignificant as expected from change in the number of parameters which showed positive $\Delta AIC_c$ value. Hence simpler model (Aiba) can be chosen to explain the kinetics.

To understand the effect of each model parameters on regression values, the input parameters of Aiba (three parameter) was varied from ±50% of their estimates, while keeping all other parameters constant. It was observed that all parameters (i.e. $\mu$, $K_s$, $K_I$) showed high sensitivity (Figure 8).

**Batch cultivation of Y. lipolytica PSA02004PP in bench-top scale bioreactor**
The batch cultivation of recombinant Y. lipolytica PSA02004PP was conducted in bench-top bioreactor in order to understand the phenotypic characteristic of strain. The initial concentration of pure xylose was 60 g/L. The strain was able to produce maximum biomass concentration of 7.3 g/L (OD$_{600}$: 34.9) with pure xylose substrate (Figure 9a). The xylose was almost completely consumed (>99%) in 84 h, which was reflected in concomitant termination of biomass, SA and AA formation. The highest SA level of 11.2 g/L with the yield of 0.18 g/g and AA was obtained at 8.5 g/L in same duration. The experiment was repeated with crude xylose-rich hydrolysate derived from sugarcane bagasse (Figure 9b). Hydrolysate after pre-treatment often contains inhibitors which can negatively impact the performance of microorganisms. The comparison was made to evaluate the robustness of strain in presence of fermentation inhibitor such as furfural and acetic acid. The cell growth (OD$_{600}$: 25.3; 5.3 g/L) was unaffected as biomass yield was almost the same in both cases. The strain accumulated 5.6 g/L SA with a yield of 0.14 g/g. In both fermentation, accumulation of substantial amount of AA along with SA resulted in significant reduction in pH. Furthermore, no accumulation of xylitol was observed during fermentation, indicating active pentose phosphate pathway resulted in enhanced biomass formation.

**Fed-batch fermentation for succinic acid production**

Based on the batch fermentation study, where the strain displayed excellent xylose uptake capability with simultaneous SA production, fed-batch fermentation was conducted to further improve SA production. The strain was evaluated in fed-batch fermentation with minimal medium under uncontrolled pH condition. The batch phase was completed in 72 h of fermentation, where the initial xylose concentration was reduced to 10.3 g/L, and the strain was in exponential phase with a maximum OD$_{600}$ of 32.0, which is equivalent to 6.7 g/L biomass concentration. The SA and AA concentration at 72 h were 10.8 g/L and 11.6 g/L, respectively. The cell metabolism coupled with accumulation of these organic acids caused reduction in pH level to 3.9 and thereafter, pH was stable till the end of fermentation. The feeding was started after 72 h to maintain a xylose level above 10 g/L (Figure 10). The cell growth was continued till 108 h, thereafter, cell reached stationary phase and
remained stable (OD$_{600}$: 50-56). Despite a low pH, synthesis of biomass and SA was continued with a smooth rate. The highest biomass concentration of 11.8 g/L was observed at 156 h of fermentation. The maximum SA concentration was 22.3 g/L, which was coincided with cell growth. The fermentation resulted in the buildup of 25.0 g/L AA, a major by-product which was obtained in higher amount than the desired product SA.

**Discussion**

SA is a top platform chemical with multitude of industrial applications, which offers access to a wide range of products with huge commercial market. In the past decade, growing interest towards clean and green production of chemicals has been witnessed. The fermentative production of SA has received attention and the potential to replace the petrochemical with a number of advantages owing to its simplicity and environmental friendliness. The route is green and sustainable due to the use of renewable feedstocks [33]. The carbon substrates used in industrial fermentations and literature report are pure sugars, mainly glucose, making the cost of bio-based SA production more expensive than the fossil-based process. The cost can be brought down by using cheap crude renewable sources, and it will eventually lead to sustainable bio-succinic acid production [15]. An economical organic acid production requires low cost LCB as a raw material which can be supplied in sufficient amount. However, the concept of LCB-based biorefineries cannot be realized without using xylose, which is a major sugar in hemicellulosic fraction. Despite advances in metabolic engineering, xylose is still considered as inferior carbon source in comparison to other fermentable carbon sources [7, 34].

In the last two decades, lots of efforts have been made towards bio-based SA production. However, there are only few reports on SA bioproduction from xylose[15, 35]. Plethora of reports are based on SA production from prokaryotes, among which A. succinogenes is considered as one of the favorite candidates for SA production, due to its high acid secreting capabilities and it can uptake a wide range of sugars. However, A. succinogenes uses reductive pathway for SA production, and it can theoretically produce 0.5 mol/mol-glucose, 0.6 mol/mol-xylose and 1 mol/mol-glycerol. In parallel, it oxidizes 2 moles of NADH and the additional reductive capacity should be supplied through other parts of metabolism [36]. Even with the higher productivity, the strain is associated with the constrain
such as inability to grow on low pH, required anaerobic condition and carboxylating agent for active reductive pathway, which makes it unsuitable for industrial level production.

Y. lipolytica is a non-conventional yeast, which is well-known for the accumulation of intracellular lipids and extracellular secretion of organic acids and polyols [37]. Also, the yeast has been explored for SA production from glycerol and glucose by our research groups [25, 38–40]. Very recently, our groups reported that SA production from co-fermentation of glucose and xylose by Y. lipolytica [29], in which pure or crude glucose and xylose from sugarcane bagasse were utilized as carbon sources. Glucose was completely consumed by the yeast; however, large fraction of xylose (50–70%) remained unutilized. The xylose utilization started only after glucose depletion, which is in agreement with our results. Little is known about xylose metabolizing ability of Y. lipolytica, and there are few literatures available on the uptake of xylose by Y. lipolytica which is quite contradictory. Majority of the studies indicates that Y. lipolytica shows a restricted uptake of xylose prior to adaptation or starvation periods. Genome mining of Y. lipolytica showed the presence of xylose pathway, however the quantitative polymerase chain reaction (qPCR) showed weak mRNA expression of XDH gene (YALI0E12463), indicating the hypothesis that weak expression of XDH is the limiting factor [27].

According to Rodriguez et al. [41], xylose pathway is in the yeast but it was poorly expressed due to cryptic genetic circuits controlling expression of key enzymes. It was found that overexpression of endogenous XDH (YALI0E12463) & XK (YALI0F10293) in Y. lipolytica Po1f strain under the control of UAS1B8-TEF_{min} promoter resulted in cell growth on xylose. In the same year, Ledesma-Amaro et al. engineered Y. lipolytica by introducing XR and XDH from S. stipitis, which serves as a model organism for xylose metabolism [29]. The engineered strain was able to produce lipids and citric acid utilizing xylose as a sole carbon source. They observed that overexpression of XR and XDH is insufficient to enable growth on xylose, and overexpression of XK allowed identical growth to wild type strain. The same group also demonstrated that Y. lipolytica can grow on xylose as well as on glucose after the expression of the three native genes XR, XDH and XK under the control of TEF promoter [42].

This is the first reported study for utilization of metabolic engineered Y. lipolytica for SA production from xylose. To this end, three enzymes, XR, XDH and XK were overexpressed under the control of
constitutive TEF promoter in Y. lipolytica PSA02004. The metabolic pathway of the xylose utilization and SA production by Y. lipolytica PSA02004PP is depicted in Fig. 4. The control strain was unable to grow on xylose as the sole carbon source, but it was able to transform xylose to xylitol in the presence of co-substrates such as glucose and glycerol (Fig. 2). Similar results were obtained by Ledesma-Amaro et al. [29] in which biotransformation of xylose into xylitol during co-fermentations with glucose was observed. The introduction of xylose pathway resulted a marked change in metabolism. The recombinant strain was able to grow (OD$_{600}$: 14.1) on xylose as the sole carbon source, as well as accumulated SA (3.8 g/L), and generated AA (4.1 g/L) as a byproduct (Fig. 3b). In case of co-fermentation with xylose and glucose/glycerol, the xylitol formation was not observed and SA production increased in comparison to the control strain, which is supported by the high XDH activity during the exponential growth phase. Walfridsson et al. reported that high XDH to XR ratio resulted in no xylitol accumulation and high ethanol formation rate in recombinant Saccharomyces cerevisiae strain integrated with multi-copy gene of xyl2 encoding xylitol dehydrogenase [43]. The carbon catabolite repression was observed with xylose and its consumption began after 48 h when glucose/glycerol was largely assimilated. This is in agreement with results obtained by Ledesma-Amaro et al. [29] and Ong et al. [31]. In the current study, we investigated the impact of initial xylose levels on Y. lipolytica using the minimal medium, and it was observed a substrate inhibition beyond 60 g/L. The xylose was completely utilized until 40 g/L and with further increased in the initial xylose concentration in cultivation medium, the amount of unutilized xylose continuously elevated (Fig. 6a). The substrate inhibition effect also negatively affected SA production. The xylitol accumulation was detected at higher xylose concentrations indicating overflow metabolism. Further XR and XDH enzymes are dependent of co-factors such as NADPH and NADH, respectively. In the presence of oxygen, mitochondrial function maintains balance between NADPH/NADH level. At high concentration of substrate, redox imbalance will be provoked and results in xylitol accumulation. Similar trend was observed by Ledesma-Amaro et al. [29]. In their study, xylose was completely consumed at 20 g/L and 30 g/L. Y. lipolytica was unable to uptake all the xylose at initial concentration between 60 g/L and 90 g/L, and 15–70% of xylose was left unconsumed at these concentrations along with xylitol
accumulation. Previously, Lin et al. [44] showed that A. succinogenes 130Z was able to tolerate 143 g/L of glucose, but the cell growth was completely inhibited at an initial glucose concentration of 158 g/L. Further, reduced SA production and prolonged lag phase were observed with the initial glucose concentration above 100 g/L. In this study, high specific growth rate (μ) of 0.017 h\(^{-1}\) at 60 g/L of initial xylose concentration was observed, and the specific growth rate is retarded at higher concentration of xylose. One possible reason for lower specific growth rate might be the possibility of exhaustion of nutrients at higher concentration of xylose, in which secretion of acetate as by product also led to an negative effect on microbial growth [46]. Previously, Robak. [47] reported that the addition of sodium acetate with other carbon sources such as glucose and glycerol resulted in stunted growth rate in Y. lipolytica. The Y. lipolytica ATCC 32338 strain exhibited specific growth rate of 0.004 and 0.002 h\(^{-1}\) at 24 and 48 h respectively, when grown in the medium containing 1% glycerol and 0.6% acetate, while the acetate mutant shown specific growth rate of 0.07 h\(^{-1}\) at 48 h grown in same medium, exhibiting higher resistance to acetate.

In order to predict the performance of the fermentation processes, it is of key importance to gain knowledge on operations involving change with respect to time and behavior of microorganism in presence of different substrate concentration. Substrate inhibition often occurs at elevated carbon source/substrate concentrations which may be due to multiple substrate molecules binding to the same site [48]. Among the various unstructured model used, three parameter Aiba model showed relatively high R\(^2\) value of 0.82 and inhibition concentration (88.87 g/L), which was closer to the experimental data. Further, the saturation constant (K\(_s\)=75.17) clearly indicates that substrate uptake is by the passive diffusion mechanism, which is common in yeast cell factory. Luthfi et al. [45] studied the inhibition kinetics of A. succinogenes 130Z on glucose-based medium by adapting Haldane model and reported that cell growth was inhibited at 78.7 g/L initial glucose concentration. Lin et al. reported exhaustion of nutrients with higher concentration of substrate led to growth retardation of A. succinogenes ZT-130 [45]. Pateraki et al. [65] used modified Monod’s model to identify both substrate and product inhibition of A. succinogenes and Basfia succiniciproducens in a xylose-rich spent sulphite
hydrolysate. They found that at substrate concentration of 67.7 g/L and 45.56 g/L, growth inhibition of A. succinogenes and Basfia succiniciproducens was evident. Further, they also found that the synergetic effect of by-product such as AA and SA have an inhibitory effect on the microbial growth. Similar findings were drawn in our study where at higher concentration of xylose, accumulation of AA showed negative effect on specific growth rate.

The scale up of data from shake flask to bioreactor level improved SA synthesis from 3.8 g/L to 11.2 g/L (Fig. 9). The results obtained with crude xylose from sugarcane bagasse hydrolysate were highly encouraging. The yeast grew robustly (OD$_{600}$: 25.3) on crude xylose despite the presence of AA and furfural at significant levels, in accordance with previous reports on agave hydrolysates [42]. The resultant SA concentration was 5.6 g/L with a yield of 0.14 g/g, which is similar to those obtained with pure xylose (0.18 g/g). The fed-batch cultivation of recombinant strain further improved the performance and yielded a biomass and SA concentration of 11.8 and 22.3 g/L, respectively at 156 h (Fig. 10). Beside cell growth and SA synthesis, AA was continuously accumulated up to 25.0 g/L. These results indicate that more carbon flux is diverted towards by-product formation indicating reduced SA production, however the strain showed resistance at lower pH condition (< 4.0). Cui et al. [25] reported excessive production of AA in glycerol fermentation using Y. lipolytica PGC1003 strain. Hyper accumulation of AA might be due to the imbalance between the flux of glycolysis and TCA cycle, which interrupts the cell growth and also affects the cell metabolism [49]. In order to understand the robustness of the recombinant Y. lipolytica PSA02004PP strain to withstand adverse condition such as low pH, the pH was not controlled in fermentations carried out in this study. The strain was able to grow and biosynthesize SA continuously even after significant reduction in pH, which shows its robustness and flexibility. The recovery and purification of SA is an obstacle for commercial production [14]. The advantage at lower pH is that most of the product fraction will be in acidic form (rather than dissociated form), resulting in simple and cost-effective downstream processes [50]. Table 4 shows SA production by different microorganisms using xylose as carbon source. It was very evident that most of the bacteria such as A. succinogenes with inherited xylose uptake metabolism have shown better SA production capabilities up to 0.8 g/g yield using xylose as a
sole carbon source. However, for the commercial implementation the strain should be able to produce titer of 100 g/L with maximum conversion rate. As compared with native succinic acid producer our studies showed the resultant SA concentration of 11.2 g/L and 22.3 g/L in batch and fed batch cultivation, respectively using xylose as sole carbon source, and about 5.6 g/L SA using xylose rich sugarcane bagasse hydrolysate. Further, much work is needed to enhance the performance required for production of a bulk chemical as succinic acid. The main focus of this study is to utilize rational metabolic engineering strategy to develop a non xylose utilization strain such as Y. lipolytica to utilize xylose which is a major sugar in hemicellulosic fraction of LCB. The experimental results reported in this study demonstrate the promising potential of engineered strain to utilize xylose in lignocellulosic hydrolysate. Unlike bacteria which require optimum pH for the growth and production needs addition of base making process non feasible. Eventually, the commercial process needs to be carried out at lower pH, which is very well demonstrated in this study. Further much of the work need to be directed towards medium and process optimization for maximizing titer and reduction of by-products.

Conclusions
The realization of biological SA production is highly dependent on utilization of low-cost renewable resources. The bio-based SA production from LCB can be a promising strategy as compared to the petrochemical route. The valorization of xylose is imperative for profitable and economical LCB-based SA production. Although the aerobic yeast Y. lipolytica is a promising industrial host, but it was unable to consume xylose, a second major sugar in LCB. In this study, a recombinant Y. lipolytica strain was designed by overexpression of XR, XDH and XK. The introduction of xylose metabolic pathway caused change in metabolism and enabled cell growth and biosynthesis of SA on xylose as the sole carbon source. Similar results in terms of cell growth and SA production from sugarcane bagasse-derived xylose-enriched hydrolysates and defined medium with pure xylose, which demonstrates the potential of Y. lipolytica for lignocellulosic biorefineries. The relationship between the cell growth and substrate concentration was clearly explained by using unstructured mathematical models. The accumulation of SA at low pH gives further advantage. In our knowledge, this is the first report on xylose-based SA production by Y. lipolytica. The work serves as a proof of concept, and it creates room for further
improvements for upcycling of agricultural residues into SA. A large amount of xylose carbon was recovered in the form of AA, which would subsequently inhibit the fermentative production of SA. Further metabolic engineering work to abolish the biosynthesis pathway of AA production is currently underway.

Methods

Materials used in this study

All chemicals used in this study were of analytical grade and purchased from Sigma Aldrich (USA) and Fischer scientific unless stated otherwise. All restriction enzymes, DNA ligase and Q5 Taq DNA polymerase used for the PCR and cloning were purchased from New England Biolabs (NEB) (USA).

Microorganism, culture maintenance and inoculum preparation

The current study made use of strain originated from adaptive evolution of engineered Y. lipolytica PSA02004 with deletion of Ylsdh5 gene encoding a sub-unit of succinate dehydrogenase [26]. The recombinant 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 minimal medium (see Section Submerged cultivations in shake flask). The flasks for seed culture were inoculated by transferring a loopful of 48 h culture grown on YPD plate. The final pH of the medium prior to sterilization was adjusted to 6.8. Cultivation was carried out for 24 h at 30 °C on a rotary shaker at an agitation speed of 250 rpm.

Cloning and expression of heterologous xylose assimilation gene in Y. lipolytica strain

Escherichia coli (DH5α) strain used for cloning and plasmid propagation. The strain was cultivated in the Lysogeny broth (LB) liquid medium at 37 °C. The gene encoding xylose reductase (XR) (YALI0D07634), xylitol dehydrogenase (XDH) (YALI0E12463) and xylulokinase (XK) (YALI0F10923) were extracted from the genome of Po1d using the appropriate primers (Table S2), the golden gateway (GG) assembly was constructed according to former studies [51,52]. The GG was constructed with Scaffold of three genes comprising three transcription units and selection marker, flanked with integration targeting sequences, constructed on a destination vector backbone. Each gene was
flanked with 396 nt of TEF promoter and 122 nt of Lip2 terminator sequences, both native to *Y. lipolytica*. URA3 (1289 nt) gene was used as selection marker in this assembly. Random integrations in *Y. lipolytica* PSA02004 were driven through zeta sequences (305 nt and 395 nt for UP and DOWN respectively). The expression vector is linearized using *NotI* enzyme and gel purified before transformation in *Y. lipolytica*. The overexpression cassette was transformed in the genome of *Y. lipolytica* using lithium acetate method described by Le Dall et al. [54]. The transformants were selected on YNBUra plates, the genomic DNA was isolated using the protocol developed by Lõoke et al. [55], and the positive transformants were identified with PCR. All the plasmids and strains used in this study are listed in Table S1 (supplementary information).

**Submerged cultivations in shake flask**

The minimal medium used for fermentation had the following composition: xylose, 20 g/L; yeast nitrogen base (YNB), 1.7 g/L; NH₄Cl, 1.5 g/L. The medium was prepared in 50 mM phosphate buffer. In case of co-fermentation with two carbon sources, each one was used at a level of 20 g/L. The initial pH was adjusted to 6.8 before inoculation by using 5 N 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 OD₆₀₀ of 0.1 and kept at 30 °C under constant shaking at 250 rpm on a rotary shaker. The xylose-rich lignocellulosic hydrolysate (with an initial xylose concentration of 42.8 g/L) from sugarcane bagasse was obtained from Nova Pangea Technologies, UK.

**Measurement of xylose reductase (XR) and xylitol dehydrogenase (XDH) activities**

For measuring the enzymatic activities, cell free extract was prepared. Initially, the cells were harvested by centrifugation at 8,000 x g and 4°C for 10 minutes. The cell pellet was then washed twice with 50 mM phosphate buffer (pH 7.2) and resuspended in the buffer. The cell disruption was performed in homogenizer by mixing the above-mentioned cells with 0.5 g (0.3 mm) glass beads and vortexed for 10 minutes. The homogenized mixture was centrifuged at 8,000 x g and 4°C for 10 minutes, the supernatant was collected and used for quantifying enzyme activities. The protein concentration was determined by the Bradford method [55].
The activities of xylose reductase (XR) and xylitol dehydrogenase (XDH) were measured using a UV spectrophotometer (Jenway 6310, UK). The molar extinction coefficient of NADPH and NAD$^+$ used for calculation is 6,220 m$^{-1}$cm$^{-1}$. The XR activity was measured by the reduction of the coenzyme NADPH at 30 °C in a reaction medium consisting of 0.17 mM NADPH, 0.17 M xylose, 0.25 mg cell extract, and the final volume was made up to 0.5 mL using 0.1 M phosphate buffer. One unit of XR enzyme activity was defined as the amount of enzyme that catalyzed the oxidation of 1 μmol of NADPH per minute at 30°C. The quantification of XDH activity was based on reduction of the coenzyme NAD$^+$ at 30°C. For XDH measurement, the reaction mixture consists of 1.5 mM NAD$^+$, 0.15 M xylitol, 0.25 mg cell extract with a total volume of 0.5 mL made up by 0.1 M Tris buffer. One unit of XDH was defined as the amount of enzyme catalyzing the oxidation of 1 μmol of NAD$^+$ per minute at 30°C [56].

**Substrate inhibition kinetic studies for recombinant Y. lipolytica**

The substrate inhibition studies were performed in shake flask using a minimal medium as mentioned in Section 2.4 at different concentration of xylose (20, 40, 60, 80, 100, 120 g/L). Specific growth rate (μ) were calculated using Equation (1):

$$
\mu = \frac{1}{X} \frac{dX}{dt} \quad \text{Equation (1)}
$$

Where X= DCW (g/L) and t= fermentation time.

Elevated levels of substrates may hamper the microbial growth and productivity of Y. lipolytica as the multiple substrate can bind to the same site. Several inhibition models derived from the Monod’s kinetics are used in this study to predict the kinetic parameters from the experimental data, and these are presented in Table 1. The parameters of different models were estimated from the experimental results using MATLAB (Mathwork R, 7.1). Since the models had non-linear coefficients, the parameters were quantified iteratively with the aid of non-linear least square algorithm.

**Comparison of models for acceptability**

High regression coefficient or low Mean Square error value for any kinetic model resulted in the best fitted model, which is achieved at a cost of complexity in the model. Since models with different
complexity (i.e. degree of freedom) were chosen, it was a key to test the model which was consistent with the experimental data[57]. In this study best fit 3 and 4 parameter models were compared using Akaike information criterion (AIC).

The Akaike information criterion (AIC) is defined by the following equation:

$$AIC = p \ln \frac{SS}{p} + 2b \quad \text{Equation (2)}$$

where “b” = prm + 1. When there are few data points, the corrected AIC (AICc) is used.

$$AICc = AIC + \frac{2b(b+1)}{p-b-1} \quad \text{Equation (3)}$$

The model with lower AICc value is more likely to be correct and the probability (pAIC) that the more complex model is correct is given by

$$pAIC = \frac{e^{-0.5\Delta AICc}}{1+e^{-0.5\Delta AICc}} \quad \text{Equation (4)}$$

**Bioreactor studies**

The batch experiments were performed in a 2.5 L bench-top bioreactor (Electrolab Bioreactors, UK) with 1.0 L working volume. The minimal medium with 60 g/L xylose was used for running bioreactor experiments. In case of lignocellulosic hydrolysate, the xylose concentration was 40 g/L. The temperature, agitation speed and aeration rate were controlled at 30 °C, 600 rpm and 2.0 L/min, respectively. The starting pH was 6.8, and it remained uncontrolled during the fermentation. For fed-batch fermentations, the residual xylose concentration was maintained at or above 10 g/L with concentrated feed containing 500 g/L xylose and 5 g/L yeast extract.

**Analytical methods**

The samples were withdrawn periodically and analyzed for OD$_{600}$, pH, residual glucose, xylose, succinic and acetic acid. 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 glucose, xylose, succinic and acetic acid 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 PVDFmembrane (Sartorious, Germany)) and eluted using Rezex ROA-Organic Acid H+ (Phenomenex, USA) column at 60 °C attached with refractive index detector (RID) and Diode Array Detector (DAD). The mobile phase and flow rate were 0.5 mM H₂SO₄ and 0.4 mL/min, respectively. All measurements were conducted in triplicates and the values were averaged. The standard deviation was no more than 10 %.

List Of Symbols

- $b$: Variable of AIC analysis (number of parameters plus one)
- $df$: Degree of freedom for a model with experimental data
- $K$: Constant, g/L
- $K_i$: Inhibition constant, g/L
- $K_M$: Monod’s constant, g/L
- $K_s$: Half-saturation constant, g/L
- $p$: Number of experimental data points
- $p_{AIC}$: Probability that model with given AICc value is correct
- $prm$: Number of parameters to be estimated in a model
- $Δ_{AICc}$: Difference in AICc value between two models
- RMSE: Root means square error

Greek letters

- $γ_s$: Initial concentration, g/L
- $γ_s^*$: Critical concentration, g/L
- $μ$: Specific growth rate, h⁻¹
- $μ_{max}$: Maximum specific growth rate, h⁻¹

Declarations

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**Contributions**

Ashish Prabhu carried out all the experimental work, analyzed the data. Vinod Kumar analyzed the data and wrote the manuscript. Frederic Coulon and Vijay Kumar Thakur was involved in proofreading the manuscript. Carol Sze Ki Lin and Rodrigo Ledesma- Amaro provided useful suggestions for experimental design 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: Variation of Monod models for the estimation of substrate inhibition kinetics.
| Model    | Equation                                                                 | Reference |
|----------|--------------------------------------------------------------------------|-----------|
| Andrew’s | $\mu = \frac{\mu_{\text{max}} \gamma_s}{(K_s + \gamma_s) \left(1 + \frac{\gamma_s}{K_I}\right)}$ | [58]      |
| Haldane  | $\mu = \frac{\mu_{\text{max}} \gamma_s}{K_s + \gamma_s + \frac{\gamma_s}{K_I}}$ | [59]      |
| Aiba     | $\mu = \frac{\mu_{\text{max}} \gamma_s}{K_s + \gamma_s} \exp \left(\frac{-\gamma_s}{K_I}\right)$ | [60]      |
| Yano     | $\mu = \frac{\mu_{\text{max}} \gamma_s}{K_s + \gamma_s + \frac{\gamma_s}{K_I} \left(1 + \frac{\gamma_s}{K}\right)}$ | [61]      |
| Edward   | $\mu = \mu_{\text{max}} \gamma_s \left[\exp \left(\frac{-\gamma_s}{K_I}\right) - \exp \left(\frac{-\gamma_s}{K_s}\right)\right]$ | [62]      |
| Webb     | $\mu = \frac{\mu_{\text{max}} \gamma_s \left(1 + \frac{\gamma_s}{K}\right)}{\gamma_s + K_s + \frac{\gamma_s^2}{K_I}}$ | [63]      |

Table 2: Estimation of Kinetic parameters for *Y. lipolytica* PSA02004PP grown at different xylose concentration.
### Table 3: Akike’s information criterion of Aiba and Yano model.

| Models | Model specific information | Akaike’s information criterion |
|--------|----------------------------|---------------------------------|
|        | prm | p | SS        | df | AICc | ΔAICc | pAICc |
| Aiba   | 3   | 7 | 0.000043  | 6  | -74.63 |       |       |
| Yano   | 4   | 7 | 4.162E-05 | 5  | -17.05 | 57.58  | 1     |

Table 4: Production of succinic acid on xylose rich feedstock by various microorganisms.
| Substrate                          | Species                        | Type of fermentation | pH control | concentration (g/L) |
|----------------------------------|--------------------------------|----------------------|------------|---------------------|
| Spent sulphite liquor            | *A. succinogenes* 130Z         | Batch                | Yes        |                     |
| Xylose                           | *A. succinogenes* 130Z         | Batch                | Yes        |                     |
| Sugarcane bagasse                | *A. succinogenes* 130Z         | Batch                | Yes        |                     |
| Corn straw hydrolysate           | *A. succinogenes* CGMCC 1593   | Batch                | Yes        |                     |
| Corn straw hydrolysate           | *A. succinogenes* CGMCC 1593   | Fed-batch            | Yes        |                     |
| Oak wood hydrolysate             | *M. succiniciproducens* MBEL55E| Batch                | Yes        |                     |
| Duckweed acid hydrolysate        | *A. succinogenes* GXAS137      | Batch                | Yes        |                     |
| Sugarcane bagasse hydrolysate    | *E.coli* BA305                 | Fed-batch            | Yes        |                     |
| Xylose                           | *A. succinogenes* CGMCC 2650   | Batch                | Yes        |                     |
| Xylose                           | *B. succiniciproducens* JF4016 | Batch                | Yes        |                     |
| Sugarcane bagasse hydrolysate    | *Y. lipolytica* PSA02004        | Batch                | No         |                     |
| Xylose                           | *Y. lipolytica* PSA02004PP      | Batch                | No         |                     |
| Sugarcane bagasse hydrolysate    | *Y. lipolytica* PSA02004PP      | Batch                | No         |                     |
| Xylose                           | *Y. lipolytica* PSA02004PP      | Fed-batch            | No         |                     |

Figures

(A) General composition of lignocellulosic biomass (adopted from Collard and Blin, 2014);
(B) Different carbon sources in hemicellulosic fraction (adopted from Finore et al., 2015).
Time course profiles of substrate consumption, OD600, pH, production of SA, AA and xylitol during shake flask culture of Y. lipolytica PSA02004 on; (A) Glucose, (B) Glucose + Xylose, (C) Glycerol and (D) Glycerol + Xylose. Symbols: filled square (glucose or glycerol), filled triangle up (OD600), empty square (SA), semi-filled right square (AA), filled circle (xylose), empty circle (xylitol) and filled star (pH).
Figure 3

(A) Golden Gate Assembly bearing pentose pathway genes dedicated to integration with Yarrowia lipolytica PSA02004 genome; (B) Time course profiles of substrate consumption, OD600, pH, production of SA and AA during shake flask culture of Y. lipolytica PSA02004PP on xylose. Symbols: filled circle (xylose), filled triangle up (OD600), empty square (SA), semi-filled right square (AA) and filled star (pH); (C) XR and XDH activity profiles during shake flask cultivation of Y. lipolytica PSA02004PP.
Figure 4

Metabolic pathway for succinic acid production from different carbon sources by engineered strain Y. lipolytica PSA02004PP.
Time course profiles of substrate consumption, OD600, pH, production of SA and AA during shake flask culture of Y. lipolytica PSA02004PP on: (A) Glucose, (B) Glucose + Xylose, (C) Glycerol and (D) Glycerol + Xylose. Symbols: filled square (glucose or glycerol), filled circle (xylose), filled triangle up (OD600), empty square (SA), semi-filled right square (AA) and filled star (pH).
Figure 6

Time course profile of Y. lipolytica PSA02004PP at different xylose levels (A) substrate consumption, (B) OD600, (C) succinic acid, (D) acetic acid, (E) xylitol. Symbols: filled circle (20 g/L), empty circle (40 g/L), filled triangle up (60 g/L), empty triangle up (80 g/L), filled square (100 g/L) and empty square (120 g/L).
Figure 7

(A) Specific growth rate as a function of initial xylose concentration; (B) Experimental and predicted data fit of various substrate inhibition kinetics as a function of xylose concentration.

Figure 8

Sensitivity analysis of Aiba model (A) Maximum specific growth rate (μmax), (B) Monod half saturation (Ks) (C) Substrate inhibition constant (KI).
Batch cultivation of Y. lipolytica PSA02004PP in bioreactor using (A) Pure xylose, (B) Xylose rich hydrolysate derived from sugarcane bagasse. Symbols: filled circle (xylose), filled triangle up (OD600), empty square (SA), semi-filled right square (AA), and filled star (pH).
Figure 10

Fed-batch kinetics of xylose uptake, cell growth, product formation and pH during Y. lipolytica PSA02004PP fermentation in bioreactor. Symbols: filled circle (xylose), filled triangle up (OD600), empty square (SA), semi-filled right square (AA), and filled star (pH).

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