

# Coordinately Express Hemicellulolytic Enzymes in *Kluyveromyces marxianus* to Improve the Saccharification and Ethanol Production From Corncobs

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## Research

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# 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, and supplement of hemicellulases can enhance performance of commercial cellulases in the enzymatic hydrolyses of lignocellulose. *K. marxianu* is an attractive yeast for cellulosic ethanol fermentation, since it has remarkable thermotolerance, high growth rate, and broad substrate spectrum etc, as well as a promising host for heterologous protein production. In this study, we attempted to coordinately express multiple hemicellulases in *Kluyveromyces 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 efficiency 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 fused at the N-terminus of *Xyn-CDBFV*. The extracellular β-mannanase activities of *IMPX* and *IMPαX* strains were 21.34 and 15.50 U/mL respectively. By contrast, the *IMPαX* strain secreted 136.17 U/mL 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 the β-D-xylosidase *RuXyn1* and *Xyn-CDBFV*, or three hemicellulolytic enzymes including *M330*, *Xyn-CDBFV* and *RuXyn1*. The *IMPαX* strain produced 1664.2 and 0.90 U/mL of extracellular β-xylanase and β-xylosidase, while the *IMPαXPαR* strain secreted 159.8, 2210.5, and 1.25 U/ml of β-mannanase, β-xylanase, and β-xylosidase in fed-batch fermentations respectively. Hemicellulolytic enzymes of these two strains enhanced the releases of both glucose and xylose from diluted acid pretreated corncobs when acted synergistically with commercial cellulases. In hybrid saccharification and fermentation (HSF) of pretreated corncobs, hemicellulases of the *IMPαXPαR* strain increased about 34.2% and 11.1% of ethanol productions at 144 and 216 h respectively .

## 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 could be used as a consolidated bioprocessing (CBP) strain for ethanol production from lignocelluloses.

# Background

Lignocellulose is the most abundant renewable resource on earth, which is recalcitrant 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 feedstock, cellulosic ethanol is manufactured from non-edible carbohydrates contained in plant cell walls, basically composing of four phases that include pretreatment, hydrolysis, fermentation, and dehydration [3]. Among these, hydrolysis (saccharification) of pretreated lignocelluloses is a critical prerequisite for ethanolic fermentation by microbes, but usually these two processes can be concurrently integrated into one step known as simultaneous saccharification and fermentation (SSF) when using enzymes to hydrolyze cellulose and hemicellulose into fermentable sugars [4].

To depolymerize lignocelluloses into fermentable sugars, at least three types of cellulases, such as  $\beta$ -1,4-endoglucanase, exocellobiohydrolase, and  $\beta$ -1,4-glucosidase, and more diversity of hemicellulases including  $\beta$ -1,4-xylanase,  $\beta$ -1,4-xylosidase,  $\beta$ -1,4-mannanase,  $\alpha$ -arabinosidases, and esterases etc are required to act synergistically [5]. 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 [6, 7]. 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 [8]. However, the conflict of optimal temperatures within saccharification and fermentation can decrease the efficiency of lignocellulose hydrolysis. To circumvent this problem, a hybrid saccharification and fermentation (HSF) is set out by hydrolyzing pretreated lignocelluloses with commercial cellulases before a CBP process [9, 10].

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 sp.*, *Aspergillus sp.*, *Fusarium oxysporum*, and *Penicillium sp.* etc, or ethanologenic microorganisms including *Saccharomyces cerevisiae*, *K. marxianus*, *Zygosaccharomyces bailii* and *Zymomonas mobilis*, by a combination of cellulase production, enzymatic hydrolysis, and microbial fermentation into a single operation [11, 12]. 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 [13, 14]. 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 as well [15]. As the most utilized yeast for ethanol fermentation, *S. cerevisiae* is unable 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 [16]. *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 [17-20]. 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 [4, 21].

To be ethanologenic CBP strains, saccharification enzymes were required to simultaneously express in one host to convert cellulose into fermentable sugars, while the genetic basis of *K. marxianus* less well understood than *S. cerevisiae*, [22]. A synthetic biology technique termed as “Promoter-based Gene Assembly and Simultaneous Overexpression (PGASO)” was developed to integrate gene cassettes in the genome of *K. marxianus* KY3 in a single step, with each gene expression regulated by an individual promoter along with a terminator [23]. Unfortunately, the double homologous recombination frequency was very low in *K. marxianus*, even flanking with long homologous fragments [24]. 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 [25, 26]. In the present study, we incorporated a different way for coexpression of multiple hemicellulases using a P2A self-processing peptide from 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 [27-29]. By assembling a  $\beta$ -mannanase M330 gene and a  $\beta$ -xylanase Xyn-CDBFV gene into a single ORF with FMDV P2A [30], the efficiency of P2A self-cleavage in secretory expression of multiple enzymes in *K. marxianus* was evaluated. Furthermore, we also investigated the capability of enzyme mixtures to improve the hydrolysis of corncobs and ethanol production by the recombinant *K. marxianus* strain.

## Methods

### Strains and plasmids

The *K. marxianus* Fim-1 $\Delta$ 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 [19].

### Expression plasmids constructions and transformations

A polycistronic gene *M330-Xyn-CDBFV* (hereafter termed the IMX gene) that the  $\beta$ -xylanase gene *Xyn-CDBF* was fused to the C terminus of the  $\beta$ -mannanase gene *M330* directly was constructed as described below. The *M330* gene was amplified from the pZP41 plasmid by the primers MF and IMXR1 (Table 1), and the *Xyn-CDBFV* gene was amplified with IMXF and XR from a pET21a/*Xyn-CDBFV* [31]. 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 [32], 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 P2A sequence alone or along with a signal sequence of *S. cerevisiae* α-factor between the *M330* and the *Xyn-CDBFV* were also constructed. The P2A sequence was added to the 3' terminus of *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 PaF1 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 the polycistronic gene *IMPαX* as described above. After cloned into pUKDN132, the resulting plasmid was then termed pUKDN132/*IMPαX*.

The polycistronic *IMPαXPaR* gene that integrated three genes into a single ORF was constructed by assembling the *IMPαX* lack of the stop codon TAG with a P2A linked α-factor signal sequence and a β-xylosidase *RuXyn1* gene. The *IMPαX* fragment and P2A linked α-factor signal sequence was amplified from pUKDN132/*IMPαX* by the primer pairs, MF/XPR and XPaF/αR1, respectively, while the *RuXyn1* gene was amplified from a pET21/*RuXyn1* vector using the primers αRF and RR [33]. These three fragments were ligated by Gibson assembly to get the full length of *IMPαXPaR*. After PCR amplification with the primers MF and RR, the *IMPαXPaR* was inserted into the *Spe I/Not I* site of pUKDN132 to obtain the plasmid pUKDN132/*IMPαXPaR*. The plasmid pUKDN132/*IXPaR* were constructed by was amplified an XPaR fragment from pUKDN132/*IMPαXPaR* by the primers IXF and RR, and then inserted into the *Spe I/Not I* site of pUKDN132.

For plasmids transformations, the *K. marxianus* Fim-1Δ*URA3* was inoculated in 5 ml YPD medium (1% Yeast Extract, 2% Peptone, 2% Glucose), 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 [34]. Transformants were then selected on SD plates containing 0.67% 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°C [35]. 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°C [31]. β-xylosidase activities were measured using *p*-nitrophenyl-β-D-xylopyranoside as we described previously [33]. 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) at 30 °C, 200 rpm for 72 h. One milliliter of cultures was harvested, and centrifuged for 10min at 5,000 rpm to detect the secretory or

intracellular expression of enzymes by Western blot. To prepare lysate samples, collected cells were suspended in 1 mL 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  $\mu$ L 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 previously described [19].

### 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 [19]. Inoculum seeds were precultured in Erlenmeyer flasks containing 150 mL YD medium at 30 °C, 220 r/min for 18 h. 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

Corncoobs, 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), and 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, diluted acid pretreated corncoobs were separated by filtration, washed with deionized water, and dried at 80 °C.

### Enzymatic saccharification and fermentation

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

HSFs were conducted in 150 mL flasks containing 10 g of the diluted acid pretreated corncoobs with 11.5 % moisture content. The corncoobs were immersed in 80 mL of 50 mM sodium citrate buffer pH 5.5 and autoclaved at 121 °C for 20 min. Following sterilization, 10 FPU CTec2 per gram corncob was added and enzymatic saccharifications were performed at 45 °C, 150 rpm. 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  $MgSO_4 \cdot 7H_2O$ , 5 g/L yeast extract, and 1 g/L  $MnSO_4$ ) [4] and 1 mL of fed-batch cultures collected at 48 h were added to the corncoobs slurries. Sterile deionized water

was supplemented to make 100 mL of the total liquid. The flasks were incubated at 30 °C without stirring, and samples were taken every 24 h for analysis of glucose, xylose, and ethanol.

## Analytical methods

Reducing sugars were determined by the DNS method [36]. 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 °C. Twenty microliters of each sample were injected and eluted with 0.01N H<sub>2</sub>SO<sub>4</sub> in water at a rate of 0.6 mL/min for 30 min.

# Results And Discussion

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

Due to the chemical diversity in structure of hemicelluloses that are heterogeneous polysaccharides with both linear and branched molecules cross-linked to cellulose microfibrils, complete degradation requires multiple hemicellulases to act synergically [37]. Aiming to facilitate the expression of 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 synthesis of a glycyl–prolyl peptide bond at the C-terminus of 2A, releases the nascent protein, and resumes the downstream translation [38]. While the efficiency of 2A self-cleavage is strongly related to the sequence contexts of upstream and downstream ORFs in the polycistrons [39]. 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 of a 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 β-mannanase and β-xylanase in both supernatants and cell lysates of the *IMX*, *IMPX*, and *IMPαX* strains, which were transformed with the plasmids pUKDN132/*IMX*, pUKDN132/*IMPX*, and pUKDN132/*IMPαX*, respectively. Unexpectedly, as a control, we observed that the *IMX* strain 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. This double-activities of the *IMX* strain provided a good reference to the P2A effect on the 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 related to the production of Xyn-CDBFV. Enzymatic determinations demonstrated that the IMPaX strains secreted 136.17 U/mL  $\beta$ -xylanase into the supernatants, and retained 39.43 U/mL intracellularly. By contrast, the supernatant  $\beta$ -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  $\beta$ -xylanase activities of IMPX and IMPaX strains were the self-cleaved 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, protein bands with approximate 57 kDa molecular weight in the supernatants of IMX strain were in accord with the theoretical prediction of the fusion protein IMX. In both supernatants of the IMPaX and IMPX strains, M330 and Xyn-CDBFV were secreted alone, while the secretory Xyn-CDBFV of IMPaX strain was much higher than that of the IMPX strain, suggesting that, in the presence of P2A and  $\alpha$ -factor signal sequence, Xyn-CDBFV could be secreted to medium more efficiently. This result was in agreement with the previous literature [40]. 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). Thus, 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 [41]. However, in our results, we also found that this ribosomal 'skipping' in co-translation would decrease in apparent the total protein express levels, especially for the downstream gene.

### 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 [42]. Cellulase supplemented with endoxylanase promoted the hydrolysis of steam-exploded feed stocks, released more glucose, accumulated higher content of xylobiose and xylooligosaccharides [43, 44]. Content of xylose, however, was not significantly elevated, which may be due to the insufficient  $\beta$ -xylosidase in most cellulase enzymes produced by filamentous fungi *Trichoderma reesei* [5, 45]. We reasoned that an ethanologenic strain co-expressed multiple hemicellulases, especially  $\beta$ -xylanase and  $\beta$ -xylosidase, would eliminate the accumulation of xylooligosaccharides and produce more fermentable xylose. A  $\beta$ -xylosidase RuXyn1 that has high capability of converting intermediate xylo-oligosaccharides into xylose was used to co-express with  $\beta$ -xylanase in *K. marxianus* [33]. The RuXyn1 coding sequence was fused to Xyn-CDBFV with a P2A and an  $\alpha$ -factor signal sequence (Fig. 2a), and the resulting IXPaR was expressed in *K. marxianus* under the unique *INU1* promoter. The IXPaR strain transformed with the pUKDN132/IXPaR produced 59.01 and 0.05 U/ml of extracellular  $\beta$ -xylanase and  $\beta$ -xylosidase in flask cultures respectively (Fig 2 b-d).

Supplements of  $\beta$ -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 [46-49]. Given critical roles of  $\beta$ -mannanase,  $\beta$ -xylanase and  $\beta$ -xylosidase in the hydrolysis of lignocellulose, we tested the feasibility of P2A for coordinately expressing these three selected enzymes in one ORF. The polycistronic gene *IMPαXPαR* that compacted *M330*, *Xyn-CDBFV* and *RuXyn1* into one ORF, each with a signal sequence (Fig. 2a). Consistent with that of the IMPαX and IXPαR strains, all activities of these three enzymes were detectable in the crude culture supernatant of the IMPαXPαR strain, which is obtained by transformation of the pUKDN132/IMPαXPαR plasmid. The  $\beta$ -mannanase,  $\beta$ -xylanase and  $\beta$ -xylosidase activities were 18.90, 61.00, and 0.07 U/mL, respectively (Fig. 2c-e). As expected, figure 2b showed three protein bands in culture supernatants of the IMPαXPαR strain that were corresponding to the 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* [19]. In this study, we evaluated the productions of multiple enzymes in fed-batch fermentation for both the IXPαR and IMPαXPαR strains. *K. marxianus* is Crabtree negative yeast that does not perform aerobic alcoholic fermentation, and but can respire even in high glucose concentrations [50, 51]. 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 [52, 53]. Additionally, ethanol fermentation could affect cell growth in *K. marxianus*, and thus decreases expressions of enzymes. 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  $\beta$ -xylanase and 0.90 U/ml  $\beta$ -xylosidase, which were about 28 and 18 folds that of in the flask cultures respectively. SDS-PAGE showed that the IXPαR strain secreted two different protein bands that represented mature forms of Xyn-CDBFV and RuXyn1 respectively. The IMPαXPαR strain produced 2210.5 U/ml of  $\beta$ -xylanase and 1.25 U/ml of  $\beta$ -xylosidase, slightly higher than that of the IXPαR strain. As well, this strain also produced 159.8 U/ml of  $\beta$ -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 [54, 55]. Using prepared hemicellulase cocktails, we next evaluated their

performances on the promotion of lignocellulose hydrolyses. We chose corncob as the feedstock for enzymatic hydrolyses because it is one of the most abundant inedible agricultural residues and consists of a relatively high content of hemicellulose (~40%) [56]. 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  $\beta$ -xylanase Xyn CDBFV and  $\beta$ -xylosidase RuXy1 performances on the enzymatic hydrolyses, 300  $\mu$ l of supernatant collected from the IXPaR strain fed-batch culture at 48 h, equal to 531.29 U  $\beta$ -xylanase and 0.22 U  $\beta$ -xylosidase, was supplemented to the Cellic® CTec2 cellulase. In accord with previous literatures on pine kraft pulp and softwood [47, 57], supplementations of xylanolytic enzymes to the Cellic® CTec2 cellulase improved the enzymatic hydrolysis of corncobs. At each sampling point, addition of the IXPaR strain culture supernatant generated higher contents of soluble sugars. After 96 h of hydrolysis, the 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  $\beta$ -mannanase M330 for the corncob hydrolysis in combination of  $\beta$ -xylanase and  $\beta$ -xylosidase was also evaluated. The culture supernatant of IMPaXPaR strain containing 49.50 U  $\beta$ -mannanase, 485.70 U  $\beta$ -xylanase, and 0.28 U  $\beta$ -xylosidase was supplemented to the Cellic® CTec2 cellulase. As shown in Figure 4a, the supplementary  $\beta$ -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 IXPaR 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  $\beta$ -mannanase could facilitate a more extensive break-down of corncobs. This promotion may be ascribed to the deep hydrolysis glucomannan by the endoglucanase TrCel5A presented in Cellic® CTec2 [47].

#### 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 more than 100 g/L [58, 59]. The hemicellulolase activities of IMPaXPaR strain would be conducive to ethanol production from pretreated lignocellulosic biomass. Subsequently, we investigated the potential of recombinant IMPaXPaR strain as a CBP strain to produce ethanol from pretreated corncobs with a high solids content, as it is more economic for cellulosic ethanol processes to generate high sugar titers necessary for high ethanol production [60, 61]. HSFs were performed by pre-hydrolyzing pretreated corncobs with 10 FPU commercial cellulase per gram solids for 72 h before inoculated with the IMPaXPaR or FIM-1 (control) strain. As shown in Figure 5a, glucose in HSFs with IMPaXPaR strain decreased more rapidly than with FIM-1 through the first 96 h. After 216 h, glucose concentrations were 5.2 and 8.7 g/L in HSFs with the IMPaXPaR and FIM-1 strains respectively. The glucose consumption rates in our *K. marxianus* strains were in apparent lower than other *K. marxianus* strains in simultaneous saccharification and fermentation of switchgrass [4, 62, 63], but the reason for this is not clear. In HSFs with both IMPaXPaR and FIM-1 strain, xylose concentrations increased

within the first 120 h, and then decreased slightly when prolonging the fermentation time (Fig. 5b). Glucose could strongly repress xylose utilization in simultaneous fermentation of them with *K. marxianus* [64], presumably, responsible for the xylose accumulation during the preceding stage of HSF. Different with the above in vitro saccharification of pretreated corncobs, no significant increase of xylose was found in the HSFs with IMPaXPaR strain comparing to that of FIM-1 strain. The reason may be due to lower glucose concentrations in the HSFs with IMPaXPaR strain that might promote its xylose assimilation.

Ethanol production profiles indicated the IMPaXPaR strain had higher efficiency of ethanol conversion rate, and produced more ethanol during fermentation. At 144h, 20.8 g/L of ethanol was obtained in HSFs with IMPaXPaR strain, which is about 34.2% more than that of FIM-1 (Fig. 5c). After prolonging fermentation time to 216 h, ethanol in HSFs with the IMPaXPaR strain increased to 23.9 g/L, while it was 21.3 g/L in that of FIM-1 strain. Considering the high glucose consumption rate in HSFs with IMPaXPaR strain, we used the sugar-ethanol conversion rates based on their initial glucose contents to present the contribution of hemicellulases produced for ethanol production. As shown in Figure 5d, the ethanol conversion rates of IMPaXPaR strain at 144, 168, 192, and 216 h were 0.54, 0.55, 0.55, and 0.55, respectively, which were higher than the theoretical ethanol conversion rate 0.51 from glucose, indicating additional amounts of glucose were produced during fermentation. While, at the same points, ethanol conversion rates for the control strain were 0.51, 0.51, 0.51, and 0.52 respectively. Consequently, we confirmed that the hemicellulolytic enzymes produced by IMPaXPaR strain improved the hydrolysis and ethanol conversion in HSFs of pretreated corncobs.

## Conclusions

The FMDV P2A showed high efficiency in self-cleavage of the polyprotein in *K. marxianus*, and could be used for secretory co-expression of multiple enzymes. Supplements of hemicellulolytic enzymes prepared by the IMPaX and IMPaXPaR strains to commercial cellulases increased both glucose and xylose yields from diluted acid pretreated corncobs. The IMPaXPaR strain that coordinately secreted three hemicellulolytic enzymes, including a  $\beta$ -mannanase M330, a  $\beta$ -xylanase Xyn CDBFV, and  $\beta$ -xylosidase RuXyn1, produced 21.3 and 23.9 g ethanol at 144 and 216 h from pretreated corncobs, which were 34.2% and 11.1% higher than that of its parent strain respectively, showing that it is a useful CBP strain for ethanol production in HSF processes.

## Abbreviations

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.

## Declarations

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## **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.

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## **Ethics declarations**

### **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.

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## Tables

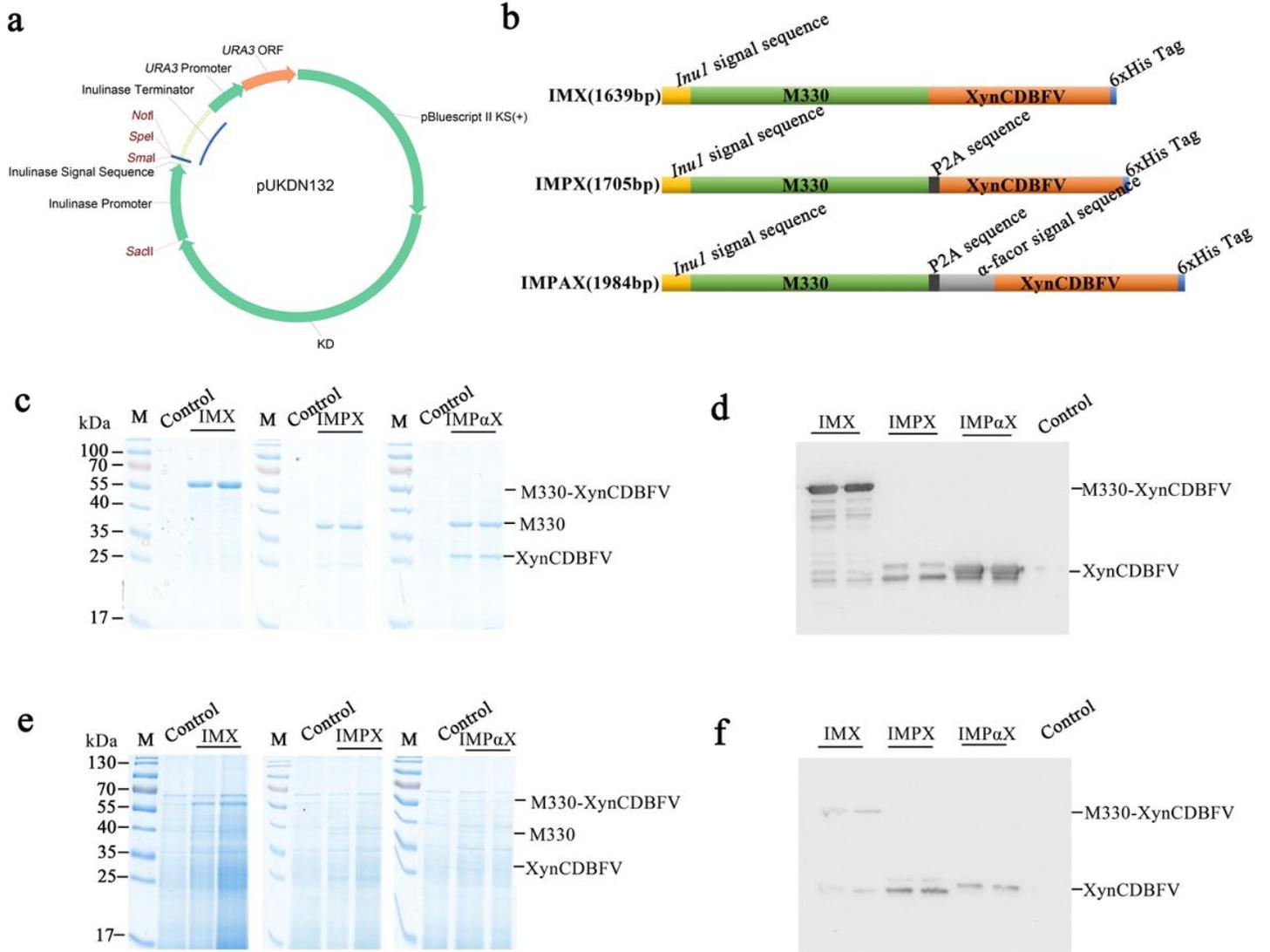
**Table1: Primer sequences used in this work**

Primers	Sequences
MF	ATGAAGTTAGCATACTCCCTCTTGC
IMXR1	GAACTACAGAACTTTGTGTAAATACGGTGGATGGTTTGGAG
IMXF	TCCACCGTATTTACACAAAGTTTCTGTAGTTCAGCTTCTC
XR	CTAGTGATGATGATGATGGTGATCACCAATGTAAACCTTTGCGTATGG
IMPR	AGGACCGGGGTTTTCTTCCACGTCTCCTGCTTGCTTTAACAGAGAGAAGT TCGTGGCTCCGGATCCTGTAAATACGGTGGATGGTTTGA
IMPXF	TGGAAGAAAACCCCGGTCTCAAAGTTTCTGTAGTTCAGCTTCTCACT
PaF1	GGAAGAAAACCCCGGTCTATGAGATTTCTTCAATTTTTACTGCAG
αXR1	GAGAAGCTGAACTACAGAACTTTGCCCGGTACGTAAGCTTCAGCCTCT
αXF1	AGAGGCTGAAGCTTACGTACCCGGGCAAAGTTTCTGTAGTTCAGCTTCTC
XPR	ATCACCAATGTAAACCTTTGCGTATG
XPαF	ACGCAAAGGTTTACATTGGTGATGGATCCGGAGCCACGAACTTCTCTC
αR1	ATAGCGTTTCTTAACTTTATCAGCCCCGGGTACGTAAGCTTCAGCCTCT
αRF	TGATAAAGTTAAGAAACGCTAT
RR	CAAAGCTTGCGGCCTTAAGCGGCCGCTTACTCATCCATGCCTTCGATGGTG
IXF	AGACGGTGACCCCGGGACTAGTATGAGATTTCTTCAATTTTTACTG

**Table 2** The  $\beta$ -mannanase and  $\beta$ -xylanase activities of the IMX, IMPX, and IMPαX strains cultured in flasks at 30°C, 220rpm for 72h.

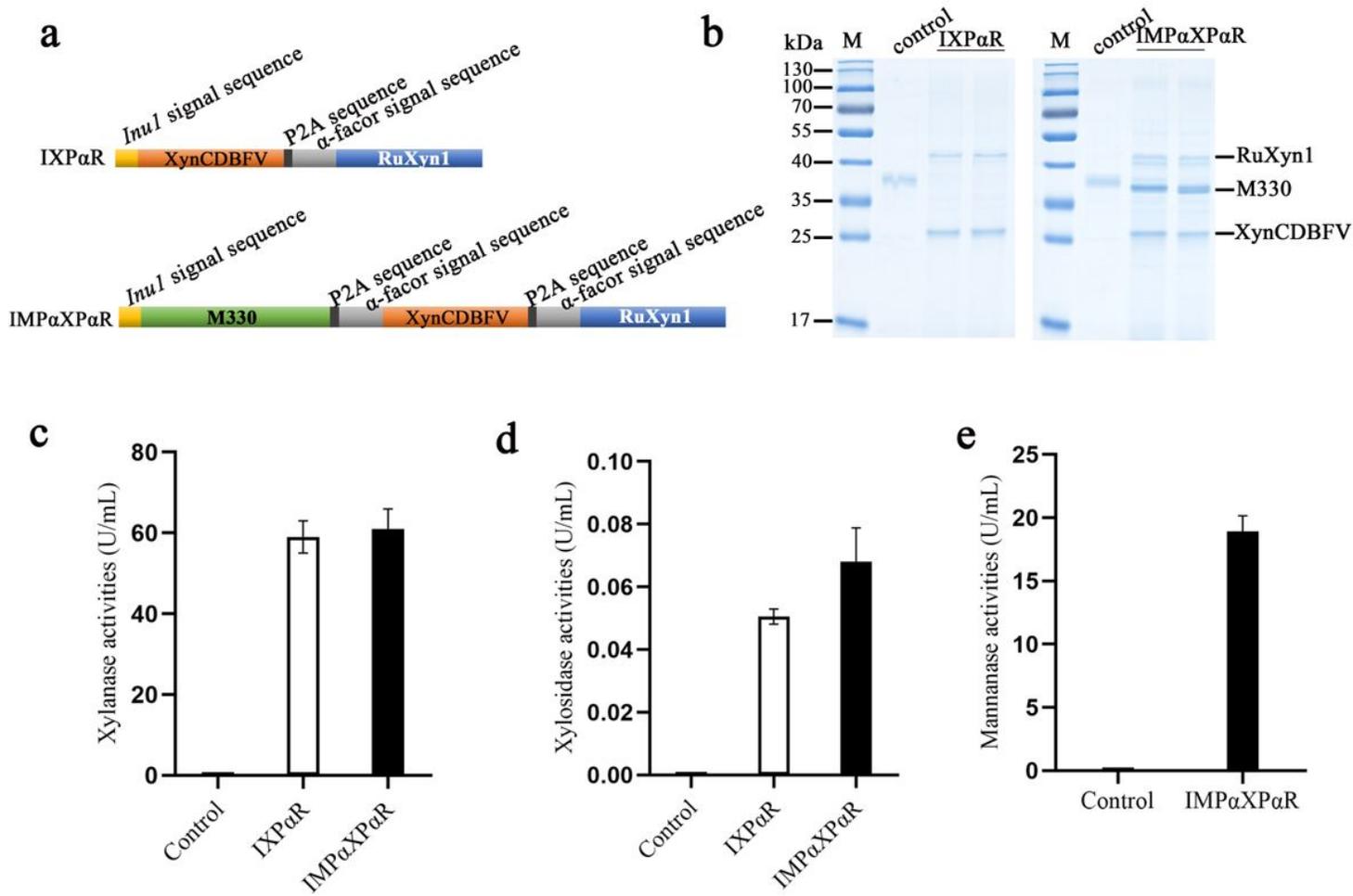
Strains	$\beta$ -Mannanase activities		$\beta$ -Xylanase activities	
	Extracellular	Intracellular	Extracellular	Intracellular
IMX	24.03±3.74	1.13±0.19	155.26±4.24	44.17±4.24
IMPX	21.34±1.37	4.50±0.75	42.07±4.99	87.59±11.41
IMPαX	15.50±1.91	4.62±0.44	136.17±15.34	39.43±4.11

## Figures



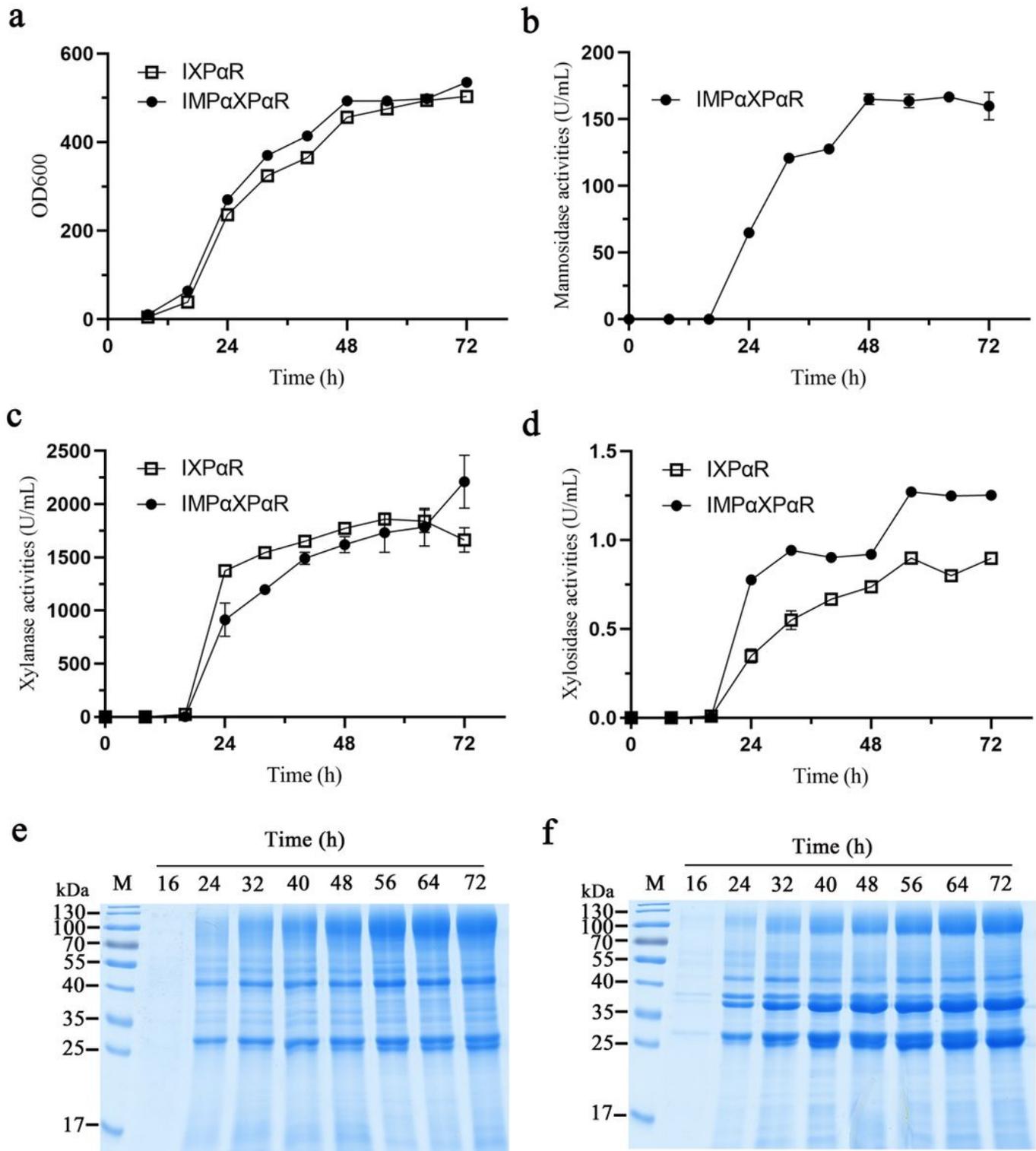
**Figure 1**

The efficiency of FMDV P2A in self-cleavage of M330 and XynCDBFV. 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.



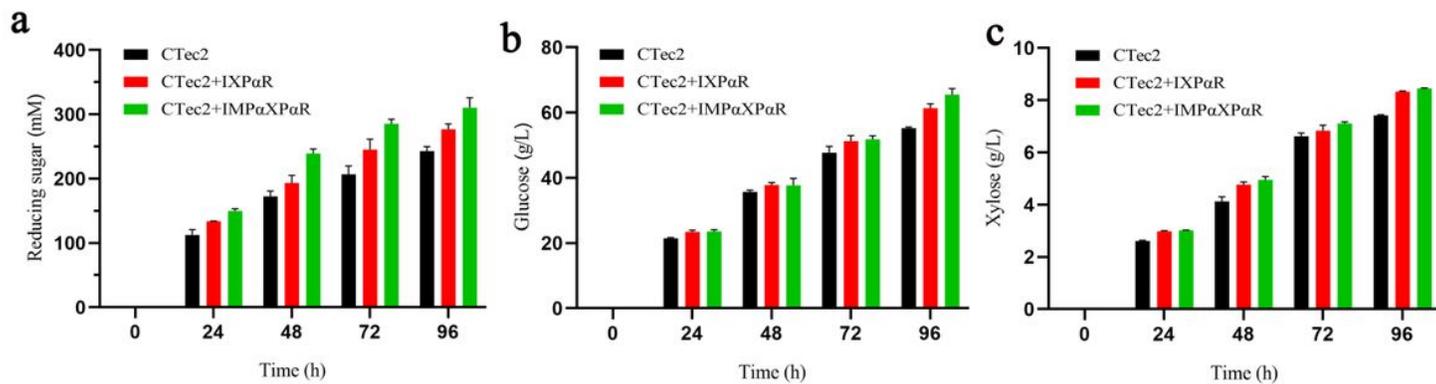
**Figure 2**

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.



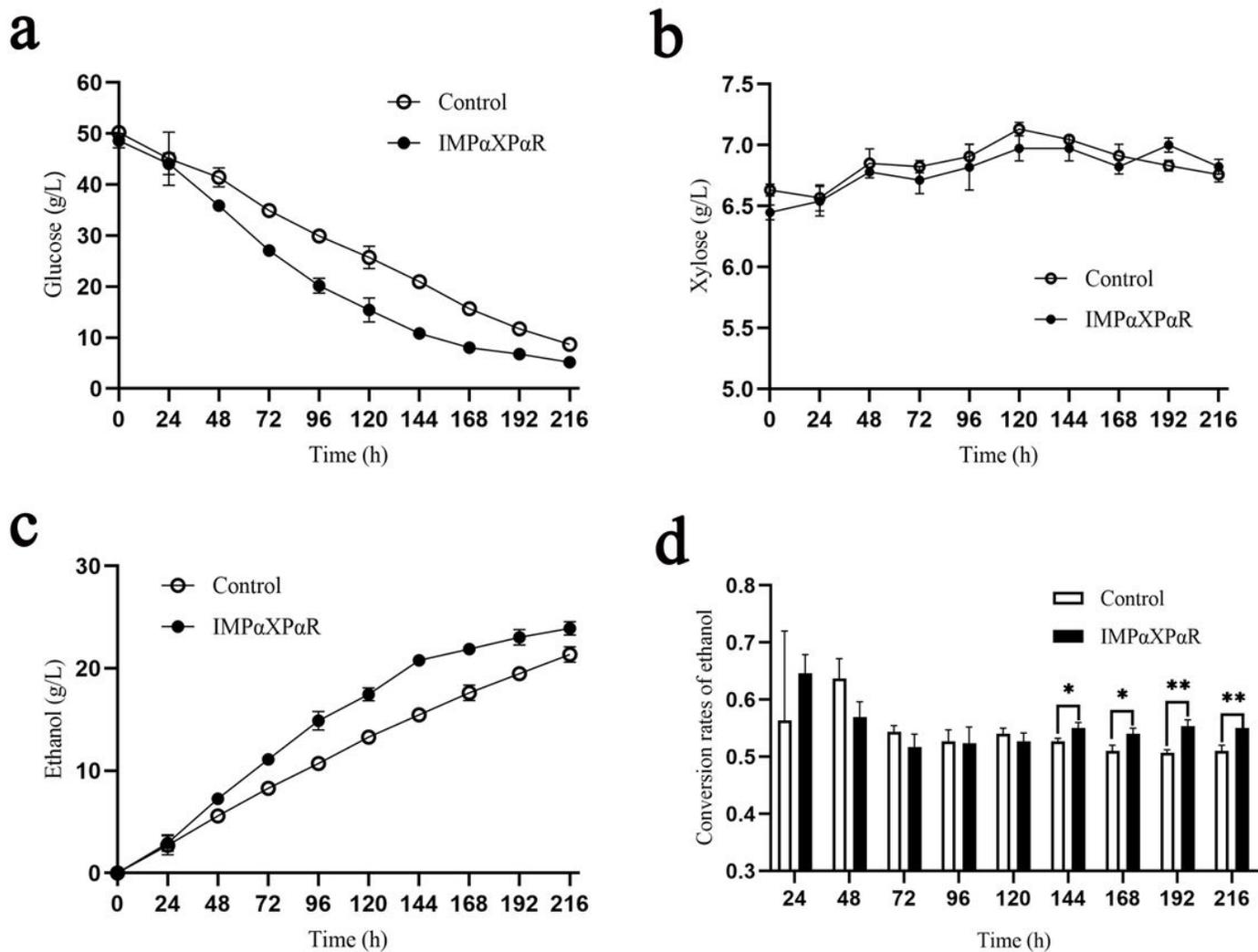
**Figure 3**

Growth curves (a) and productions of  $\beta$ -mannanase (b),  $\beta$ -xylanase (c), and  $\beta$ -xylosidase (d) in fed-batch fermentation of the IMPaX and IMPaXPaR strains. Supernatant samples at the indicated times of the IMPaX (e) and IMPaXPaR 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 pretreated corncobs.



**Figure 5**

Concentrations of glucose (a), xylose (b), and ethanol (c), and sugar-ethanol conversion rates (d) during HSFs with the IMPaXPaR and Fim-1(control) strains. Conversion rates were calculated as following: ethanol yields / (initial glucose concentration-residual glucose concentration). \*, p<0.05; \*\*, p<0.01.