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Effects of Oxygen Limitation on Xylose Fermentation, Intracellular Metabolites, and Key Enzymes of *Neurospora crassa* AS3.1602

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Abstract The effects of oxygen limitation on xylose fermentation of *Neurospora crassa* AS3.1602 were studied using batch cultures. The maximum yield of ethanol was 0.34 g/g at oxygen transfer rate (OTR) of 8.4 mmol/L·h. The maximum yield of xylitol was 0.33 g/g at OTR of 5.1 mmol/L·h. Oxygen limitation greatly affected mycelia growth and xylitol and ethanol productions. The specific growth rate (μ) decreased 82% from 0.045 to 0.008 h^{-1} when OTR changed from 12.6 to 8.4 mmol/L·h. Intracellular metabolites of the pentose phosphate pathway, glycolysis, and tricarboxylic acid cycle were determined at various OTRs. Concentrations of most intracellular metabolites decreased with the increase in oxygen limitation. Intracellular enzyme activities of xylose reductase, xylitol dehydrogenase, and xylulokinase, the first three enzymes in xylose metabolic pathway, decreased with the increase in oxygen limitation, resulting in the decreased xylose uptake rate. Under all tested conditions, transaldolase and transketolase activities always maintained at low levels, indicating a great control on xylose metabolism. The enzyme of glucose-6-phosphate dehydrogenase played a major role in NADPH regeneration, and its activity decreased remarkably with the increase in oxygen limitation.

Keywords *Neurospora crassa* · Oxygen limitation · Xylose · Xylitol · Ethanol

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Introduction

Lignocellulosics are the most abundant renewable resource in the world, and bioconversion of lignocellulosics into fuel ethanol could contribute to renewable energy supplies. Hemicelluloses, the second most abundant polysaccharides in nature, represents about 20–30% of agricultural residues [1]. The utilization of hemicellulose is essential for whole components utilization of lignocellulosic materials, as well as for the economy of the bioconversion process in industrial applications.

Xylose is the main product of enzymatic hydrolysis of angiosperm hemicellulose. In the past, it was regarded that xylose was unfermentable, until Wang et al. [2] found that some microbes were able to utilize xylose to produce ethanol in 1980. Now, more than 100 kinds of microbes that are capable of fermenting xylose have been found, including bacteria, fungi, and yeasts [3].

In fungi, xylose is reduced to xylitol by NADH- or NADPH-dependent xylose reductase (XR) and thereafter is oxidized to xylulose by NAD^+-dependent xylitol dehydrogenase (XDH). The xylulose is phosphorylated, channeled into the pentose phosphate pathway [3]. XR of most fungi, including most yeasts, prefers NADPH to NADH. Because of the cofactor preference of XR (NADPH) and XDH (NAD^+), redox imbalance occurs under anaerobic condition [4]. Therefore, the oxygen-limited rather than anaerobic condition is ideal for bioconversion of xylose to ethanol, so that the accumulated reduced cofactor can be oxidized to reach redox balance. A critical level of oxygen should exist for the highest ethanol yield and productivity.

Since the 1980s, there have been many studies on yeast xylose fermentation [5–8] but fewer studies on filamentous fungi. *Neurospora crassa* has both abilities of producing cellulase and hemicellulase and fermenting glucose and xylose; therefore, it maybe can be used in a consolidated processing [9–11]. In this study, the effects of oxygen limitation on xylose fermentation of *N. crassa* have been investigated. Comparisons of the metabolic rates, intracellular metabolite concentrations, and key enzyme activities under various oxygen-limited conditions were carried out.

Materials and Methods

Microorganism

N. crassa AS3.1602 (orn^-) was purchased from the China General Microbiological Culture Collection Center (Beijing, China) and was routinely maintained on potato dextrose agar slant (potato extract, 200 g/L; dextrose, 20 g/L; agar, 1.5–2.5%) at 4 °C.

Medium and Cultivation Conditions

The culture medium composed of the following (g/L): KH_2PO_4, 2.0; $MgSO_4$, 0.3; $CaCl_2$, 0.3; peptone, 5.0; yeast extract, 3.0; $FeSO_4{\cdot}7H_2O$, 0.005; $ZnSO_4$, 0.0014; $MnSO_4{\cdot}4H_2O$, 0.0016; $CoCl_2$, 0.002; pH 5.0. The carbon source was described in specific later.

The mycelia were inoculated directly from the potato slants into 300-mL flasks containing 100 mL of culture medium added with 1.0% xylose as carbon source. The inocula were precultured for 48 h at 30 °C and shaken at 200 rpm. After 48 h, the precultivated broth was transferred into flasks containing 2.0% xylose and cultivated under various oxygen-limited conditions as described later, at 37 °C and 150 rpm. Samples were

taken every 24 h for analysis of cell and metabolite concentrations in fermentation broth. Mycelia were collected at the exponential phase of fermentation for assays of intracellular intermediate metabolite concentrations and key enzyme activities.

Determination of Oxygen Transfer Rate

Oxygen transfer rate (OTR) was determined using the sodium sulfite oxidation method [12]. OTRs were measured for standard Erlenmeyer flasks of 500-mL flask, 200-mL flask, 100-mL flask, 100-mL serum flask, and 100-mL serum flask filled with nitrogen, containing 100 mL of medium, respectively, shaken at 150 rpm at 37 °C. The OTRs of different oxygen-limited conditions were 12.6, 8.4, 5.1, 3.3, and 0 mmol/L·h, respectively.

Substrate and Products Analysis

Xylose, xylitol, and ethanol were analyzed using LC-10AD high-performance liquid chromatography (Shimadzu, Japan), equipped with an HPX-87H Aminex column (Bio-Rad) and a RID-10A refractive index detector. The column was maintained at 55 °C and eluted with 5 mmol/L H_2SO_4 at a flow rate of 0.4 mL/min.

Intracellular Metabolite Analysis

The extraction of intracellular metabolites was performed according to the method of Ruijter and Visser [13]. The samples for phosphorylated carbohydrate and organic acid analysis were concentrated using solid phase extraction (SPE) and vacuum evaporation, respectively, and stored at −70 °C before assays. The SPE was performed according to the method of Smits et al. [14].

Intracellular metabolites were analyzed using Dionex DX 500 high-pressure anion exchange chromatography (Dionex, USA) equipped with a Dionex GP40 gradient pump and a Dionex ED40 electrochemical detector.

Malate, citrate and isocitrate, pyruvate, 3-phosphoglycerate, and phosphoenolpyruvate (PEP) were analyzed using an AS11-HC column (250×4 mm, Dionex) and a conductivity detector. Solvent A of H_2O and solvent B of 100 mmol/L of NaOH were used to make a time-dependent gradient elution solution: initial 95% of A and 5% of B (0–5 min), followed by a linear decrease in A to 60% and a linear increase in B to 40% (5–43 min), then, a linear increase in A to 95% and a linear decrease in B to 5% (43–45 min), and finally, 95% of A and 5% of B (45–50 min). The flow rate for each gradient was 0.9 mL/min.

Glucose-6-phosphate (G6P), fructose-6-phosphate (F6P), fructose-1,6-bisphosphate (FBP), ribose-5-phosphate (R5P), and erythrose-4-phosphate (E4P) were analyzed using a CarboPac PA20 column (150×3 mm, Dionex) and a pulsed amperometric detector. The detector was equipped with a working gold electrode and a reference electrode consisting of a Ag/AgCl combination. The following potential–time sequences were used: 0.1 V (0–0.4 s), −2.0 V (0.41–0.42 s), 0.6 V (0.43 s), −0.1 V (0.44–0.5 s). The gradient elution system was according to Panagiotou et al. [15].

The protein content was measured using the Bradford method [16].

Enzyme Assays

For the measurement of intracellular enzyme activities, mycelia were collected at the exponential growth phase of fermentation. Mycelia were washed three times with 0.9%

NaCl and lysed using a FastPrep FP120 cell disrupter (Qbiogene, USA). After centrifugation at 4 °C, the supernatants were stored at −70 °C for further assays.

XR (EC 1.1.1.21), XDH (EC 1.1.1.9), xylulokinase (XK, EC 2.7.1.17), and ethanol dehydrogenase (ADH, EC 1.1.1.1) activities were measured as described by Eliasson [17].

FBP aldolase (ALD, EC 4.1.2.13), pyruvate kinase (PK, EC 2.7.1.40), transaldolase (TAL, EC 2.2.1.2), $NADP^+$-dependent isocitrate dehydrogenase (IDH, EC 1.1.1.41), G6P dehydrogenase (G6PDH, EC 1.1.1.49), and malate dehydrogenase (MDH, EC 1.1.1.37) activities were measured as described by Bergmeryer [18].

The activities of pyruvate decarboxylase (PDC, EC 4.1.1.1), phosphofructokinase (PFK, EC 2.7.1.11), and transketolase (TKL, EC 2.2.1.1) were measured as described by Postma et al. [19], Tian et al. [20], and Selivanov et al. [21], respectively.

All assays were carried out at 37 °C, and the samples were measured using UV2550 spectrophotometer (Simadzu, Japan) at 340 nm wavelength.

Enzyme units (U) are defined as micromoles of oxidized or reduced coenzymes per minute under assay conditions. Specific activities are expressed as U/mg total cell protein.

Biomass Measurement

The biomass was measured using the perchloric acid method [22].

Results and Discussion

Xylose Fermentation of *N. crassa* at Various Oxygen-limited Conditions

Xylose fermentation was carried out at OTRs of 12.6, 8.4, 5.1, 3.3, and 0 mmol/L·h, respectively. Xylose uptake rates of exponential growth phase decreased with the increase in oxygen limitation. At OTR of 12.6 mmol/L·h, cell growth was fast, and there was little xylose remained after 24 h (Fig. 1). However, at anaerobic conditions (OTR of 0 mmol/L·h), cell growth was limited, almost no increase in cell concentration appeared, and 12.2 g/L of xylose remained after 10 days of fermentation.

Oxygen limitation had great effects on ethanol and xylitol productions. As shown in Table 1, the maximum conversion of xylose to ethanol was 66% at OTR of 8.4 mmol/L·h, with the ethanol concentration of 6.7 g/L and ethanol yield of 0.34 g/g. The maximum conversion of xylose to xylitol was 34% at OTR of 5.1 mmol/L·h, with the xylitol concentration of 6.7 g/L and xylitol yield of 0.33 g/g. It has been reported that the ratio of ethanol to xylitol is influenced by oxygen [23–25]. Schvester et al. [26] found that during xylose fermentation using *Pachysolen tannophilus*, ethanol began to accumulate only after oxygen was exhausted. In the present research on *N. crassa*, it was found that OTR of 8.4 mmol/L·h was optimal for ethanol production, while OTR of 5.1 mmol/L·h was optimal for xylitol production. More oxygen was needed for production of ethanol than xylitol to reach the maximum conversion and yield. *Candida boidinii* NRRL Y-17213 showed a similar pattern in flask cultivation [27]. Whereas, *C. boidinii* NRRL Y-17213 got the highest ethanol yield at OTR of 10 mmol/L·h and the highest xylitol yield at OTR of 14 mmol/L·h, when OTR varied from 10 to 30 mmol/L·h in fermentor [4].

At low xylose level, ethanol concentration reduced rapidly (Fig. 1). No byproducts of acetic acid and lactic acid were found (data not shown), which suggested that ethanol was

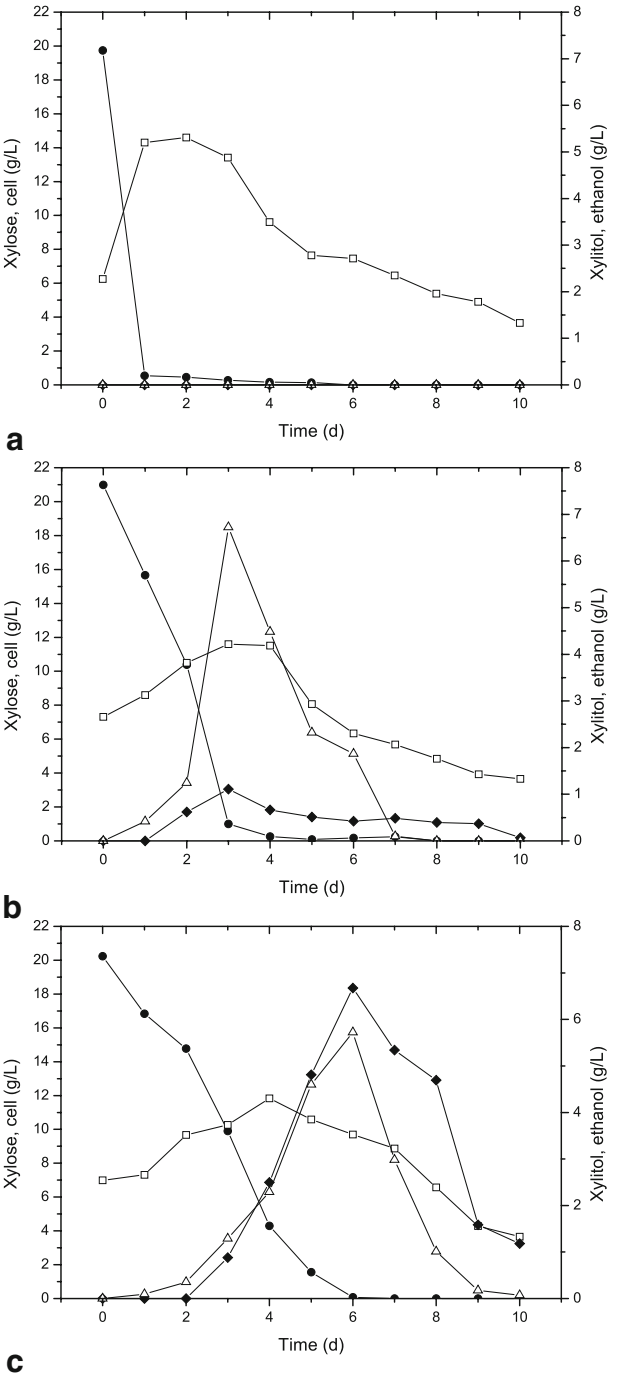
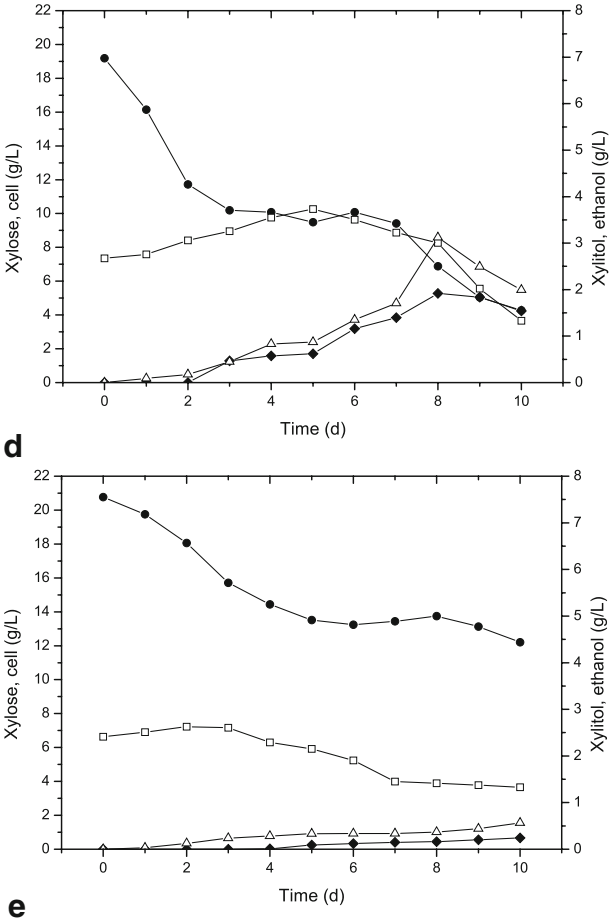


Fig. 1 The time course of substrate, products and cell concentrations (g/L) for *N. crassa* batch cultures using xylose as carbon source. The values are the averages of at least four measurements from two fermentations. OTR: 12.6 (**a**), 8.4 (**b**), 5.1 (**c**), 3.3 (**d**), and 0 mmol/L·h (**e**). Xylose (*filled circles*), cell (*open squares*), xylitol (*filled diamonds*), and ethanol (*open triangles*)

Fig. 1 (continued)



utilized by the cells. Xylitol uptake rate was low at high ethanol levels. When ethanol concentration decreased to a lower level, xylitol uptake rate increased obviously (Fig. 1), which indicated that xylitol uptake was inhibited by ethanol.

Intracellular Metabolite Profiles at Various Oxygen-limited Conditions

Xylose fermentation was carried out at OTRs of 12.6, 8.4, and 0 mmol/L·h, respectively. Mycelia were collected at the exponential growth phase, and the intracellular metabolites were assayed.

As shown in Fig. 2, intracellular metabolite concentrations decreased with the increase in oxygen limitation, except for G6P/E4P and pyruvate. PEP was never detected in all tested conditions, same with the results obtained using *Candida tropicalis* [28].

To compare the values at the same level of xylose uptake rate, the intracellular metabolite concentrations were divided by the corresponding xylose uptake rates [29]. These calculations (Fig. 3) showed accumulation of all intracellular metabolites with the increase in oxygen limitation, indicating a faster metabolism at higher OTRs. When OTRs

Table 1 The influence of oxygen limitation on xylose fermentation of *N. crassa*.

OTR (mmol/L·h)	Xylose consumption[a] (mmol/L·h)	Ethanol				Xylitol			
		Maximal concentration (g/L)	Volumetric productivity (g/L·h)	Yield (g/g)	Conversion[b] (%)	Maximal concentration (g/L)	Volumetric productivity (g/L·h)	Yield (g/g)	Conversion (%)
12.6	5.4±0.1[c]	0	0	0	0	0	0	0	0
8.4	1.5±0.04	6.7±0.4	0.093±0.006	0.34±0.02	66±4	1.1±0.1	0.015±0.001	0.06±0.01	5.6±0.5
5.1	0.94±0.05	5.7±0.4	0.040±0.002	0.28±0.02	56±4	6.7±0.2	0.046±0.001	0.33±0.02	34±2
3.3	0.84±0.03	3.1±0.2	0.016±0.001	0.25±0.02	50±3	1.9±0.03	0.010±0.0001	0.16±0.02	16±2
0	0.28±0.03	0.57±0.06	0.0024±0.0002	0.066±0.003	13±1	0.24±0.01	0.0010±0.0001	0.028±0.002	2.9±0.2

[a] Xylose consumption during exponential growth.

[b] The ethanol conversion rate was calculated with 1 mol xylose producing 1.67 mol ethanol.

[c] The values are the averages and standard deviations of at least four measurements from two fermentations.

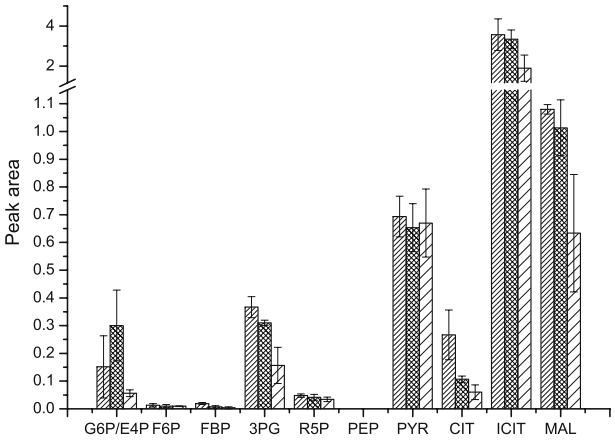


Fig. 2 Intracellular metabolite profiles of *N. crassa* batch cultures at various OTRs using xylose as carbon source. OTR: 12.6 (▨), 8.4 (▩), and 0 mmol/L·h (▨). Bars represent the peak areas divided by the protein concentration. The values are the averages and standard deviations of at least four measurements from two fermentations. *G6P* Glucose-6-phosphate, *E4P* erythrose-4-phosphate, *F6P* fructose-6-phosphate, *FBP* fructose-1,6-bisphosphate, *3PG* 3-phosphoglycerate, *R5P* ribose-5-phosphate, *PEP* phosphoenolpyruvate, *PYR* pyruvate, *CIT* citrate, *ICIT* isocitrate, *MAL* malate

varied from 12.6 to 0 mmol/L·h, the accumulations of all detected intracellular metabolites increased more than 2.8-fold. The most remarkable accumulation happened on pyruvate, F6P, R5P, and malate, whose accumulations increased more than tenfold. These four metabolites were of glycolysis, the pentose phosphate pathway, and the tricarboxylic acid cycle, respectively, which showed that oxygen limitation affected the whole cell metabolism.

Intracellular Enzyme Activities at Various Oxygen-limited Conditions

Xylose fermentation was carried out at five OTRs, and mycelia were collected at the exponential growth phases for intracellular enzyme assays. The results are shown in Fig. 4. The cells incubated at low OTRs might not have fully adapted to the aeration conditions,

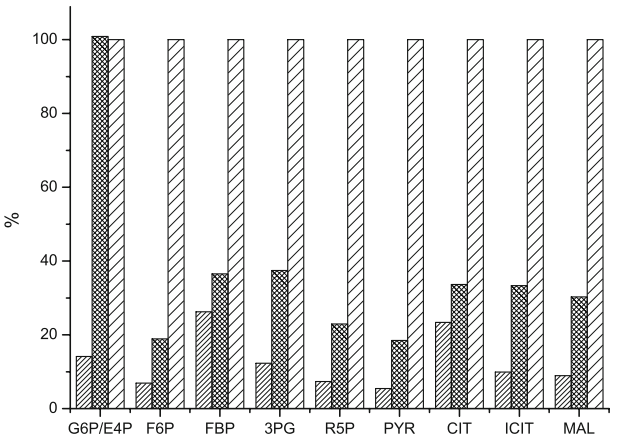


Fig. 3 Intracellular metabolite concentrations divided by corresponding xylose uptake rate at various OTRs, normalized by the values at OTR of 0 mmol/L·h as 100%. OTR: 12.6 (▨), 8.4 (▩), and 0 mmol/L·h (▨)

and the enzyme titers might not be representative.

The activities of XR, XDH, and XK decreased with the increase in oxygen limitation (Fig. 4a), which could be a reason for the decrease in xylose uptake rate under oxygen-limited conditions.

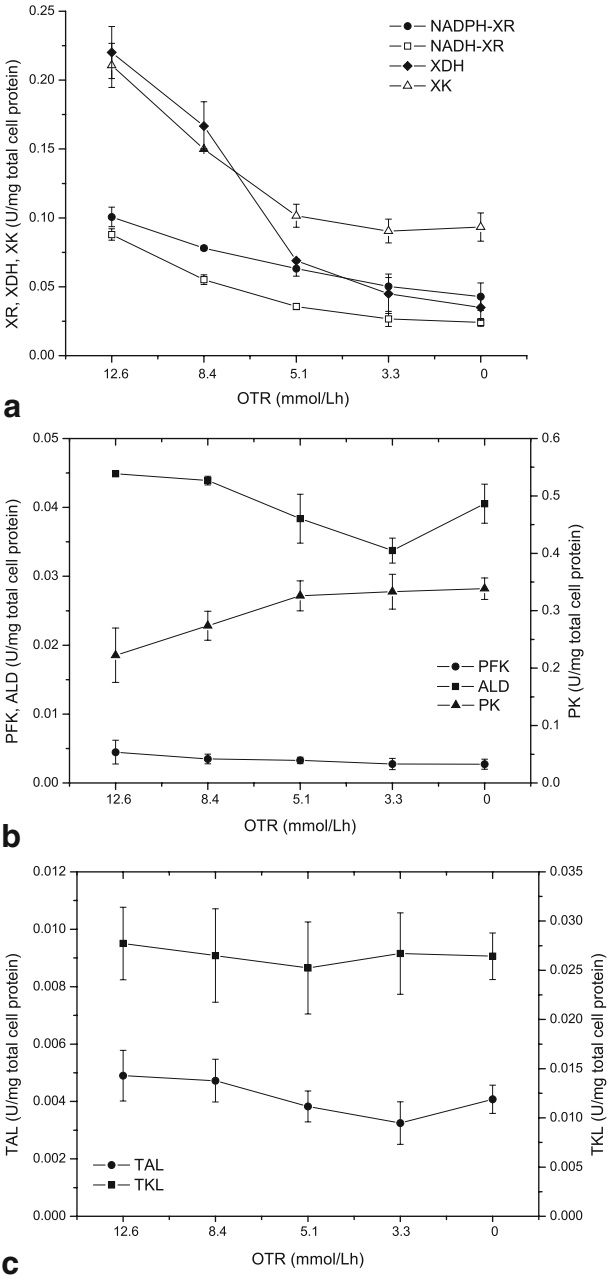
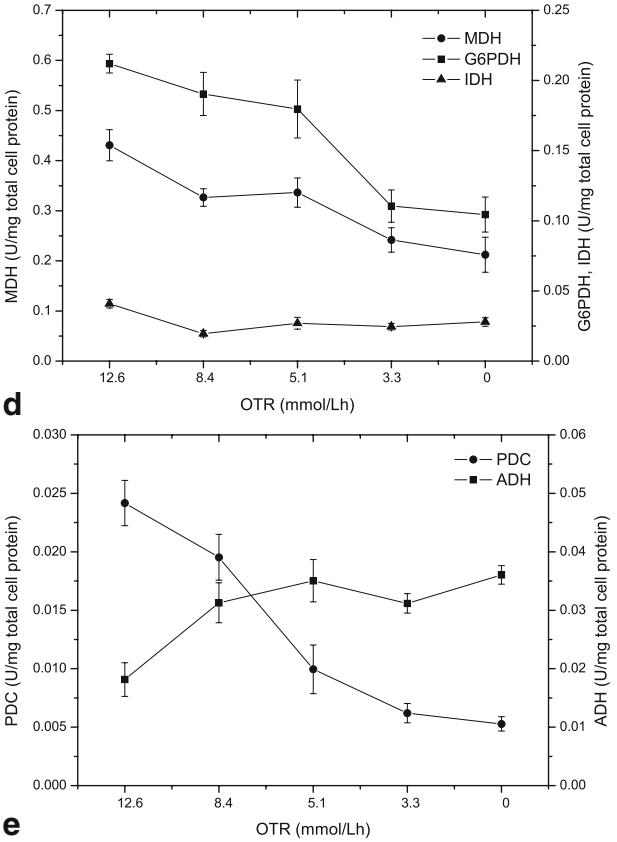


Fig. 4 a–e Specific intracellular enzyme activity profiles of *N. crassa* at various OTRs using xylose as carbon source. The values are the averages and standard deviations of at least four measurements from two fermentations. *XR* Xylose reductase, *XDH* xylitol dehydrogenase, *XK* xylulokinase, *PFK* phosphofructokinase, *ALD* fructose-1,6-bisphosphate aldolase, *PK* pyruvate kinase, *TAL* transaldolase, *TKL* transketolase, *MDH* malate dehydrogenase, *IDH* isocitrate dehydrogenase, *G6PDH* glucose-6-phosphate dehydrogenase, *PDC* pyruvate decarboxylase, *ADH* ethanol dehydrogenase

Fig. 4 (continued)



XR and XDH are the first two enzymes in the xylose metabolic pathway. Xylose is converted to xylitol by NADPH-XR or NADH-XR. Then, xylitol is converted to xylulose by NAD^+-XDH. The ratio of NADH-XR to NADPH-XR activity of *N. crassa* was lower than 1 (Fig. 5), which is the same as most of the fungi. At OTR of 12.6 mmol/L·h, the ratio was 0.87. The ratio decreased with the increase in oxygen limitation and remained at 0.55 when OTR was below 5.1 mmol/L·h. For most xylose-fermenting fungi, XR has a preference for NADPH to NADH, although there is an exception reported by Vandeska et al. to have the NADH-XR/NADPH-XR activity ratio larger than 1 for *C. boidinii* [4]. At OTR of 14 mmol/L·h, the NADH-XR/NADPH-XR activity ratio and xylitol yield were reported being the highest values of 5.88 and 0.48 g/g, respectively [4].

At OTRs of 12.6 and 8.4 mmol/L·h, the ratios of NADH-XR/XDH and NADPH-XR/XDH were low, about 0.45. However, the ratio increased rapidly, especially for NADPH-XR/XDH, with the increase in oxygen limitation. At anaerobic conditions (OTR of 0 mmol/L·h), the ratio of NADPH-XR/XDH reached 1.22, which was 165% larger than the value at OTR of 12.6 mmol/L·h (Fig. 5).

The activities of XR and XDH decreased with the increase in oxygen limitation (Fig. 4a), which indicated that an increase in XR and XDH activities could lead to the increase in xylose metabolic rate. Karhumaa et al. [30] increased XR and XDH activities in *Saccharomyces cerevisiae* by genetic manipulation, which significantly increased ethanol but decreased xylitol productions. Not only XR and XDH activities but also the XR/XDH

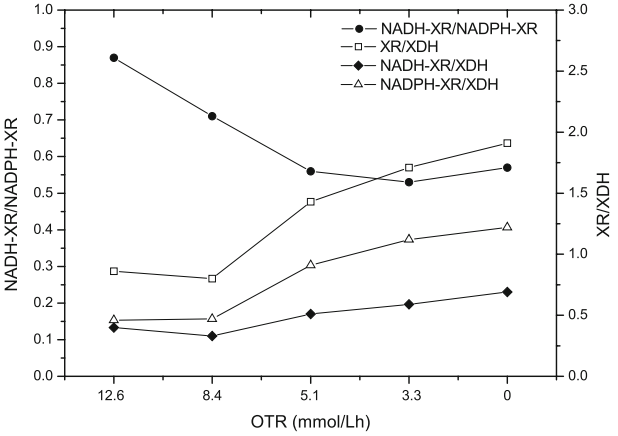


Fig. 5 Effects of oxygen limitation on the NADH-XR and NADPH-XR ratio and the XR/XDH ratio

ratio is important for xylose metabolism. It was reported that a XR/XDH ratio of 1:10 was optimal in minimizing xylitol formation in xylose-utilizing *S. cerevisiae* [31]. For *N. crassa*, the XR/XDH ratio is much higher than 1:10, which indicated that the activity of XDH was low for efficient ethanol fermentation, and the increase in XDH activity might decrease the xylitol accumulation.

XK activity decreased with the increase in oxygen limitation (Fig. 4a). XK is a key enzyme in xylose metabolism and fulfills the initial steps of xylose metabolism together with XR and XDH, to convert xylose to xylitol, and then to xylulose in series. After that, xylulose is phosphorylated and channeled into the pentose phosphate pathway. The decrease in XK activity could be another reason for the decrease in xylose uptake rate under oxygen-limited conditions. It was reported that overexpression of the *XKS1* gene encoding xylulose kinase significantly increased xylose utilization and ethanol production in recombinant *S. cerevisiae* [32].

Oxygen limitation had little influence on the intracellular enzyme activities of PFK and ALD that were in the upstream of glycolysis, while activated PK that was in the downstream of glycolysis converting PEP to pyruvate (Fig. 4b). PK is an important regulatable enzyme in glycolysis and is inhibited by adenosine triphosphate (ATP) [33]. The decreased ATP production rate under oxygen-limited conditions could alleviate the inhibiting effect of ATP on PK.

Oxygen limitation had little effect on the activities of TAL and TKL. When OTR varied from 0 to 12.6 mmol/L·h, TAL and TKL activities always maintained at low levels (Fig. 4c). TAL and TKL activities always maintained at low levels, to show big control effects on xylose metabolism. Walfridsson et al. [34] overexpressed TAL in XR- and XDH-expressing *S. cerevisiae*, which increased the cell growth but not ethanol production. Karhumaa et al. [30] found that increased XR and XDH activities redirected the production from xylitol to ethanol, whereas the rate of xylose consumption was governed by the overexpressed nonoxidative pentose phosphate pathway.

MDH activity reduced by 51%, decreased from 0.43 to 0.21 U/mg total cell protein when OTR varied from 12.6 to 0 mmol/L·h, with the decrease in intracellular malate concentration of 42% (Fig. 2).

Both G6PDH and IDH could generate NADPH. Activity of G6PDH decreased with the increase in oxygen limitation, while oxygen had no effect on IDH. G6PDH activity was much higher than IDH activity (Fig. 4d), which indicated that G6PDH was the main

enzyme in NADPH regeneration. IDH was not regulated by oxygen, similar with the results obtained using *Debaryomyces hansenii* [35].

Oxygen limitation showed reverse effects on PDC and ADH (Fig. 4e). The former decreased and the latter increased with the increase in oxygen limitation. At OTR of 8.4 mmol/L·h, the activities of both enzymes were high, and ethanol yield was the highest.

Conclusion

In this study, the effects of oxygen limitation on xylose fermentation of *N. crassa* AS3.1602 were investigated in batch cultures. With the increase in oxygen limitation, xylose uptake and cell growth rates decreased. Oxygen had great effects on ethanol production. With the increase in oxygen limitation, the metabolic fluxes of ethanol production changed gradually. At OTR of 8.4 mmol/L·h, the productivity and final concentration of ethanol reached the highest values.

Intracellular metabolites were determined at various OTRs. Concentrations of most of the intracellular metabolites decreased with oxygen limitation. The cells incubated at low aeration rates might not have fully adapted to the aeration conditions, and the enzyme titers might not be representative. Intracellular enzyme activities of XR, XDH, and XK, the first three enzymes in the xylose metabolic pathway, decreased with the increase in oxygen limitation, resulting in the decreased xylose uptake rate. When OTR varied from 12.6 to 0 mmol/L·h, TAL and TKL activities always maintained at low levels, indicating a great control on xylose metabolism. The enzyme of G6PDH played a major role in NADPH regeneration, and its activity decreased remarkably with the increase in oxygen limitation.

This work provides intracellular information of xylose metabolism of *N. crassa* AS3.1602 under oxygen-limited conditions, which is useful for the understanding of the metabolic controls for future genetic modification or condition optimization for improvement of ethanol production of this strain.

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