Enhancement of xylanase expression by *Komagataella phaffii* through pexophagy inhibition

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**ABSTRACT**

Xylanase is widely used in agriculture and food industry. To increase heterologous xylanase expression by *Komagataella phaffii* under the condition of mixed carbon source feeding, the *atg30* gene was knocked out from this yeast to inhibit pexophagy for increasing sorbitol consumption. *Komagataella phaffii atg30Δ* was constructed through homologous recombination that resulted in the inhibition of pexophagy. When compared with the control, the yield of secretory xylanase increased by 4.1 ± 0.2%, 5.7 ± 0.5%, 11.8 ± 0.4% and 11.4 ± 0.7% under the conditions of 1.5% methanol and 0.5, 1, 1.5 and 2% sorbitol addition, respectively. The residual sorbitol analysis showed that sorbitol consumed by *Komagataella phaffii atg30Δ* increased by 121.3 ± 9.3%, 66.8 ± 15.2% and 31.2 ± 3.2% following 1, 1.5 and 2 sorbitol addition, respectively. Thus, non-methanol carbon source, co-feeding with methanol, provided more energy without pexophagy by *atg30* gene knockout. Pexophagy inhibition increased the heterologous protein expression by *Komagataella phaffii*, which provided basis for increased yield of xylanase through pexophagy monitoring.

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**Introduction**

*Komagataella phaffii* (previously known as *Pichia pastoris*) is a notable heterologous protein producer with advantages such as strong and strictly regulated alcohol oxidase1 promoter (*P_{AOX1}*), high yield of heterologous protein, and high cell density fermentation. However, low methanol metabolism flux of *K. phaffii* leads to low energy supply for protein production [1] and high cell death ratio (up to 35%) during high cell density fermentation [2]. Different studies have considered mixed carbon source feeding, such as methanol mixed with glucose, glycerol [3], lactic acid [4], sorbitol [5], etc., as a reasonable strategy to enhance the carbon metabolism of *K. phaffii*. However, the major issue associated with the mixed carbon source feeding strategy is the inhibition of *P_{AOX1}* in the presence of non-methanol carbon source, resulting in pexophagy [6]. Therefore, there is an urgent need to increase the heterologous protein yield of *K. phaffii* by unlocking the inhibition of *P_{AOX1}* in the presence of non-methanol carbon source. Interestingly, sorbitol is used as a non-repressing carbon source to supply energy for heterologous protein expression. However, the specific growth rate and final biomass of *K. phaffii* in the presence of sorbitol as the carbon source have been reported to be lower than those achieved in the presence of glycerol [7]. To increase sorbitol metabolism and heterologous protein production, *atg30* knockout recombinant *K. phaffii* was constructed to inhibit pexophagy because protein Atg30, encoded by *atg30*, is an important component of pexophagy [8]. In our previous study, xylanase, an important food-grade enzyme used in food industry, from *Aspergillus niger* ATCC 1015 was expressed by recombinant *K. phaffii* [9]. Hence, in the present study, the yield of xylanase was enhanced for its wide applications.

**Materials and methods**

**Strains, vectors and media**

*A. niger* ATCC 1015 was purchased from American Type Culture Collection (Manassas, VA, USA). *Escherichia coli* Top10 competent cells were obtained...
from Takara Biotechnology Co. Ltd (Dalian, China). *K. phaffii* GS115, pPIC9K and zeocin were bought from Life Technologies (Carlsbad, CA, USA). All the restriction enzymes, DNA markers and biological kits were purchased from Takara Biotechnology Co., Ltd (Dalian, China). All the primers in this study (Table 1) were synthesized by Sangon Biotech Inc (Shanghai, China), and all the chemicals were purchased from Sino Chemical Co. (Shanghai, China).

YPD medium contained yeast extract, 10 g/L; peptone, 20 g/L; glucose, 20 g/L and agar powder (if necessary), 15 g/L. BMGY medium consisted of yeast extract, 10 g/L; peptone, 20 g/L; PBS buffer (pH 7.0), 100 mmol/L; glycerol, 20 g/L; YNB, 13.4 g/L and biotin, 4 \( \times \) \( 10^{-4} \) g/L. BMMY medium contained yeast extract, 10 g/L; peptone, 20 g/L; PBS (pH 7.0), 100 mmol/L; methanol, 5 mL/L; \((\text{NH}_4)_2\text{SO}_4\), 20 g/L and biotin, 4 \( \times \) \( 10^{-4} \) g/L. MD medium consisted of YNB, 13.4 g/L; biotin, 4 \( \times \) \( 10^{-4} \) g/L; glucose, 10 g/L and ethanol, 10 mL/L. MM medium comprised (per L) YNB, 13.4 g/L; biotin, 4 \( \times \) \( 10^{-4} \) g/L and methanol, 5 mL/L.

**Construction of *K. phaffii* atg30\( \Delta \), complement strain and xylan expression strain (*K. phaffii* xynB/atg30\( \Delta \))**

The general molecular operations were conducted according to Molecular Cloning [10]. Molecular manoeuvre in *K. phaffii* was performed according to EasySelect Pichia Expression Kit Manual (Thermo Fisher Scientific). The atg30 upstream fragment with 5’ BglII site and 3’ XhoI site was amplified by polymerase chain reaction (PCR) using primers up-atg30-F and up-atg30-R. Furthermore, primers down-atg30-F and down-atg30-R were used for PCR amplification of the atg30 downstream fragment with 3’ BglII site according to the genome sequence of *K. phaffii* GS115. Then, the upstream and downstream fragments were used as templates, and up-atg30-F and up-atg30-R were employed as primers to obtain homologous arm with a BglII site between atg30 upstream and downstream fragment. After adding PolyA by using rTaq enzyme, the homologous arm was ligated with vector pMD19-T and transformed into *E. coli* Top10 cells. The recombinant vector was digested using XhoI and ligated with XhoI-digested Kanamycin-HIS4 fragment obtained from pPIC9K using kan-his-F and kan-his-R as primers, and transformed into *E. coli* Top10 cells to obtain a recombinant vector pMD-atg30\( \Delta \). The primers in-atg30-F and in-atg30-R were used for the verification of atg30 of pMD-atg30\( \Delta \). After linearisation by BglII digestion, the recombinant vector pMD-atg30\( \Delta \) was transformed into *K. phaffii* and *K. phaffii* xynB competent cells to obtain *K. phaffii* atg30\( \Delta \) and *K. phaffii* xynB/atg30\( \Delta \) strain, which were screened from HIS-plate and 200 \( \mu \)g/mL G418 MD plate, respectively.

For complement strain construction, the atg30 gene was obtained from the *K. phaffii* genome using atg30-F and atg30-R as primers. The obtained atg30 gene fragment was fused with pPICZA after BglII and XhoI digestion to produce recombinant pPICZA-atg30. The pPICZA-atg30 vector, after linearisation by DraI digestion, was transformed into *K. phaffii* atg30\( \Delta \) competent cells to obtain the complement strain, which was verified by atg30 amplification using in-atg30-F and in-atg30-R as primers, and sequencing. The cloning and expression of xylanase gene (xynB) from *A. niger* ATCC 1015 has been described in our previous study [9].

**Table 1. Primers (Restriction endonuclease sites were marked by bold-underline).**

| Name        | Sequence (5’-3’)                      | Restriction endonuclease |
|-------------|---------------------------------------|--------------------------|
| up-atg30-F  | CCGTTGATAGATTATGATCCTAGTTTCTCTCC      | Bgl II                   |
| up-atg30-R  | ATTCTCGAGATCAGATCAATACTCCAAAGCAACTG   | Xho I                    |
| down-atg30-F| ATCTGAATATCAATTTGCAACCC               |                         |
| down-atg30-R| TGAAATTATGATCAGATCAATAACCGTACATTGCCAAGT | Bgl II                   |
| atg30-F     | CTTGGAGATCAGATCAATAAGGTATAGGATGAGAAA  | Bgl II                   |
| atg30-R     | CCGCCGCGCTAGAGGTTTAATCTCCTGTTGAGC    | Xho I                    |
| in-atg30-F  | TGGACGACATACGACATAGA                  |                          |
| in-atg30-R  | GGAAGAGGGAGGAGGAGG                   |                          |
| kan-his-F   | GCATGATCTCAGATCGATCGATGTTATACAGT     | Xho I                    |
| kan-his-R   | GCATGATCTCAGATCGATCGATCGGCGATTTAATCCAA | Xho I                  |

**Cultivation of *K. phaffii* strains for pexophagy identification**

The recombinant *K. phaffii* strains were cultivated in 50-mL tubes containing 5 mL of YPD medium at 30°C and 200 rpm for 8–24 h. Then, the YPD supernatant was discarded by centrifugation (8437 \( g \)) to obtain the cell pellet. The cell pellet was transferred into MM medium and incubated for another 24 h for induction. Then, the cell pellet was transferred into a 250-mL flask containing 50 mL of MD medium (containing glucose for micropexophagy, containing ethanol for macropexophagy) for cultivation at 30°C and 200 rpm. Alcohol oxidase (AOX) and formate dehydrogenase
(FDH) activities were determined at 2.0, 4.0 and 6.0 h cultivation.

To obtain the growth curves of the recombinant K. phaffii strains, the strains were cultivated in 50-mL tubes containing 5 mL of YPD medium at 30 °C and 200 rpm for 8–24 h. Subsequently, the culture broths were transferred into a 250-mL flasks containing 100 mL of YPD medium and incubated at 30 °C and 200 rpm for 48 h.

**Cultivation optimization for xylanase expression by recombinant K. phaffii strains**

The recombinant K. phaffii strains were cultivated in 50 mL of YPD medium at 30 °C and 200 rpm for 18–24 h to an optical density at 600 nm (OD600) of 2.0–8.0, and then transferred into 250-mL flasks containing 100 mL of BMGY medium at 4% inoculation ratio and incubated at 30 °C and 200 rpm for 24 h. The recombinant K. phaffii cells were harvested by centrifugation (8437 g, 5 min) and resuspended in 100 mL of BMGY medium in 250-mL flasks with different methanol concentrations. The default (control) cultivation conditions were pH of 5.0, 30 °C and 4% inoculation ratio.

For cultivation with sorbitol addition, the recombinant K. phaffii strains were cultured in 50 mL of YPD medium at 30 °C and 200 rpm for 18–24 h to an OD600 of 2.0–8.0, and then transferred into 100 mL of BMGY medium at 4% inoculation ratio and incubated at 30 °C and 200 rpm for 24 h. The recombinant K. phaffii cells were harvested by centrifugation (8437 g, 5 min) and suspended in 100 mL BMGY medium with 1.5% methanol for 24 h. Subsequently, 0.5, 1, 1.5 and 2% sorbitol was respectively added and 1.5% methanol was added every 24 h. The default (control) cultivation conditions were pH of 5.0, 30 °C and 4% inoculation ratio.

**Analysis**

The cell density of K. phaffii cells was measured using a spectrophotometer (U-1100, Hitachi Ltd., Tokyo, Japan) against distilled water at 600 nm after appropriate dilution. Determination of alcohol oxidase (AOX) and formate dehydrogenase (FDH) activities, ATP, ADP and AMP concentrations, and energy charge (EC) was conducted according to our previous study [11]. Xylanase B activity was ascertained as described in our previous work [9], and sorbitol determination process has been presented in an earlier study [12].

**Results and discussion**

**Construction of K. phaffii atg30Δ, complement strain and K. phaffii xynB/atg30Δ**

The entire process of atg30 gene knock out and complementation is shown in Supplementary material, Figure S1. PCR amplification generated fragments 600 and 650 bp for upstream and downstream fragments, respectively (Supplementary material, Figure S1(A)). These two fragments were ligated into a 1250-bp fragment by overlap PCR (Supplementary material, Figure S1(B)). After polyA generation using rtTaq enzyme, this 1250-bp fragment was ligated with vector pMD19-T to obtain pMD19-atg30Δ-DU. A 4110-bp Kanamycin-HIS4 fragment (Supplementary material, Figure S1(C)) was generated by PCR from vector pPIC9K. Both pMD19-atg30Δ-UD and the Kanamycin-HIS4 fragment were ligated after Xhol digestion to obtain pMD19-atg30Δ, which was confirmed by Xhol digestion and double enzymes (BglII and Xhol) digestion, resulting in a band of about 4000 bp and two bands of 7400 and 600 bp, respectively (Supplementary material, Figure S1(D)). After linearisation using BglII, pMD19-atg30Δ was transformed into K. phaffii GS115 and K. phaffii xynB to generate recombinant K. phaffii atg30Δ and K. phaffii xynB/atg30Δ, respectively, which were screened using MD plates with G418. The entire process of gene knockout and complementation is shown in Supplementary material, Figure S1(E,F).

Furthermore, the atg30 gene was complemented into K. phaffii atg30Δ to confirm atg30 gene knockout of K. phaffii atg30Δ. Briefly, the complete atg30 gene (2380 bp) was amplified from the K. phaffii GS115 genome (Supplementary material, Figure S1(G)) and ligated with vector pPICZA after digestion by BglII and Xhol. Subsequently, after linearisation by Drai, pPICZA-atg30 was transformed into K. phaffii atg30Δ, and screened using MD plate with 100 μg/mL zeocin. The genome of complement K. phaffii strain was used as the template to amplify part of atg30 (350 bp) (Supplementary material, Figure S1(H)). The results confirmed that the atg30 gene was knocked out successfully.

**Characterisation of K. phaffii atg30Δ and K. phaffii atg30Δ complement**

The growth curves of these K. phaffii strains were similar to that of K. phaffii GS115 (Figure 1), suggesting
that \textit{atg30} knockout did not significantly influence \textit{K. phaffii} growth.

Figure 2 illustrates the AOX and FDH activities during the two pexophagy conditions (micropexophagy and macropexophagy). In the \textit{K. phaffii atg30Δ} complement, the AOX activity significantly decreased during both the pexophagy conditions (Figure 2(A,C)), whereas the FDH activity significantly decreased only during micropexophagy (Figure 2(B,D)). These changes in the enzyme activities were similar to those of \textit{K. phaffii} GS115 during pexophagy [13], confirming that the \textit{atg30} gene was successfully complemented into the \textit{K. phaffii atg30Δ} genome. In \textit{K. phaffii atg30Δ}, the AOX activity did not decrease significantly under both the conditions of pexophagy (Figure 2(A,C)), indicating that the \textit{atg30} gene knockout inhibited pexophagy in the presence of non-methanol carbon source.

**Effect of methanol addition on xylanase expression by \textit{K. phaffii} strains**

The effect of methanol addition on the cell density of \textit{K. phaffii xynB} and \textit{K. phaffii xynB/atg30Δ} was similar (Figure 3(A,C)). The cell densities of the strains increased with the increasing of induction time. After 96 h of induction, the cell densities of \textit{K. phaffii xynB} increased from 13.0 ± 0.3, 13.0 ± 0.3, 13.5 ± 0.3 and 13.5 ± 0.3 OD$_{600}$ to 20.2 ± 0.8, 20.9 ± 1.3, 19.2 ± 0.1 and 18.9 ± 0.6 OD$_{600}$ at the conditions of 0.5, 1.0, 1.5 and 2.0% methanol addition, respectively. The cell densities of \textit{K. phaffii xynB/atg30Δ} increased from 13.8 ± 0.6, 13.7 ± 0.5, 14.0 ± 0.4 and 14.6 ± 0.6 OD$_{600}$ to...
21.1 ± 0.5, 21.8 ± 0.4, 22.6 ± 0.5 and 21.2 ± 0.3 OD600 at the conditions of 0.5, 1.0, 1.5 and 2.0% methanol addition, respectively, after 96 h of induction. In both the K. phaffii strains, the xylanase activities continuously increased with time till 48 h of induction. Subsequently, the xylanase activities of K. phaffii-xynB remained stable at 48–96 h of induction, whereas those of K. phaffii-xynB/atg30Δ continued to increase till 72 h of induction. The xylanase activities in the two K. phaffii strains increased with increasing methanol concentration of up to 1.5% methanol, and did not increase further under the condition of 2% methanol addition. The highest xylanase activities achieved in K. phaffii-xynB and K. phaffii-xynB/atg30Δ were 864.4 ± 36.1 and 956.1 ± 34.7 U/mL at 1.5% methanol, respectively (Figure 3(B,D)).

The effect of sorbitol addition on the cell density of the two K. phaffii strains was similar as shown in Figure 4(A,C). The cell densities of K. phaffii-xynB increased from 14.1 ± 0.4, 15.0 ± 0.2, 15.3 ± 0.6 and 15.1 ± 0.7 OD600 to 22.6 ± 0.5, 23.5 ± 0.4, 23.5 ± 0.4 and 24.0 ± 0.5 OD600 at the conditions of 0.5, 1.0, 1.5 and 2.0% sorbitol addition, respectively, after 96 h of induction. The cell densities of K. phaffii-xynB/atg30Δ increased from 15.0 ± 0.2, 14.9 ± 0.4, 15.1 ± 0.2 and 14.7 ± 0.2 OD600 to 22.9 ± 0.2, 22.4 ± 0.6, 23.2 ± 0.4 and 22.8 ± 0.2 OD600 at the conditions of 0.5, 1.0, 1.5 and 2.0% sorbitol addition, respectively, after 96 h of induction. Furthermore, the cell densities of both the K. phaffii strains grown in the presence of different concentrations of sorbitol showed similar curves. The xylanase activities in the two K. phaffii strains increased with time following the addition of different concentrations of sorbitol (Figure 4(B,D)), and, in particular, addition of 0.5% and 1% sorbitol led to higher xylanase activities. The final xylanase activities of K. phaffii-xynB/atg30Δ after 96 h of 0.5, 1, 1.5 and 2% sorbitol addition were 1365.3 ± 55.2, 1340.6 ± 79.5, 1115.2 ± 11.9 and 1140.7 ± 40.9 U/mL, which increased by 4.1 ± 0.2%, 5.7 ± 0.5%, 11.8 ± 0.4% and 11.4 ± 0.7%, respectively, with the highest activity of xylanase (1365 U/mL ± 55.2) achieved following 0.5% sorbitol and 0.5% methanol addition.

As an indicator of pexophagy, EC remained at a relatively constant value of 0.5, demonstrating non-pexophagy conditions with sorbitol addition (Figure 5(A,B)). The residual sorbitol content in the two recombinant K. phaffii cultures showed that more sorbitol was consumed by K. phaffii-xynB/atg30Δ, which provided more energy for protein expression.
Figure 4. Effect of sorbitol and 0.5% methanol addition on cell density and xylanase activity of recombinant *K. phaffii* strains. Cell density (A) and xylanase activity (B) of *K. phaffii xynB*; Cell density (C) and xylanase activity (D) of *K. phaffii xynB/atg30Δ*.

Figure 5. EC and residual sorbitol content during recombinant *K. phaffii* cultivation with methanol and sorbitol. EC of *K. phaffii xynB* (A) and *K. phaffii xynB/atg30Δ* (B); Residual sorbitol (C).
Furthermore, whereas the residual sorbitol level in the two recombinant K. phaffii cultures was similar under the condition of 0.5% sorbitol addition, it was lower in the K. phaffii xynB/atg30Δ culture under the conditions of 1, 1.5 and 2% sorbitol addition, when compared with that in the K. phaffii xynB culture. After 96 h induction, the final sorbitol concentrations in the cultures of K. phaffii xynB and K. phaffii xynB/atg30Δ were 1.8 ± 0.1, 3.3 ± 0.2, 4.1 ± 0.1, 4.5 ± 0.1%, and 1.9 ± 0.2, 2.7 ± 0.1, 2.8 ± 0.1, 3.4 ± 0.1%. The amount of sorbitol consumed by K. phaffii xynB/atg30Δ increased by 121.3 ± 9.3%, 66.8 ± 15.2% and 31.2 ± 3.2% under the condition of 1, 1.5, and 2 sorbitol addition, respectively (Figure 5(C)).

**Prospects**

Research on the application of K. phaffii for heterologous proteins and metabolites production has attracted numerous researchers and industrial partners [14,15]. However, a major limitation of heterologous protein production by K. phaffii is the dissolved oxygen content, with low dissolved oxygen content causing methanol toxicity to K. phaffii cells, and high dissolved oxygen content resulting in high cell death ratio owing to low methanol concentration [16]. Although mixed carbon source feeding has been reported to increase the carbon metabolism flux and energy generation, it inhibits \( P_{\text{AOX1}} \), resulting in pexophagy [6], which decreases heterologous protein production [17]. Atg30 has been identified as an initial acceptor of the pexophagy process [8]. Therefore, we knocked out the \( \text{atg30} \) gene to interrupt pexophagy (Figure 2), causing retention of peroxisomes in K. phaffii cells under the condition of non-methanol carbon source addition, which demonstrated the significance of \( P_{\text{AOX1}} \) (Figure 4(D)) although the relationship between pexophagy and \( P_{\text{AOX1}} \) inhibition is not clear. Furthermore, under the condition of 1, 1.5 and 2% sorbitol addition as a non-methanol carbon source following \( \text{atg30} \) gene knockout in K. phaffii cells, the consumption of sorbitol increased by 89.7, 66.4 and 31.1%, respectively, revealing that carbon metabolism increased with pexophagy inhibition. As a non-repressing carbon source, sorbitol has been often employed for K. phaffii cultivation in previous studies [18], with higher sorbitol consumption resulting in increased heterologous protein production [19]. Accordingly, in the present study, \( \text{atg30} \) gene knockout in K. phaffii cells further increased the sorbitol consumption resulting in higher xylanase yield.

Xylanase is widely used in food, animal feed, brewing, paper and pulp industry, and components extraction from biomass, because of its breaking of xylan [20]. Therefore, xylanase is highly demanded to utilize all kinds of biomass as a renewable source. Recently, several kinds of xylanases were discovered to endeavour a range of diverse applications [21]. Heterologous expression of xylanase is a hot topic for researchers and industrial engineers [9,22]. One important property of xylanase for its further application is its safety. Aspergillus niger ATCC1015 is a safe fungal species of GRAS state approved by the United States’ Food and Drug Administration (FDA) [23]. High-level expression of Aspergillus niger ATCC 1015 xylanase is a benefit for its application, even in food industry. K. phaffii is advantageous for its high cell density fermentation and high level of heterologous protein expression. In this study, the xylanase yield was increased by 4.1 ± 0.2%, 5.7 ± 0.5%, 11.8 ± 0.4% and 11.4 ± 0.7%, through 0.5, 1.0, 1.5 and 2% sorbitol addition. These results showed a promising further high-level xylanase production at the condition of K. phaffii high cell density fermentation.

**Conclusions**

The sorbitol consumption of recombinant K. phaffii was increased by \( \text{atg30} \) gene knockout to enhance xylanase expression as heterologous protein. This result was attributed to pexophagy inhibition of K. phaffii at the condition of supplemental carbon source addition. Our results showed the potential of heterologous protein biosynthesis by recombinant K. phaffii, and further application of xylanase.

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**Disclosure statement**

No potential conflict of interest was reported by the authors.

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