Coordinately Express Hemicellulolytic Enzymes in *Kluyveromyces Marxianus* to Improve the Saccharification and Ethanol Production from Corncobs

Qing Lan
Fudan University
Yitong Duan
Fudan University
Pingping Wu
Fudan University
Xueyin Li
Fudan University
Yao Yu
Fudan University
Bo Shi
Chinese Academy of Agricultural Sciences
Jungang Zhou (zhoujg@fudan.edu.cn)
Fudan University https://orcid.org/0000-0001-9618-1683
Hong Lu
Fudan University

Research

Keywords: Kluyveromyces marxianus, hemicellulases, ribosomes skipping, enzymatic hydrolysis, ethanol

Posted Date: September 2nd, 2021

DOI: https://doi.org/10.21203/rs.3.rs-151980/v2

Version of Record: A version of this preprint was published at Biotechnology for Biofuels on November 22nd, 2021. See the published version at https://doi.org/10.1186/s13068-021-02070-1.

Abstract

Background

Hemicelluloses act as one factor contributing to the recalcitrance of lignocelluloses that prevent cellulases to degrade the cellulose efficiently even in low quantities. Supplement of hemicellulases can enhance performance of commercial cellulases in the enzymatic hydrolyses of lignocellulose. *Kluyveromyce marxianu* is an attractive yeast for cellulosic ethanol fermentation, as well as a promising host for heterologous protein production, since it has remarkable thermotolerance, high growth rate, and broad substrate spectrum etc. In this study, we attempted to coordinately express multiple hemicellulases in *K. marxianus* through a 2A-mediated ribosomes skipping to self-cleave polyproteins, and investigated their capabilities for saccharification and ethanol production from corncobs.

Results

Two polycistronic genes *IMPX* and *IMPαX* were constructed to test the self-cleavage of P2A sequence from Foot and Mouth Disease virus (FMDV) in *K. marxianus*. The *IMPX* gene consisted of a β-mannanase gene *M330* (without the stop codon), a P2A sequence and a β-xylanase gene *Xyn-CDBFV* in turn, while in the *IMPαX* gene there was an additional α-factor signal sequence in frame with the N-terminus of *Xyn-CDBFV.* The extracellular β-mannanase activities of IMPX and IMPαX strains were 21.34 and 15.50 U/mL repectively. By contrast, the IMPαX strain secreted 136.17 U/mLof the β-xylanase, which was much higher than that of IMPX strain 42.07 U/mL. Based on these, two recombinant strains, the IXαR and IMPαXPαR, were constructed to coordinately and secretorily express two xylantic enzymes a β-D-xylosidase RuXyn1 and Xyn-CDBFV, or three hemicellulolytic enzymes including M330, Xyn-CDBFV and RuXyn1. In fed-batch fermentations, extracellular activities of β-xylanase and β-xylosidase in the IMPαX strain were 1664.2 and 0.90 U/mL, while productions of secretory β-mannanase, β-xylanase, and β-xylosidase in the IMPαXPαR strain were 159.8, 2210.5, and 1.25 U/ml of respectively. Hemicellulolytic enzymes of these two strains enhanced the yields of both glucose and xylose from diluted acid pretreated (DAP) corncobs when acted synergistically with commercial cellulases. In hybrid saccharification and fermentation (HSF) of DAP corncobs, hemicellulases of the IMPαXPαR strain increased the ethanol yields by 8.7% at 144 h. When using aqueous ammonia pretreated (AAP) corncobs as HSF feedstocks, the IMPαXPαR strain increased both ethanol and xylose yields, which were about 12.7% and 18.2% more than that of the control at 120 h. Our results indicated that coordinately expression of hemicellulolytic enzymes in *K. marxianus* could promote the saccharification and ethanol production from corncobs.

Conclusions

The FMDV P2A sequence showed high efficiency in self-cleavage of polyproteins in *K. marxianus*, and could be used for secretory expression of multiple enzymes in present of their own signal sequences. The IMPαXPαR strain that coexpressed three hemicellulolytic enzymes improved the saccharification and ethanol production from corncobs, and could be used as a promising strain for ethanol production from lignocelluloses.

Background

Lignocellulose is the most abundant renewable resource on earth, which is recalcitrance and compact biomass that composes of directly interlinked cellulose, hemicelluloses and lignin [1]. Utilization of lignocellulosic biomass is a feasible solution to avoid the excessive reliance on fossil fuels, and alleviates the global warming and environmental pollution events [2]. Unlike first-generation biofuels used edible feedstocks, cellulosic ethanol is manufactured from non-edible carbohydrates contained in plant cell walls, [3]. Bioethanol production from lignocelluloses instead of edible feedstocks is expected to avert the competition for food and energy demand concurrent with the growth of world population, which has exerted great stress on current agriculture, and provided more environmental benefits since it was carbon-neutral that avoids an increase of greenhouse gases in atmosphere [4, 5]. Unlike biodiesel and biogas, two types of biofuels made from living matter that suffered the uncertain combustion standardization due to the different cetane numbers and the variable gelatinization temperatures depending on the source of lipids or the variable methane contents varied with the substrate composition and digestion method, bioethanol is a more uniform and cleaner source of fuel that can be used as alone, or mixed in varying amounts with gasoline [6]. Bioethanol production basically composes of four phases that include pretreatment, hydrolysis, fermentation and dehydration. Hydrolysis (saccharification) of pretreated lignocelluloses is a critical prerequisite for ethanolic fermentation by microbes, but usually it can be concurrently integrated with fermentation, known as simultaneous saccharification and fermentation (SSF), when using enzymes to hydrolyze cellulose and hemicellulose into fermentable sugars [7].

To depolymerize lignocelluloses into fermentable sugars, at least three types of cellulases, such as β–1,4-endoglucanase, exocellobiohydrolase, and β–1,4-glucosidase, and more diversity of hemicellulases including β–1,4-xylanase, β–1,4-xylosidase, β–1,4-mannanase, α-arabinosidases, and esterases etc are required to act synergistically [8]. But the less catalytic efficiency and high cost of enzymes made the cellulose hydrolysis become the major bottleneck for bringing down the production cost of biofuel from lignocelluloses[9, 10]. A consolidated bioprocessing (CBP) strategy that integrated enzyme production, saccharification, and fermentation in one step is well accepted as an attractive approach to reduce the cost of biofuel production [11]. However, conventional yeast and bacteria for separated hydrolysis and fermentation (SHF) and SSF processes are well established, while the use of ideal CBP is still on the way. Naturally, a hybrid saccharification and fermentation (HSF), also called hybrid hydrolysis and fermentation (HHF), is set out by hydrolyzing pretreated lignocelluloses with cellulases before a CBP or SSF process [12, 13].

CBP microbes for cellulosic ethanol production were genetically modified from either natural cellulolytic bacteria, such as *Cellulolytic thermophiles, Caldicellulosiruptor bescii,* and *Thermoanaerobacterium saccharolyticum* etc, and filamentous fungi, such as *Trichoderma reesei, Aspergillus niger, Fusarium oxysporum,* and *Penicillium oxalicum* etc, or ethanologenic microorganisms including *Saccharomyces cerevisiae, Kluyveromyces marxianus, Zygosaccharomyces bailii* and *Zymomonas mobilis,* by a combination of cellulase production, enzymatic hydrolysis, and microbial fermentation into a single operation [14, 15]. But the low ethanol tolerance is an actual inferiority of cellulolytic microbes, since

distillation of ethanol is an energy-intensive process and it consumes more of heat to separate ethanol from a lower concentration fermentation [16, 17]. Co-fermentation of pentoses, xylose and arabinose, is a reasonable way to raise the bioethanol concentration from lignocellulosic biomass and reduce the cost of cellulosic ethanol at the same time [18]. As the most utilized yeast for ethanol fermentation, *S. cerevisiae* isunable to assimilate xylose and other C5 sugars, which impedes the efficient ethanol conversion from lignocellulose, even with efficient glucose fermentation and high ethanol productivity and tolerance [19]. *K. marxianus* is regarded as another attractive yeast for ethanolic fermentation due to its abilities of fastest growth, remarkable thermotolerance, and broad substrate spectrum including glucose, mannose, galactose, lactose, cellobiose, the pentose sugars xylose and arabinose, which virtually presented in all enzymatic hydrolysates of pretreated lignocelluloses [20–23]. Factually, either in SSF or HSF, high-temperature fermentation can significantly elevate the efficiency of lignocellulose hydrolysis, decrease the risk of contamination, and curtail the ethanol production phase [7, 24].

To be ethanologenic CBP strains, saccharification enzymes converting cellulose into fermentable sugars were required to simultaneously express in one host, while the genetic basis of *K. marxianus* less well understood [25]. A synthetic biology technique termed as “Promoter-based Gene Assembly and Simultaneous Overexpression (PGASO)" was developed to integrate gene cassettes into *K. marxianus* KY3 genome in a single step, with each gene expression regulated by an individual promoter along with a terminator [26]. Unfortunately, the double homologous recombination frequency was very low in *K. marxianus,* even flanking with long homologous fragments [27]. On the contrary, it has a high activity of non-homologous end-joining (NHEJ) that can efficiently integrate non-homologous DNA fragments into chromosome via fusing two DNA strands together in the absence of specific sequences [28, 29]. This feature is disadvantageous to integrate expression cassettes into the specific target loci. In the present study, we incorporated a different way for coexpression of multiple hemicellulases in one replicative plasmid using a P2A self-processing peptide form foot-and-mouth disease virus (FMDV) in *K. marxianus.* P2A sequences are relatively short oligopeptides located between the P1 and P2 proteins in some *picornavirus* viruses, and it can undergo an enzyme-independent self-cleavage at its own C terminus during protein translation, enabling the ribosome skipping to the next codon to continue the translation [30–32]. By assembling a β-mannanase M330 gene and a β-xylanase Xyn-CDBFV gene into a single ORF with the FMDV P2A [33], the efficiency of P2A self-cleavage in secretory expression of multiple enzymes in *K. marxianus* was evaluated. Results showed that FMDV P2A could efficiently secrete three hemicellulolytic enzymes. More importantly, the saccharification and ethanol production in HSFs of corncobs were improved by using the engineered strain as a fermentation starter. Our findings demostrate that the 2A-mediated ribosomes skipping is a good tool for secretory co-expression of multiple enzymes in *K. marxianus,* which is greatly beneficial to the construction of CBP strains for cellulosic ethanol production.

Results And Discussion

Self-cleavage of polyprotein with FMDV P2A in *K. marxianus*

Due to the chemical diversity of hemicellulose structure that heterogeneous polysaccharides with both linear and branched molecules are cross-linked to cellulose microfibrils, complete degradation requires multiple hemicellulases to act synergically [34]. Aiming to facilely express multiple enzymes in ethanologenic *K. marxianus* for the hemicellulose degradation, we resorted to a 2A-mediated ribosomes skipping for co-translational cleavage of the polyprotein. The 2A-mediated cleavage is a common phenomenon in eukaryotic cells that it skips the glycyl–prolyl peptide bond synthesis at the C-terminus of 2A, releases the nascent protein, and resumes the downstream translation [35]. While the 2A self-cleavage efficiency strongly relies on the sequence contexts of upstream and downstream ORFs in the polycistrons [36]. Given that we tested the efficiency of FMDV P2A self-cleaving in *K. marxianus* by expression of three polycistronic genes *IMX, IMPX,* and *IMPαX (*Fig1a and b). The *IMX* gene consisted ofa M330 coding sequence (*INU1* signal peptide+mature protein coding sequence) and a C-terminal 6xHis-tagged Xyn-CDBFV mature protein coding sequence fused in-frame directly. In the *IMPX* gene, the P2A sequence was incorporated between M330 and Xyn-CDBFV without stop codon. The *IMPαX* gene had an extra α-factor signal sequence between P2A and Xyn-CDBFV. These three polycistronic genes were all cloned into the vector pUKDN132, in which their expressions were all driven by an *INU1* promoter from *K. marxianus.*

After cultured in flasks, expressions of M330 and Xyn-CDBFV were detected by measuring the activities of β-mannanase and β-xylanase in both supernatants and cell lysates of the IMX, IMPX, and IMPαX strains transformed with the plasmids pUKDN132/IMX, pUKDN132/IMPX, and pUKDN132/IMPαX, respectively. Unexpectedly, the IMX strain, as a control, produced high activities of both β-mannanase and β-xylanase in the supernatant, with approximately 24.03 U/ml and 155.26 U/ml respectively (Table 2), suggesting that these two genes fused directly did not impair their catalytic activities. The double-activities of the IMX strain provided a good reference to the effect of P2A on expression of downstream Xyn-CDBFV. The extracellular β-mannanase activities of IMPX and IMPαX strains were about 21.34 and 15.50 U/mL respectively, which were slightly lower than that of the IMX strain, whereas the intracellular activities of M330 in the two strains were higher, inferring that fusion of Xyn-CDBFV to the C-terminus of M330 with P2A slightly decreased the secretory expression of M330.

In our constructs, the efficiency of FMDV P2A self-cleavage was associated with the production of Xyn-CDBFV. Enzymatic determinations demonstrated that the IMPαX strains secreted 136.17 U/mL β-xylanase into the supernatants, and retained 39.43 U/mL intracellularly. By contrast, the supernatant β-xylanase of IMPX strain was 42.07 U/mL, which was far less than the intracellular activity 87.59 U/mL. To confirm whether the β-xylanase activities of IMPX and IMPαX strains were the self-cleavaged Xyn-CDBFV by the 2A-mediated ribosomes skipping during translation, these samples were further analyzed by SDS-PAGE and western blot. As shown in figure 1c and e, these were protein bands with approximate 57 kDa molecular weight in the supernatants of IMX strain, which were in accord with the theoretical prediction of the fusion protein IMX. But in both supernatants of the IMPαX and IMPX strains, M330 and Xyn-CDBFV were secreted alone, and the secretory Xyn-CDBFV in IMPαX strain was much higher than that of the IMPX strain, suggesting that, in the presence of P2A and α-factor signal sequence, Xyn-CDBFV could be secreted to medium more efficiently. This result was in agreement with the previous literature [37]. Furthermore, western blot assays for the His-tagged Xyn-CDBFV in the above samples were in

compliance with the enzymatic assays and SDS-PAGE above (Fig 1 d and f). Nevertheless, to extracellularly express two proteins via FMDV P2A self-cleavage, an extra signal sequence should be included at the N-terminus of downstream gene. The 2A-mediated ribosomal 'skipping' is an attractive alternative to the internal ribosomal entry site (IRES), first identified in the *encephalomyocarditis* virus, since it can express multiple cistrons at equimolar levels [38]. However, in our results, we also found ribosomal 'skipping' in co-translation decreased in apparent the total level of expressed proteins, especially for the downstream one.

Coexpression of hemicellulolytic enzymes with FMDV P2A

Hemicelluloses act as one important factor contributing to the recalcitrance of lignocelluloses, and they, even in low quantities, can prevent cellulases to degrade the cellulose efficiently [39]. Cellulase supplemented with endoxylanase promoted the hydrolysis of steam-exploded feed stocks, released more glucose, accumulated higher content of xylobiose and xylooligosaccharides [40, 41]. Xylose yield, however, was not significantly elevated, which may be due to the insufficient β-xylosidase in most cellulase enzymes produced by filamentous fungi *Trichoderma reesei [*8, 42]. We reasoned that an ethanologenic strain co-expressed multiple hemicellulases, especially β-xylanase and β-xylosidase, would eliminate theaccumulation of xylooligosaccharides and produce more fermentable xylose. A β-xylosidase RuXyn1 with high capability of converting intermediate xylo-oligosaccharides into xylose was used to co-express with β-xylanase in *K. marxianus* [43]. The RuXyn1 coding sequence was fused to Xyn-CDBFV with a P2A and an α-factor signal sequence (Fig. 2a), and then the resulting IXPαR was expressed in *K. marxianus* under the unique *INU1* promoter. The IXPαR strain transformed with the pUKDN132/IXPαR produced 59.01 and 0.05 U/ml of extracellular β-xylanase and β-xylosidase in flask cultures respectively (Fig 2 b-d).

Supplements of β-mannanase facilitated the total enzymatic hydrolysis of lignocellulose feedstock and brewery's by-product, such as beech sawdust, spruce, Douglas fir wood and chips spent grain [44–47]. Given critical roles of β-mannanase, β-xylanase and β-xylosidase in the hydrolysis of lignocellulose, we tested the feasibility of P2A for coordinately expressing three selected enzymes in one ORF. A polycistronic gene *IMPαXPαR* compacted *M330, Xyn-CDBFV* and *RuXyn1* into one ORF was contructed, each with a signal sequence (Fig. 2a). Consistent with the IMPαX and IXPαR strains, activities of all three enzymes were detectable in the crude supernatant of the IMPαXPαR strain that was obtained by transformation of the pUKDN132/IMPαXPαR plasmid. The activities of β-mannanase, β-xylanase and β-xylosidase were 18.90, 61.00, and 0.07 U/mL, respectively (Fig. 2c-e). As expected, figure 2b showed three protein bands in the culture supernatant of IMPαXPαR strain, which were corresponding to the predicted molecular weights of M330, Xyn-CDBFV and RuXyn1, confirming that FMDV P2A is applicable for secretory co-expression of multiple enzymes in *K. marxianus.*

Preparation of hemicellulolase mixtures by recombinant *K. marxianus* strains

We have previously developed a high-cell density fed-batch fermentation for single hemicellulolytic enzyme production in *K. marxianus* [22]. In this study, we evaluated the productions of multiple enzymes

in fed-batch fermentation for both the IXPαR and IMPαXPαR strains. *K. marxianus* is a Crabtree negative yeast that does not perform aerobic alcoholic fermentation, and but can respire even in high glucose concentrations [48, 49]. However, high concentration of glucose could adversely cause respiratory repression and turn to alcoholic fermentation especially in high-cell density, probably due to the insufficient oxygen supply. Similar to *S. cerevisiae,* a Crabtree positive yeast that predominantly produces ethanol in high glucose even in sufficient oxygen levels, it is practicable to guide *K. marxianus* to utilize glucose for respiratory metabolism and convert carbon resources into cell biomass, as glucose can be fed slowly to maintain a concentration below the threshold value in fed-batch fermentation [50, 51]. Additionally, ethanol fermentation could affect cell growthin *K. marxianus,* and thus it decreases expression of enzyme. To circumvent this, we controlled the dissolved O_2 above 10% by limiting the fed rate of glucose during fermentation. Cell densities of both strains reached more than 450 (OD_{600nm}) after 48 h (Fig. 3a). Productions of secretory proteins synchronized with the cell growths, and all enzymes were dramatically accumulated during the stages from 16 h to 48 h (Fig. 3b-d). After 72 h, the IXPαR strain secreted 1664.2 U/ml of l β-xylanase and 0.90 U/ml β-xylosidase, which were about 28 and 18 folds that of in the flask cultures respectively. SDS-PAGE showed that the IXαR strain secreted two different protein bands that represented mature forms of Xyn-CDBFV and RuXyn1. The IMPαXPαR strain produced 2210.5 U/ml of β-xylanase and 1.25 U/ml of β-xylosidase, slightly higher than that of the IXαR strain. As well, this strain also produced 159.8 U/ml of β-mannanase concurrently, and all enzymes were secreted extracellularly as their mature forms (Fig. 3e and f).

Enzymatic hydrolyses of pretreated corncobs

Hemicellulases supplementation to commercial cellulases enhanced the enzymatic hydrolyses of lignocellulose significantly [52, 53]. Using prepared hemicellulase cocktails, we next evaluated their performances on promotion of lignocellulose hydrolyses. We chosed corncob as feedstocks for the enzymatic hydrolyses because it is one of the most abundant inedible agricultural residues and consists of a relatively high content of hemicellulose (~40%) [54]. Enzymatic hydrolyses were conducted with 10 % (w/v) corncobs pretreated by aqueous dilute acid, and 5 FPU of Cellic® CTec2 cellulase per gram solids. After 96 h, about 300 mM soluble sugars were released from the pretreated corncobs. To test the β-xylanase Xyn CDBFV and β-xylosidase RuXy1 performances on the enzymatic hydrolyses, 300 μl of supernatant collected from the IXPαR strain fed-batch culture at 48 h, equal to 531.29 U β-xylanase and 0.22 U β-xylosidase, was supplemented to the Cellic® CTec2 cellulase. In accord with previous literatures on pine kraft pulp and softwood [45, 55], supplementations of xylanolytic enzymes to the Cellic® CTec2 cellulase improved the enzymatic hydrolysis of corncobs. At each sampling point, addition of the IXPαR strain culture supernatant generated higher contents of soluble sugars. After 96 h of hydrolysis, release of soluble sugars increased by 15.7% (Fig 4a). Similarly, the amounts of monomeric glucose and xylose increased to 8.32 and 61.39 g/L respectively, which were 11.2% and 11.1% higher than that of Cellic® CTec2 cellulase alone (Fig 4b and c).

The role of β-mannanase M330 for the corncob hydrolysis in combination of β-xylanase and β-xylosidase was also evaluated. The culture supernatant of IMPαXPαR strain containing 49.50 U β-mannanase,

485.70 U β-xylanase, and 0.28 U β-xylosidase was supplemented to the Cellic® CTec2 cellulase. As shown in Fig. 4a, the supplementary β-mannanase increased the amount of total soluble sugars over time. At 96 h, about 12.1% more soluble sugars were obtained comparing to that of the IXPαR strain, and the glucose and xylose contents were increased to 65.48 and 8.45 g/L (Fig 4b and c), which were 11.9% and 11.4% higher than that of xylanolytic enzymes respectively, showing that β-mannanase could facilitate a more extensive break-down of corncobs. This promotion may be ascribed to the deep hydrolysis glucomannan by synergistic action of β-mannanase with endoglucanase TrCel5A of *T. reesei* presented in Cellic® CTec2, a crude cellulase produced by the ascomycete fungus *T. reesei,* since TrCel5A has minor hydrolytic activity towards glucomannans [45].

HSFs of ethanol from pretreated corncobs

Besides the application in expression of heterologous protein, the *K. marxianus* strain used in this study can produce ethanol from multiple substrates, including glucose, xylose, lactose, and inulin, with a maximum ethanol concentration higher than 100 g/L [56, 57]. Hemicellulolase expressed by the IMPαXPαR strainwould be conducive to ethanol production from pretreated lignocellulosic biomass. Subsequently, we investigated the potential of recombinant IMPαXPαR strain as a fermentation starter to produce ethanol from both the DAP and AAP corncobs. HSFs were performed by pre-hydrolyzing pretreated corncobs with 10 FPU commercial cellulase per gram solids at a solid-to-liquid (S/L) ratio of 1:10 (irrespective of the moisture content) for 72 h before inoculated with the IMPαXPαR or FIM–1 (control) strain. Considering that, *K. marxianus* is more strictly Crabtree-negative than the model organism species *Kluyveromyces lactis* and other known Crabtree-negative yeasts that it cannot grow under strictly anaerobic conditions and its ethanol fermentation exclusively relies on oxygen limitation [58–60]. Meanwhile, dissolved oxygen tension is a key factor for the production of inulinase in *K. marxianus [*61, 62]. Since expressions of hemicellulolytic enzymes were drived by the inulinase promoter in the IMPαXPαR strain, to ensure the hemicellulolytic enzymes were highly expressed and sufficient for HSFs, HSFs starters were prepared by high-cell-density fed-batch cultures in 5L fermenters under aeration and agitation. In addition, the mode of pretreatment might have a great effect on the structure and composition of corncobs [63, 64], which may effect the performance of IMPαXPαR strain in HSFs. Consequently, corncobs pretreated by the diluted acid and aqueous ammonia, containing 57.4% and 48.9% of glucan, and 8.2% and 32.1% of xylan respectively, were both used for HSFs. After pre-hydrolysis by Ctec2 cellulase, about 80% glucan and 84% xylan of DAP corncobs were degraded into monosaccharides.

After prehydrolysis with Cellic® CTec2, about 80% glucan and 84% xylan of DAP corncobs were degraded into monosaccharides, liberating 48.5 g/L glucose and 7.2 g/L xylose. Ethanol fermentations were started inoculating the cell cultures of IMPαXPαR and control prepared by fed batch fermentation. As shown in Figure 5a, glucose and xylose contents in HSFs with IMPαXPαR strain were slightly higher than with FIM–1strain during the first 72 h when using the DAP corncobs, which were in agreement with the enzymatic saccharification described above. Similar to the glucose and xylose productions, using the DAP corncobs, ethanol yields from IMPαXPαR were slightly higher than the control. At 144 h, the ethanol

concentration in HSF with the IMPαXPαR strain was 16.4 g/L, and it was about 8.7% higher than the control 15.1 g/L. But for xylitol production, there was no significantly different between the IMPαXPαR HSFs and the control, since the xylitol concentrations were very low in all HSFs, which was below 1g/L even after 240 h fermentation. The reason for this might be strongly repression of xylose utilization by glucose in simultaneous fermentation of them with *K. marxianus [*65].

But in the case of AAP corncobs, only 60–70% glucan and 62–67% xylan were hydrolyzed by Ctec2 before HSFs. Due to a higher saccharification of AAP corncobs by the Cellic® CTec2 cellulase in the control HSFs, glucose and xylose contents in samples for IMPαXPαR HSFs were 26.4 g/L and 17.9 g/L, which were lower than those in control, 30.9 g/L and 19.4 g/L respectively. As a result, during the preceding 48 h of HSFs, both glucose and xylose contents in the IMPαXPαR HSFs were lower than in the control (Fig. 5b). However, their contents in the IMPαXPαR HSFs became apparently higher than the control at 72 h, as there were 3.3 g/L of glucose and 16.9 g/L of xylose in the IMPαXPαR HSFs, and 1.68 g/L of glucose and 15.0 g/L of xylose in the control, respectively. After 120 h, glucose was depleted in all HSFs, but the xylose content in the IMPαXPαR HSFs were still 17.5 g/L, which was 18.2% higher than in the control. The reason for this was that *K. marxianus* cannot assimilate xylose to form ethanol under anaerobic condition [66]. Similarly, ethanol yield in the IMPαXPαR HSFs was 14.2 g/L at this time point, which was 12.7% higher than that of the control. Before 144 h, xylitol yield with the IMPαXPαR strain was not different than the control. Consequently, we confirmed that the hemicellulolytic enzymes produced by IMPαXPαR strain improved the hydrolysis and ethanol conversion in HSFs of pretreated corncobs, especially with high xylan content of feedstocks. Comparing with HSFs of DAP corncobs, glucose consumption rate was in apparent higher when using AAP corncobs. Additionally, xylose was consumed to form xylitol before 48h in all HSFs of AAP corncobs, while its consumption did not occur until 144 h in HSFs of DAP corncobs. A recent study demostrated that the addition of nitrogen increased the fermentative capacity of *K. marxianus* in ethanol production [67]. Presumably, the residual ammonia in AAP corncobs was conducive to the glucose uptake and ethanol production and turned to affecting the xylose ultilization.

Conclusion

In this study, we used a 2A-mediated ribosomes skipping strategy to coordinately express hemicellulolytic enzymes in *K. marxianus,* andinvestigated the performances of the multiple expressed enzymes in saccharification and ethanol production from pretreated corncobs. We showed high efficiency of the FMDV P2A in secretory co-expression of multiple enzymes in *K. marxianus,* and that three hemicellulolytic enzymes, including a β-mannanase M330, a β-xylanase Xyn CDBFV, and β-xylosidase RuXyn1, were coordinately secreted in the IMPαXPαR strain. Multiple enzymes of the recombinant *K. marxianus* strains increased both glucose and xylose yields from DAP corncobs when acted with the commercial cellulases, indicating that strengthening of the hemicellulolytic activity could improve the enzymatic saccharification of lignocellulose. Considering the effect of oxygen limition on the expression of multiple enzymes in *K. marxianus* during ethanol fermentation*,* as well as a compromise of ethanol productivity with enzyme productions, HSFs of pretreated corncobs were conducted using fed-batch

cultures grown under aeration and agitation. Ethanol yield in HSF of DAP corncobs with the IMPαXPαR strain was about 8.7% higher than the control, while it was 12.7% higher when using AAP corncobs. When using DAP corncobs, there was no difference in the productions of xylose along with xylitol between the IMPαXPαR and control strain. By contrast, in HSF of AAP corncobs with higher xylan contents, the xylose yield in the IMPαXPαR HSFs was 18.2% higher than in the control at 120 h, suggesting that promotions of ethanol and xylose yields in HSFs by hemicellulases were closed to the content of hemicellulose in feedstocks. Our findings demostrate that the 2A-mediated ribosomes skipping is a good tool for secretory co-expression of multiple enzymes in *K. marxianus,* which is greatly beneficial to the construction of CBP strains for cellulosic ethanol production.

Methods

Strains and plasmids

The *K. marxianus* Fim−1*URA3* strain is uracil auxotrophic that derived from FIM−1 deposited in China General Microbiological Culture Collection Center (CGMCC No.10621). The expression plasmid pUKDN132 was constructed as described previously [22].

Expression plasmids constructions and transformations

A polycistronic gene *M330-Xyn-CDBFV (*hereafter termed the IMX gene) that a β-xylanase (EC 3.2.1.8) gene *Xyn-CDBFV* from *Neocallimastix patriciarum* was directly fused to the C terminus of β-mannanase (EC. 3.2.1.78) gene *M330* from *Bacillus sp.* N16−5 was constructed as described below. The *M330* gene was amplified from the pZP41plasmid by the primers MF and IMXR1 (Table 1), and the *Xyn-CDBFV* gene was amplified with IMXF and XR from a pET21a/Xyn-CDBFV[68]. After purification with a SanPrep Column DNA Gel Extraction Kit (B518131, Sangon Biotech, Shanghai, China), the two PCR fragments were ligated together by Gibson assembly [69], and then used as a template to amplify the fused hybrid gene *IMX* with the primers MF and XR. The resulting PCR amplicon was ligated with the *Spe* I and *Not* I linearized pUKDN132 by Gibson assembly, and generated the plasmid pUKDN132/IMX.

Two polycistronic genes, *IMPX* and *IMPαX,* that contained a P2A sequence between *M330* and *Xyn-CDBFV* alone or with a α-factor signal sequence from *S. cerevisiae* were also constructed. The *P2A* sequence was added to the 3' terminusof *M330* by PCR using the primers MF and IMPR. The *Xyn-CDBFV* sequence was amplified by the primer pair IMPXF/XR, and ligated with the *P2A*fused *M330.* After that the full-length *IMPX* was amplified by the primers MF and XR, and then inserted into the pUKDN132, obtaining the pUKDN132/IMPX plasmid. When assembling the *IMPαX* gene,the α-factor signal sequence was amplified from the plasmid pPIC9 (Invitrogen, USA) using the primers PαF1 and αXR1, and the *Xyn-CDBFV* sequence was amplified by the primers αXF1 and XR from the pET21a/Xyn-CDBFV. Three fragments including the *P2A*fused *M330,* α-factor signal sequence, and *Xyn-CDBFV* were ligated together to assemble thepolycistronic gene *IMPαX* as describedabove. After cloned into pUKDN132, the resulting plasmid was then termed pUKDN132/IMPαX.

The polycistronic *IMPαXPαR* gene that integrated three genes into a single ORF was constructed by assembling the *IMPαX* lack of the stop codon TAG, a *P2A* linked α-factor signal sequence and a β-xylosidase (EC.3.2.1.37) gene *RuXyn1* from uncultured Yak rumen microorganism. The *IMPαX*fragment and the *P2A* linked α-factor signal sequence were amplified from pUKDN132/IMPαX by the primer pairs, MF/XPR and XPαF/αR1, respectively, while the *RuXyn1* fragment was amplified from a pET21/RuXyn1vector using the primers αRF and RR [43]. Three fragments were ligated by Gibson assembly to get the full length of *IMPαXPαR.* After PCR amplification with the primers MF and RR, the *IMPαXPαR* was inserted into the *Spe* I/*Not* I site of pUKDN132 to obtain the plasmid pUKDN132/IMPαXPαR. The plasmid pUKDN132/IXPαR were constructed by inserted the XPαR fragment, amplified from pUKDN132/IMPαXPαR by the primers IXF and RR, into the*Spe*I/*Not* I site of pUKDN132.

For plasmid transformations, the *K. marxianus* Fim−1 *URA3* was inoculated in 5 ml YPD medium (1% Yeast Extract, 2% Peptone, 2% Glucose, pH 6.5), and cultured at 30°C, 200 rpm for 20 h. Yeast cells were collected by centrifugation, and all plasmid transformations were conducted according to the method by Antunes et al [70]. Transformants were then selected on synthetic defined (SD) plates (pH 5.5) containing 0.67% yeast nitrogen base without amino acids (YNB), 2% glucose, and 2% agar.

Enzymatic assays

The activity of β-mannanase was determined with 0.5% locust bean gum (G0753, Sigma-Aldrich, USA) in 50 mM acetate buffer pH 5.5 at 68 ℃ [71]. Quantitative assays of β-xylanase were performed using 1% wheat arabinoxylan (P-WAXYL, Megazyme, Bray, Ireland) buffered with 50 mM acetate pH 5.5 at 50 ℃[68]. β-xylosidase activities were measured using *p*-nitrophenyl-β-D-xylopyranoside as we described previously [43]. One unit (U) of enzyme activity was defined as the amount of enzyme releasing 1 μmol of reducing sugars or *p*-nitrophenol per minute.

Western blot assays

Transformants were grown in YG mediums (2% yeast extract, 4% glucose, pH 6.0) at 30 ℃, 200 rpm for 72 h. One milliliter of cultures was harvested and centrifuged for 10 min at 5,000 rpm to detect the secretory or intracellular expression of enzymes by western blot. To prepare lysate samples, cells were suspended in 1mL lysis buffer (50 mM HEPES pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X−100, 0.1% Na-deoxycholate), and then disrupted by a bead-beater (FastPrep−24, MP, California, USA) at 6 m/s for 2 min with 400 μL acid-washed glass beads (G8772, Sigma-Aldrich, Missouri, USA). Western blots were carried out using an Anti-His Tag antibody (M30111, Abmart, Shanghai, China) and a horseradish peroxidase-conjugated goat-anti-mouse secondary antibody (074−1806, KPL, USA) as described previously [22].

Fed-batch fermentations

All fermentations were performed in 5 L bioreactors (BXBIO, Shanghai, China) with an initial working volume of 1.5 L as described previously [22]. Inoculum seeds were precultured in Erlenmeyer flasks containing 150 mL YG medium at 30 °C, 220 rpm for 18 h [22]. After sterilization and cooling, temperatures of the bioreactors were set to 30 °C. Batch fermentations were started by inoculating with 150 mL inoculum seeds. After glucose was completely depleted, concentrated mediums consisting of 600 g/L glucose, 5 mg/L biotin, 100 mg/L calcium pantothenate, and 100 mg/L niacin were fed into the reactors at rates of 20–35 mL/h depending on the dissolved oxygen (DO), which should be maintained above 10%. The pHs were controlled automatically at 5.5 with ammonium hydroxide. Samplings at given intervals were determined for cell densities (OD_{600} nm), wet cell weights (WCW), and enzymes activities.

Pretreatment of the corncobs

Corncobs, purchased from Bei Piao Bang Bang Corncob Development Company (Beijing, China), were ground to a particle size range of 0.25–0.45 mm (40–60 meshes). For dilute acid pretreatment, corncobs were immersed in an aqueous solution of 2% diluted sulfuric acid at a solid-to-liquid (S/L) ratio of 1:5. The mixtures were autoclaved at 121°C for 1 h. After neutralization with 0.1 N NaOH, the pretreated corncobs were separated by filtration under vacuum, washed with deionized water, and dried at 80 ℃. Aqueous ammonia pretreatment were performed by soaking corncobs with 15% ammonia in a screw-capped bottle at a solid–liquid ratio of 1:7 at 60 °C for 24 h. After pretreatment, the pretreated corncobs were diluted with four volumes of deionized wate, filtrated under vacuum, and washed with deionized water until the pH reached around 7.0 before drying at 80 ℃. Compositions of pretreated corncobs were determined according to National Renewable Energy Laboratory (NREL) procedures LAP–002 and –005[72, 73].

Enzymatic saccharification and fermentation

Enzymatic saccharifications were performed in 150 mL Erlenmeyer flasks with 2 g pretreated corncobs in 20 mL of 50 mM sodium citrate buffer pH 5.5. The corncobs slurries were autoclaved at 121 ℃ for 30 min. After addition of 5 FPU CTec2 per g corncob or coupled with 300 µL supernatant of the fed-batch fermentation cultures, flasks were stirred in an air incubator shaker at 45 ℃, 150 rpm. At given intervals, hydrolysates were sampled for sugar analyses.

HSFs were conducted in 150 mL flasks each containing 10 g of the diluted acid pretreated (DAP) corncobs (with 4.5 % moisture content) or aqueous ammonia pretreated (AAP) corncobs (with 18.6 % moisture content). The corncobs were immersed in 80 mL of 50 mM sodium citrate buffer pH 5.5 and autoclaved at 121 ℃ for 20 min. Following sterilization, 10 FPU CTec2 per gram corncob was added and enzymatic saccharifications were performed at 45 ℃, 150 rpm [74]. After 72 h, 10 mL of filter-sterilized media (20 g/L KH_2PO_4, 20 g/L $(NH_4)_2SO_4$, 10 g/L MgSO4·$7H_2O$, 5 g/L yeast extract, and 1 g/L $MnSO_4$)[7] and 1 mL of fed-batch cultures, collected at 48 h and adjusted to equal cell densities (OD_{600nm}, 300) with sterile deionized water, were added to the corncobs slurries. Sterile deionized water was supplemented to make 100 mL of the total liquid volume. The flasks were incubated at 30 ℃ without stirring. Every 24 h,

200 µl liquid of each sample was taken, centrifuged and the supernatants were analyzed for glucose, xylose, xylitol, and ethanol.

Analytical methods

Reducing sugars were determined by the DNS method [75]. HPLC analyses of glucose, xylose, xylitol, and ethanol were performed using a MetaCarb 87H column (300 × 7.8 mm) (Agilent, USA) with a refractive index detector at 35 ℃. Twenty microliters of each sample were injected and eluted with 0.01N H_2SO_4 in water at a rate of 0.6 mL/min for 30 min.

Abbreviations

SHF: Separated Hydrolysis and Fermentation; CBP: consolidated bioprocessing; HSF: hybrid saccharification and fermentation; SSF: simultaneous saccharification and fermentation; FMDV: Foot-and-Mouth Disease virus; DO: the dissolved oxygen; WCW: wet cell weights; FPU: filter paper unit; ORF: open read frame; IRES: the internal ribosomal entry site; DAP: diluted acid pretreated; AAP: aqueous ammonia pretreated.

Declarations

Acknowledgements

Not applicable.

Funding

This project was sponsored by National Key Research and Development Program of China (2021YFC2100203), Tianjin Synthetic Biotechnology Innovation Capacity Improvement Project (TSBICIP-KJGG–006), National Natural Science Foundation of China (31200022), and Science and Technology Research Program of Shanghai (19DZ2282100).

Contributions

JZ and HL conceived the study and wrote the manuscript. QL, YD, PW, and XL performed the experiments, acquisition and interpretation of data. YY, BS, JZ, and QL guided the study, analyzed the data, and edited the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors have given their consent for the publication.

Conflict of Interest

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 this published article.

References

1. Lorenci Woiciechowski A, et al. Lignocellulosic biomass: Acid and alkaline pretreatments and their effects on biomass recalcitrance - Conventional processing and recent advances. Bioresour Technol. 2020;304:122848.

2. Francois JM, et al. Engineering microbial pathways for production of bio-based chemicals from lignocellulosic sugars: current status and perspectives. Biotechnol Biofuels. 2020;13:118.

3. Mosier N, et al. Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresour Technol. 2005;96(6):673-86.

4. Hattori T, Morita S. Energy crops for sustainable bioethanol production; which, where and how? Plant Prod Sci. 2010;13(3):221-34.

5. Sethupathy S, et al. Harnessing microbial wealth for lignocellulose biomass valorization through secretomics: a review. Biotechnol Biofuels. 2021;14(1):154.

6. Beschkov V. Biogas, biodiesel and bioethanol as multifunctional renewable fuels and raw materials. Frontiers in Bioenergy and Biofuels. 2017.

7. Faga BA, et al. Ethanol production through simultaneous saccharification and fermentation of switchgrass using *Saccharomyces cerevisiae* D(5)A and thermotolerant *Kluyveromyces marxianus* IMB strains. Bioresour Technol. 2010;101(7):2273-9.

8. Alvira P, et al. Effect of endoxylanase and alpha-L-arabinofuranosidase supplementation on the enzymatic hydrolysis of steam exploded wheat straw. Bioresour Technol. 2011;102(6):4552-8.

9. Shi J, et al. Dynamic changes of substrate reactivity and enzyme adsorption on partially hydrolyzed cellulose. Biotechnol Bioeng. 2017;114(3):503-15.

10. Lynd LR, et al. How biotech can transform biofuels. Nat Biotechnol. 2008;26(2):169-72.

11. Jiang Y, et al. Consolidated bioprocessing performance of a two-species microbial consortium for butanol production from lignocellulosic biomass. Biotechnol Bioeng. 2020;117(10):2985-95.

12. Kim SM, et al. Promise of combined hydrothermal/chemical and mechanical refining for pretreatment of woody and herbaceous biomass. Biotechnol Biofuels. 2016;9:97.

13. Chang JJ, et al. Constructing a cellulosic yeast host with an efficient cellulase cocktail. Biotechnol Bioeng. 2018;115(3):751-61.

14. Liu H, et al. Engineering microbes for direct fermentation of cellulose to bioethanol. Crit Rev Biotechnol. 2018:1-17.

15. Branduardi P, et al. The yeast : a new host for heterologous protein production, secretion and for metabolic engineering applications. FEMS Yeast Res. 2004;4(4-5):493-504.

16. Liu H, et al. Engineering microbes for direct fermentation of cellulose to bioethanol. Crit Rev Biotechnol. 2018;38(7):1089-105.

17. Lassmann T, et al. Simulation of the downstream processing in the ethanol production from lignocellulosic biomass with ASPEN Plus® and IPSEpro. Energy Sustain Soc. 2014;4(1).

18. Binder JB, Raines RT. Fermentable sugars by chemical hydrolysis of biomass. Proc Natl Acad Sci U S A. 2010;107(10):4516-21.

19. Matsushika A, et al. Ethanol production from xylose in engineered *Saccharomyces cerevisiae* strains: current state and perspectives. Appl Microbiol Biotechnol. 2009;84(1):37-53.

20. Fonseca GG, et al. The yeast *Kluyveromyces marxianus* and its biotechnological potential. Appl Microbiol Biotechnol. 2008;79(3):339-54.

21. Radecka D, et al. Looking beyond *Saccharomyces*: the potential of non-conventional yeast species for desirable traits in bioethanol fermentation. FEMS Yeast Res. 2015;15(6).

22. Zhou J, et al. Improved secretory expression of lignocellulolytic enzymes in *Kluyveromyces marxianus* by promoter and signal sequence engineering. Biotechnol Biofuels. 2018;11:235.

23. Mehmood N, et al. *Kluyveromyces marxianus*, An attractive yeast for ethanolic fermentation in the presence of imidazolium ionic liquids. Int J Mol Sci. 2018;19(3).

24. Castro RC, Roberto IC. Selection of a thermotolerant *Kluyveromyces marxianus* strain with potential application for cellulosic ethanol production by simultaneous saccharification and fermentation. Appl Biochem Biotechnol. 2014;172(3):1553-64.

25. Lobs AK, et al. CRISPR-Cas9-enabled genetic disruptions for understanding ethanol and ethyl acetate biosynthesis in *Kluyveromyces marxianus*. Biotechnol Biofuels. 2017;10:164.

26. Jui-Jen Chang C-YH, Feng-Ju Ho, Tsung-Yu Tsai, Huei-Mien Ke, Christine H-T Wang, Hsin-Liang Chen, Ming-Che Shih, Chieh-Chen Huang and Wen-Hsiung Li. PGASO: A synthetic biology tool for engineering a cellulolytic yeast. Biotechnol Biofuels. 2012;5(1):53.

27. Choo JH, et al. Deletion of a KU80 homolog enhances homologous recombination in the thermotolerant yeast *Kluyveromyces marxianus*. Biotechnol Lett. 2014;36(10):2059-67.

28. Abdel-Banat BM, et al. Random and targeted gene integrations through the control of non-homologous end joining in the yeast *Kluyveromyces marxianus*. Yeast. 2010;27(1):29-39.

29. Nonklang S, et al. High-temperature ethanol fermentation and transformation with linear DNA in the thermotolerant yeast *Kluyveromyces marxianus* DMKU3-1042. Appl Environ Microbiol. 2008;74(24):7514-21.

30. Fang J, et al. Stable antibody expression at therapeutic levels using the 2A peptide. Nat Biotechnol. 2005;23(5):584-90.

31. Ryan MD, et al. Analysis of the aphthovirus 2A/2B polyprotein 'cleavage' mechanism indicates not a proteolytic reaction, but a novel translational effect: a putative ribosomal 'skip'. J of Gen Virol. 2001;82(5):1013-25.

32. de Felipe P, et al. Co-translational, intraribosomal cleavage of polypeptides by the foot-and-mouth disease virus 2A peptide. J Biol Chem. 2003;278(13):11441-8.

33. Chen Y-L, et al. Directed evolution to produce an alkalophilic variant from a Neocallimastix patriciarum xylanase. Can J Microbiol. 2001;47(12):1088-94.

34. Shallom D, Shoham Y. Microbial hemicellulases. Curr Opin Microbiol. 2003;6(3):219-28.

35. Doronina VA, et al. Site-specific release of nascent chains from ribosomes at a sense codon. Mol Cell Biol. 2008;28(13):4227-39.

36. Souza-Moreira TM, et al. Screening of 2A peptides for polycistronic gene expression in yeast. FEMS Yeast Res. 2018;18(5).

37. Roongsawang N, et al. Coexpression of fungal phytase and xylanase utilizing the cis-acting hydrolase element in *Pichia pastoris*. FEMS Yeast Res. 2010;10(7):909-16.

38. Trichas G, et al. Use of the viral 2A peptide for bicistronic expression in transgenic mice. BMC Biol. 2008;6:40.

39. Ohgren K, et al. Effect of hemicellulose and lignin removal on enzymatic hydrolysis of steam pretreated corn stover. Bioresour Technol. 2007;98(13):2503-10.

40. García-Aparicio MP, et al. Xylanase contribution to the efficiency of cellulose enzymatic hydrolysis of barley straw. Appl Biochem Biotechnol. 2007;137-140(1-12):353-65.

41. Várnai A, et al. Restriction of the enzymatic hydrolysis of steam-pretreated spruce by lignin and hemicellulose. Enzyme Microb Tech. 2010;46(3-4):185-93.

42. Saitoh S, et al. Co-fermentation of cellulose/xylan using engineered industrial yeast strain OC-2 displaying both beta-glucosidase and beta-xylosidase. Appl Microbiol Biotechnol. 2011;91(6):1553-9.

43. Zhou J, et al. Biochemical and kinetic characterization of GH43 beta-D-xylosidase/alpha-L-arabinofuranosidase and GH30 alpha-L-arabinofuranosidase/beta-D-xylosidase from rumen metagenome. J Ind Microbiol Biotechnol. 2012;39(1):143-52.

44. Banerjee G, et al. Rapid optimization of enzyme mixtures for deconstruction of diverse pretreatment/biomass feedstock combinations. Biotechnol Biofuels. 2010;3:22.

45. Varnai A, et al. Synergistic action of xylanase and mannanase improves the total hydrolysis of softwood. Bioresour Technol. 2011;102(19):9096-104.

46. Inoue H, et al. Effect of beta-mannanase and beta-mannosidase supplementation on the total hydrolysis of softwood polysaccharides by the *Talaromyces cellulolyticus* cellulase system. Appl Biochem Biotechnol. 2015;176(6):1673-86.

47. Katsimpouras C, et al. A thermostable GH26 endo-beta-mannanase from *Myceliophthora thermophila* capable of enhancing lignocellulose degradation. Appl Microbiol Biotechnol. 2016;100(19):8385-97.

48. Cornelis, et al. Effect of benzoic acid on metabolic fluxes in yeasts: A continuous-culture study on the regulation of respiration and alcoholic fermentation. Yeast. 1992.

49. Karim A, et al. *Kluyveromyces marxianus*: An emerging yeast cell factory for applications in food and biotechnology. Int J Food Microbiol. 2020;333:108818.

50. Postma E, et al. Enzymic analysis of the crabtree effect in glucose-limited chemostat cultures of *Saccharomyces cerevisiae*. Appl Environ Microbiol. 1989;55(2):468.

51. Dashko S, et al. Why, when, and how did yeast evolve alcoholic fermentation? FEMS Yeast Res. 2014;14(6):826-32.

52. Remond C, et al. Combination of ammonia and xylanase pretreatments: impact on enzymatic xylan and cellulose recovery from wheat straw. Bioresour Technol. 2010;101(17):6712-7.

53. Shin HD, et al. Novel *Aspergillus* hemicellulases enhance performance of commercial cellulases in lignocellulose hydrolysis. Biotechnol Prog. 2011;27(2):581-6.

54. Liu K, et al. High concentration ethanol production from corncob residues by fed-batch strategy. Bioresour Technol. 2010;101(13):4952-8.

55. Tenkanen M, et al. Investigation of lignin-carbohydrate complexes in kraft pulps by selective enzymatic treatments. Appl Microbiol Biotechnol. 1999;51(2):241-8.

56. Gao J, et al. Transcriptional analysis of *Kluyveromyces marxianus* for ethanol production from inulin using consolidated bioprocessing technology. Biotechnol Biofuels. 2015;8:115.

57. Mo W, et al. *Kluyveromyces marxianus* developing ethanol tolerance during adaptive evolution with significant improvements of multiple pathways. Biotechnol Biofuels. 2019;12:63.

58. Heck BL, et al. Ethanol formation and enzyme activities around glucose-6-phosphate in *Kluyveromyces marxianus* CBS 6556 exposed to glucose or lactose excess. FEMS Yeast Res. 2010(7):691-8.

59. Visser W, et al. Oxygen requirements of yeasts. Appl Environ Microbiol. 1990.

60. Fonseca GG, et al. The yeast *Kluyveromyces marxianus* and its biotechnological potential. Appl Microbiol Biotechnol. 2008;79(3):339-54.

61. Santharam L, et al. Effect of aeration and agitation on yeast inulinase production: a biocalorimetric investigation. Biopro Biosyst Eng. 2019;42(6):1-13.

62. Hensing MC, et al. Production of extracellular inulinase in high-cell-density fed-batch cultures of *Kluyveromyces marxianus*. Appl Microbiol Biotechnol. 1994;42(4):516-21.

63. Xin, et al. Comparison of aqueous ammonia and dilute acid pretreatment of bamboo fractions: Structure properties and enzymatic hydrolysis. Bioresour Technol. 2015.

64. Kim TH, et al. Pretreatment of corn stover by aqueous ammonia. Bioresour Technol. 2003;90(1):39-47.

65. Hua Y, et al. Release of glucose repression on xylose utilization in *Kluyveromyces marxianus* to enhance glucose-xylose co-utilization and xylitol production from corncob hydrolysate. Microb Cell Fact. 2019;18(1):24.

66. Rodrussamee N, et al. Growth and ethanol fermentation ability on hexose and pentose sugars and glucose effect under various conditions in thermotolerant yeast *Kluyveromyces marxianus*. Appl Microbiol Biotechnol. 2011;90(4):1573-86.

67. Gschaedler A, et al. Use of non-*Saccharomyces* yeasts in cider fermentation: Importance of the nutrients addition to obtain an efficient fermentation. Int J Food Microbiol. 2021;347.

68. You C, et al. Potential hydrophobic interaction between two cysteines in interior hydrophobic region improves thermostability of a family 11 xylanase from *Neocallimastix patriciarum*. Biotechnol Bioeng. 2010;105(5):861-70.

69. Gibson, et al. Enzymatic assembly of DNA molecules up to several hundred kilobases. Nat Methods. 2009.

70. Antunes DF, et al. A simple and rapid method for lithium acetate-mediated transformation of *Kluyveromyces marxianus* cells. World J Microb Biot. 2000;16(7):653-4.

71. Pan X, et al. High level expression of a truncated beta-mannanase from alkaliphilic *Bacillus sp.* N16-5 in *Kluyveromyces cicerisporus*. Biotechnol Lett. 2011;33(3):565-70.

72. Sluiter A, et al. Determination of sugars, byproducts, and degradation products in liquid fraction process samples. NREL Analytical Procedure National Renewable Energy Laboratory, Golden, CO. 2006.

73. Sluiter A, et al. Determination of structural carbohydrates and lignin in biomass. NREL Analytical Procedure National Renewable Energy Laboratory, Golden, CO. 2004.

74. Rehman O, et al. Optimization of low-temperature energy-efficient pretreatment for enhanced saccharification and fermentation of Conocarpus erectus leaves to produce ethanol using *Saccharomyces cerevisiae*. Biomass Convers Bior. 2019;10(4):1269-78.

75. Miller GL. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem. 1959;31(3):426-8.

Tables

Table1: Primer sequences used in this work

Primers	Sequences
MF	ATGAAGTTAGCATACTCCCTCTTGC
IMXR1	GAACTACAGAAACTTTGTGTAAATACGGTGGATGGTTTGGAG
IMXF	TCCACCGTATTTACACAAAGTTTCTGTAGTTCAGCTTCTC
XR	CTAGTGATGATGATGATGGTGATCACCAATGTAAACCTTTGCGTATGG
IMPR	AGGACCGGGGTTTTCTTCCACGTCTCCTGCTTGCTTTAACAGAGAGAAGT TCGTGGCTCCGGATCCTGTAAATACGGTGGATGGTTTGGA
IMPXF	TGGAAGAAAACCCCGGTCCTCAAAGTTTCTGTAGTTCAGCTTCTCACT
PαF1	GGAAGAAAACCCCGGTCCTATGAGATTTCCTTCAATTTTTACTGCAG
αXR1	GAGAAGCTGAACTACAGAAACTTTGCCCGGGTACGTAAGCTTCAGCCTCT
αXF1	AGAGGCTGAAGCTTACGTACCCGGGCAAAGTTTCTGTAGTTCAGCTTCTC
XPR	ATCACCAATGTAAACCTTTGCGTATG
XPαF	ACGCAAAGGTTTACATTGGTGATGGATCCGGAGCCACGAACTTCTCTC
αR1	ATAGCGTTTCTTAACTTTATCAGCCCCGGGTACGTAAGCTTCAGCCTCT
αRF	TGATAAAGTTAAGAAACGCTAT
RR	CAAAGCTTGCGGCCTTAAGCGGCCGCTTACTCATCCATGCCTTCGATGGTG
IXF	AGACGGTGACCCCGGGACTAGTATGAGATTTCCTTCAATTTTTACTG

Table 2 The β-mannanase and β-xylanase activities of the IMX, IMPX, and IMPαX strains cultured in flasks at 30 ℃, 220 rpm for 72 h.

Strains	β-Mannanase activities		β-Xylanase activities	
	Extracellular	Intracellular	Extracellular	Intracellular
IMX	24.03±3.74	1.13±0.19	155.26±4.24	44.17±4.24
IMPX	21.34±1.37	4.50±0.75	42.07±4.99	87.59±11.41
IMPαX	15.50±1.91	4.62±0.44	136.17±15.34	39.43±4.11

Figures

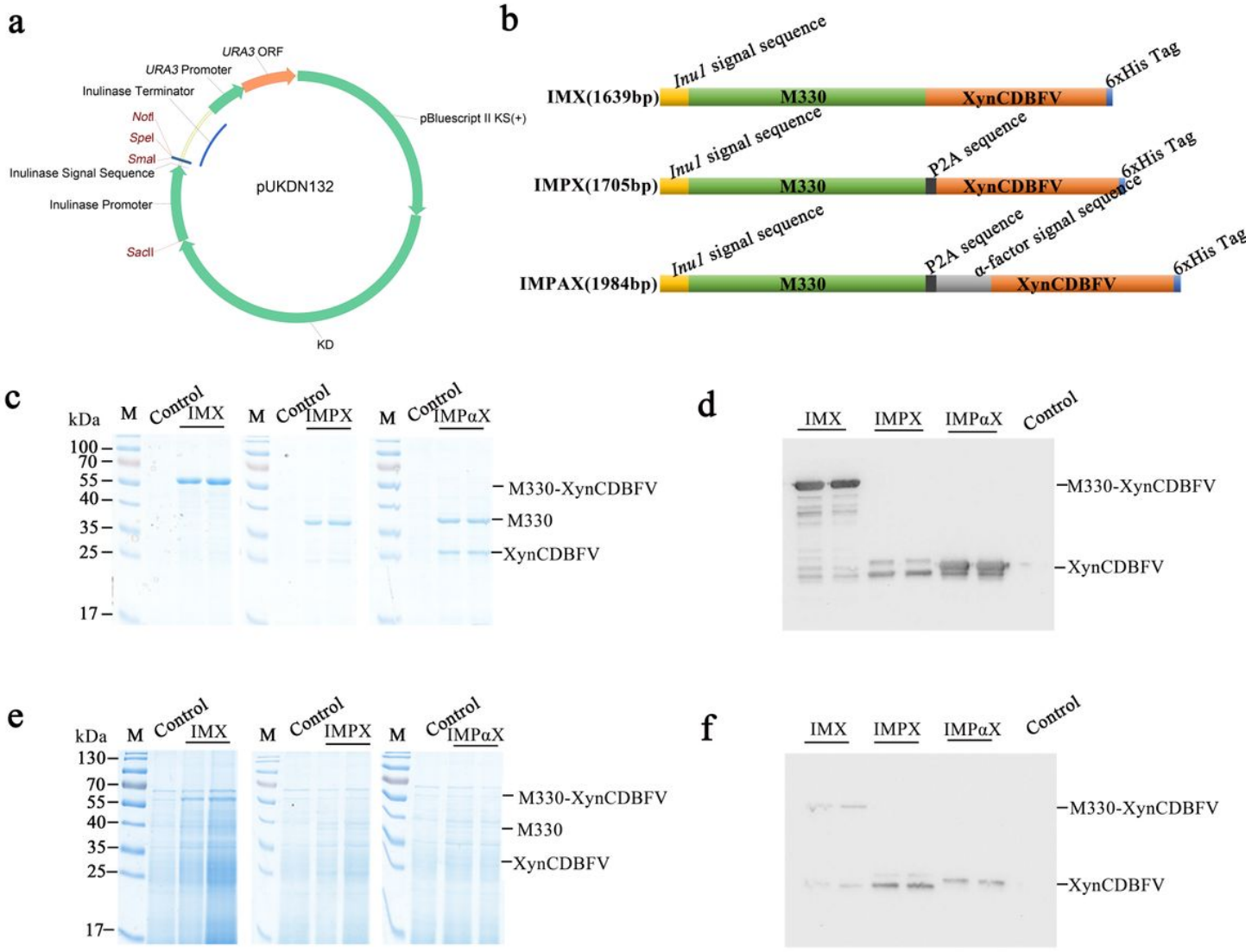

Figure 1

The efficiency of FMDV P2A in self-cleavage of M330 and XynCBDFV. a Map of the expression vector pUKDN132; b Illustrations of the polycistronic genes IMX, IMPX, and IMPαX; SDS-PAGE and western blots of the supernatants (c and d) and cell lysates (e and f) of flask cultures.

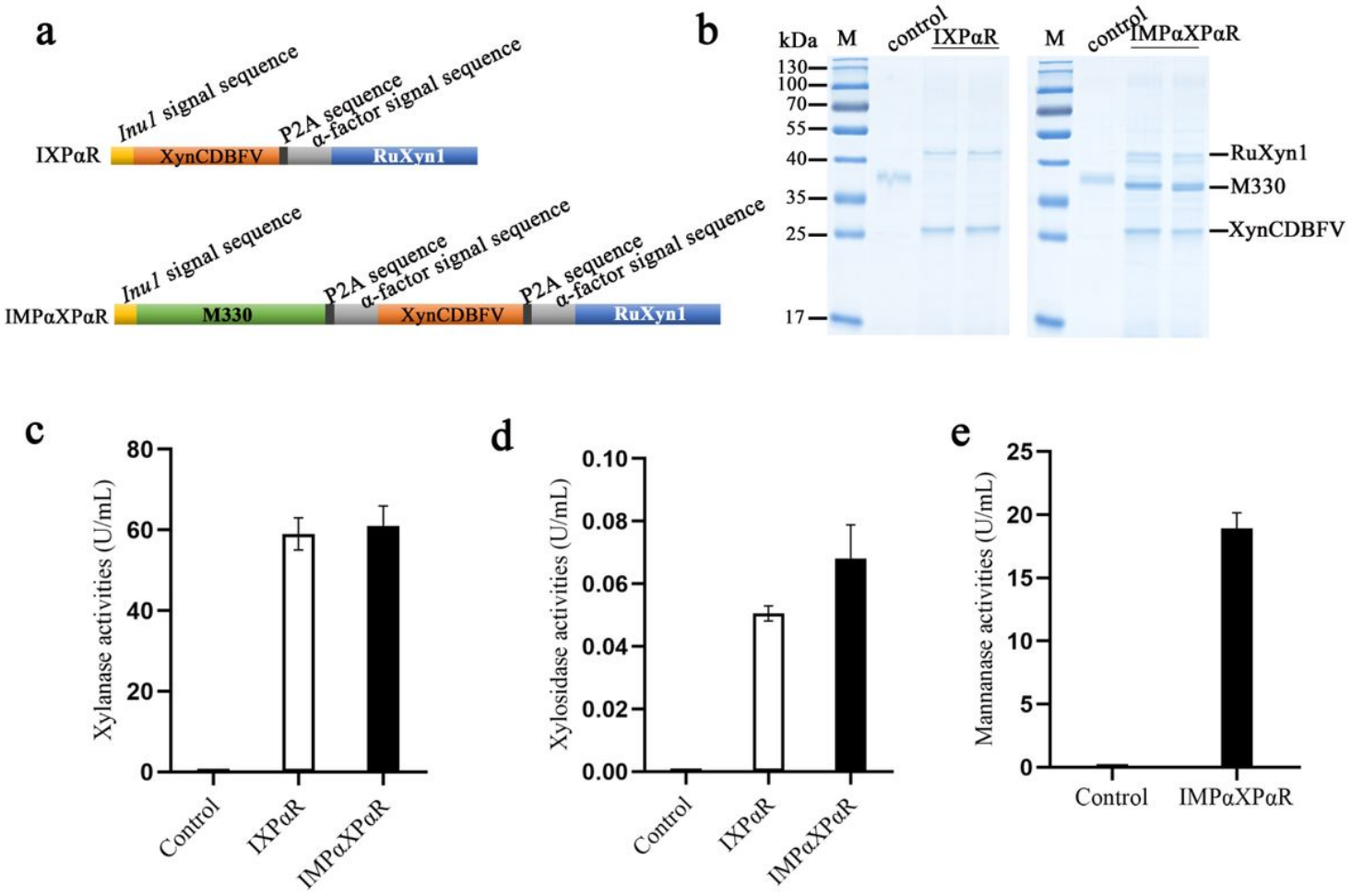

Figure 2

Secretory expression of hemicellulolytic enzymes in K. marxianus. a Constructions of the polycistronic genes IMPαX and IMPαXPαR; SDS-PAGE (b) and activities of β-xylanase (c), β-xylosidase (d), and β-mannanase (e) for the supernatants of IMPαX and IMPαXPαR strains.

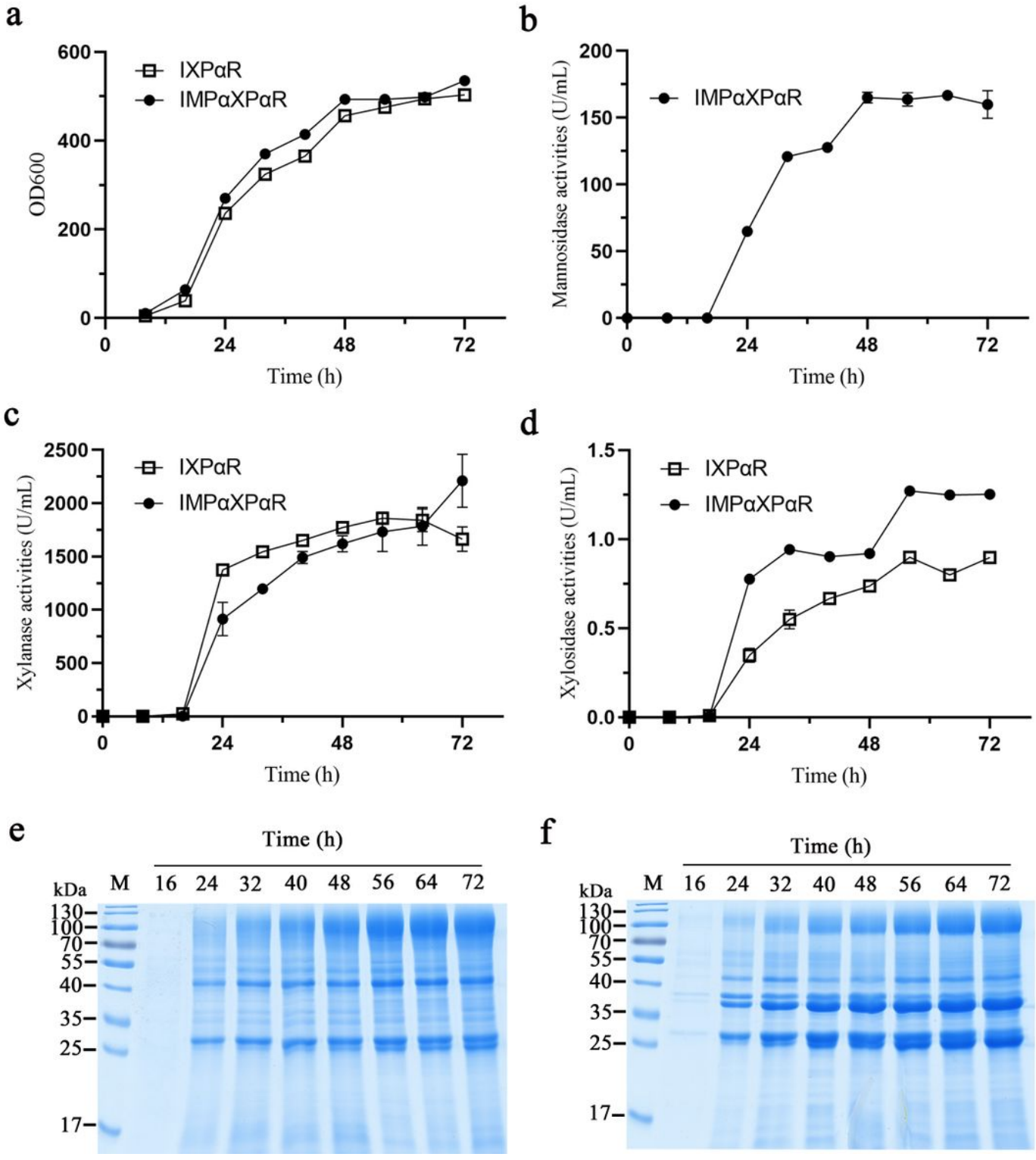

Figure 3

Growth curves (a) and productions of β-mannanase (b), β-xylanase (c), and β-xylosidase (d) in fed-batch fermentation of the IMPαX and IMPαXPαR strains. Supernatant samples at the indicated times of of the IMPαX (e) and IMPαXPαR strains were also analyzed by SDS-PAGE.

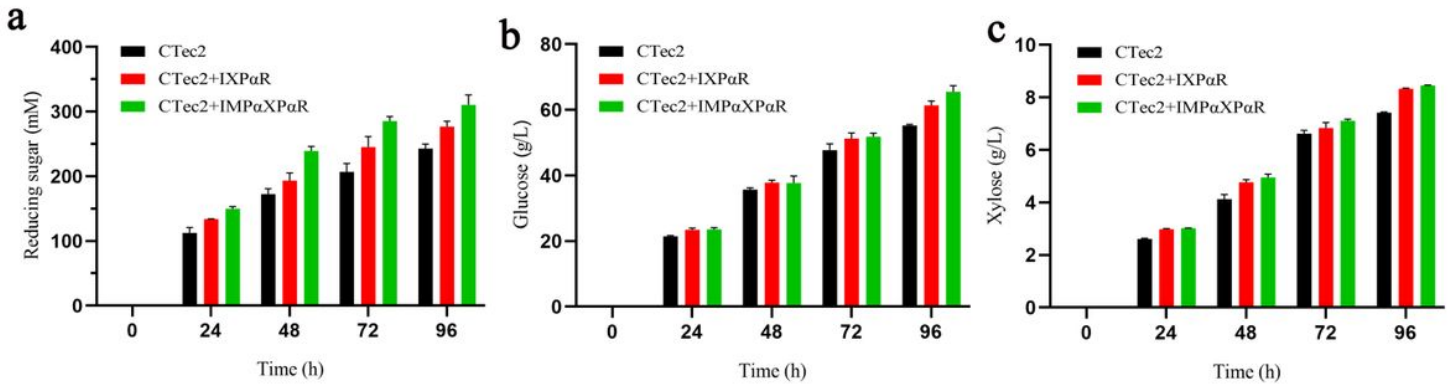

Figure 4

Concentrations of the reducing sugars (a), glucose (b), and xylose (c) over time in the hydrolysis of DAP corncobs.

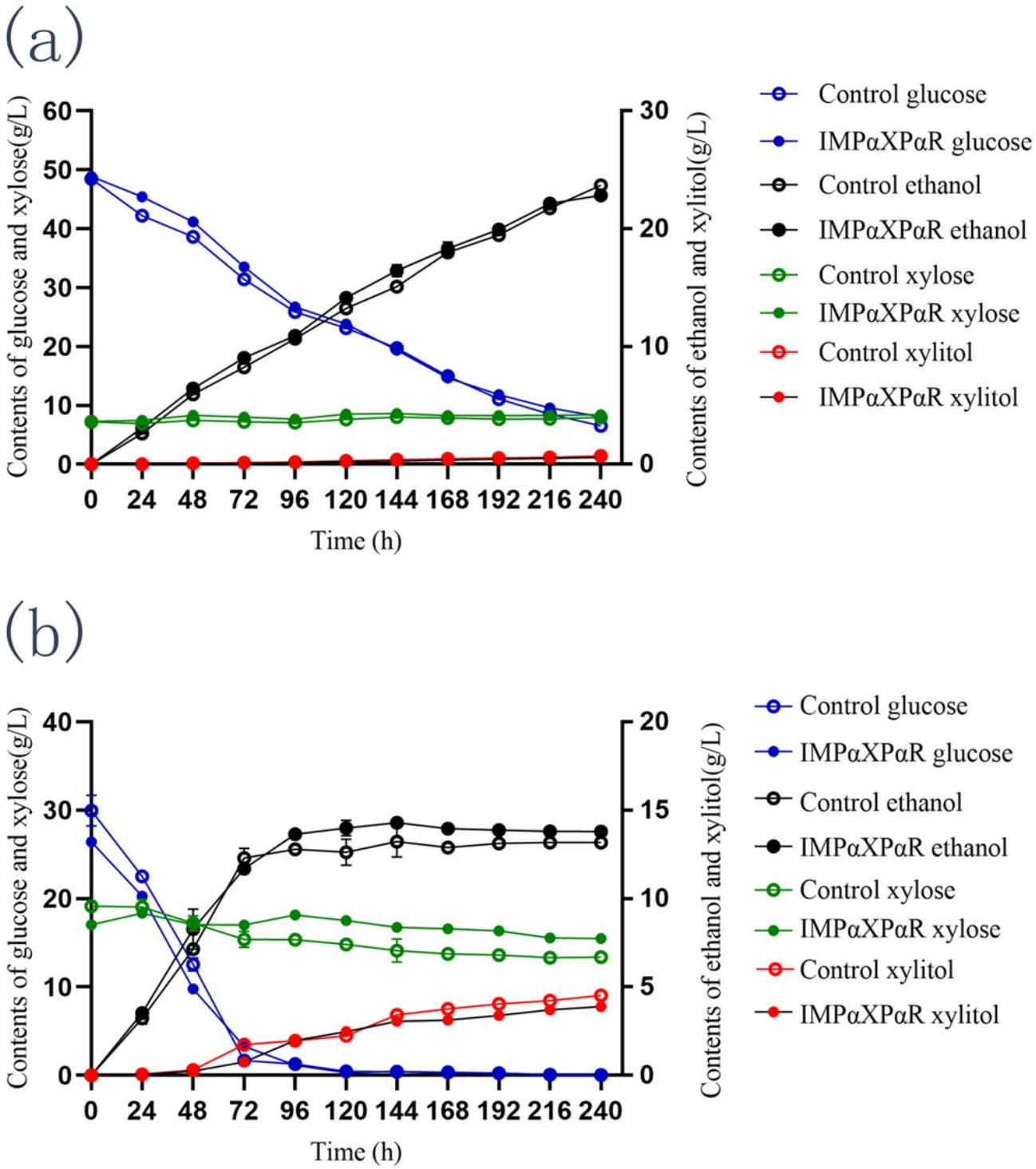

Figure 5

Profiles of glucose, xylose, ethanol, and xylitol in HSFs of the diluted acid (a) and aqueous ammonia (b) pretreated corncobs.