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Oxalic acid production by citric acid-producing *Aspergillus niger* overexpressing the oxaloacetate hydrolase gene *oahA*

Keiichi Kobayashi · Takasumi Hattori · Yuki Honda · Kohtaro Kirimura

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Abstract The filamentous fungus *Aspergillus niger* is used worldwide in the industrial production of citric acid. However, under specific cultivation conditions, citric acid-producing strains of *A. niger* accumulate oxalic acid as a by-product. Oxalic acid is used as a chelator, detergent, or tanning agent. Here, we sought to develop oxalic acid hyperproducers using *A. niger* as a host. To generate oxalic acid hyperproducers by metabolic engineering, transformants overexpressing the *oahA* gene, encoding oxaloacetate hydrolase (OAH; EC 3.7.1.1), were constructed in citric acid-producing *A. niger* WU-2223L as a host. The oxalic acid production capacity of this strain was examined by cultivation of EOAH-1 under conditions appropriate for oxalic acid production with 30 g/l glucose as a carbon source. Under all the cultivation conditions tested, the amount of oxalic acid produced by EOAH-1, a representative *oahA*-overexpressing transformant, exceeded that produced by *A. niger* WU-2223L. *A. niger* WU-2223L and EOAH-1 produced 15.6 and 28.9 g/l oxalic acid, respectively, during the 12-day cultivation period. The yield of oxalic acid for EOAH-1 was 64.2 % of the maximum theoretical yield. Our method for oxalic acid production gave the highest yield of any study reported to date. Therefore, we succeeded in generating oxalic acid hyperproducers by overexpressing a single gene, i.e., *oahA*, in citric acid-producing *A. niger* as a host.

K. Kobayashi · T. Hattori · Y. Honda · K. Kirimura (✉)
Department of Applied Chemistry, Faculty of Science and Engineering, Waseda University, 3-4-1 Ohkubo, Shinjuku-ku, Tokyo 169-8555, Japan
e-mail: kkohtaro@waseda.jp

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Introduction

Oxalic acid is a valuable chemical used as a chelator, detergent, or tanning agent. Currently, oxalic acid is produced through chemical processes, for example, by heating sodium formate followed by H_2SO_4 treatment [21]. Oxalic acid is detected as a metabolite in many microorganisms [3, 19]. The availability of microorganisms producing oxalic acid has been recognized in the field of metal bioleaching, i.e., microbial solubilization and metal recovery [4, 28]. However, in the citric acid-producing fungus *Aspergillus niger*, oxalic acid is a by-product and, in some cases, a contaminant in the recovery of citric acid [13, 27].

There are two major pathways for oxalic acid biosynthesis in microorganisms. One pathway involves the oxidation of glyoxylic acid by glyoxylate dehydrogenase (GDH), and the other involves the hydrolysis of oxaloacetic acid by oxaloacetate hydrolase (OAH; EC. 3.7.1.1) [2, 6, 14]. In the wood-rotting basidiomycete *Fomitopsis palustris*, oxalic acid is produced via both pathways [19]. On the other hand, in *A. niger*, no GDH activity [18], only OAH activity, was detected [14, 18], as shown in Fig. 1. The enzyme OAH, which has a high molecular weight of approximately 420,000 Da [15], is a multimer of single subunits [22, 23] and localizes in the cytoplasm of *A. niger* [14]. OAH cleaves the C–C bond between the second and third carbons of oxaloacetic acid, resulting in the formation of oxalic acid and acetic acid [14].

Rujiter et al. [24] reported that the mutant strain showing no OAH activity in *A. niger* is a non-oxalic acid-producing

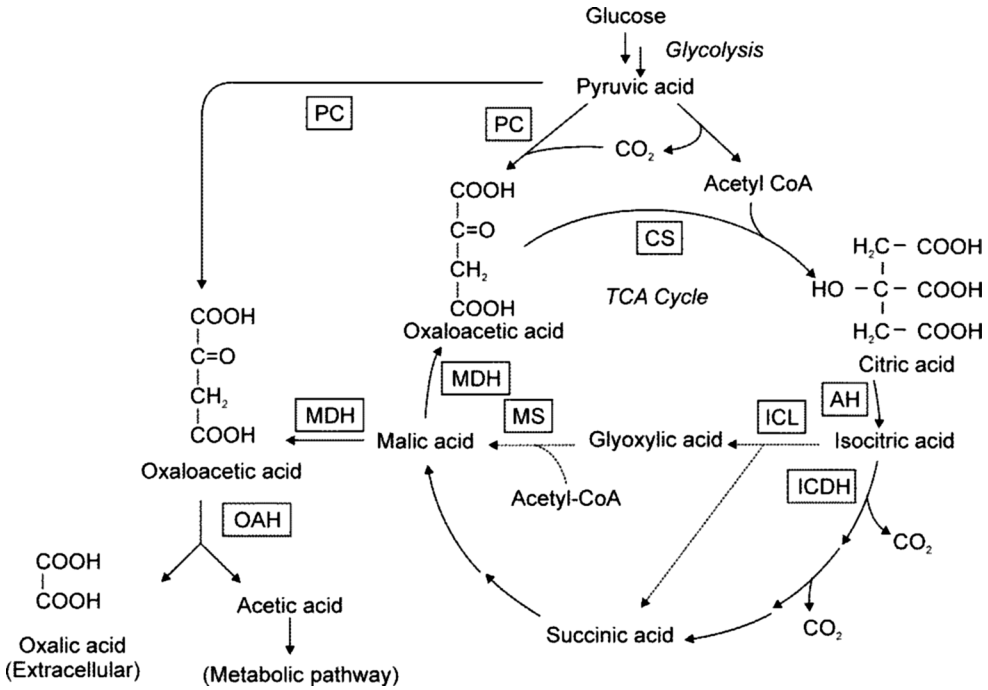

Fig. 1 Metabolic pathway for production of oxalic acid and citric acid in *A. niger*. *AH* aconitate hydratase, *CS* citrate synthase, *ICDH* isocitrate dehydrogenase, *ICL* isocitrate lyase, *MDH* malate dehydrogenase, *MS* malate synthase, *OAH* oxaloacetate hydrolase, *PC* pyruvate carboxylase. Part of the glyoxylate cycle is shown by *dotted arrows*

strain. In α-amylase-producing *A. niger* BO-1, to overcome the problem of oxalic acid formation as a by-product, the gene encoding OAH was cloned and then disrupted; the resulting disruptant strain produced no oxalic acid [22, 23]. These results indicate that the biosynthesis of oxalic acid in *A. niger* strains is only due to the hydrolysis of oxaloacetate by OAH. However, complete genome sequence analysis of *A. niger* CBS 513.88 has revealed the presence of *oahA* homolog genes encoding OAH-like proteins (GenBank Accession Nos. CAK48221, CAK42659). CAK48221 and CAK42659 share the same domains (phosphoenolpyruvate mutase and isocitrate lyase domains) with OAH. In particular, the nucleotide sequence of the gene encoding CAK48221 shows 73 % homology to the gene Pc18g05100, an OAH homolog gene in *Penicillium chrysogenum* [5]. Moreover, since some strains belonging to black *Aspergillus* containing *A. niger* are able to be classified into subgroups [25, 29], distribution of oxalic acid productivity in black *Aspergillus* strains has begun to be studied.

Although pathways for oxalic acid biosynthesis in microorganisms have been investigated, limited reports concerning hyperproducers of oxalic acid have been published [16, 18]. The bioproduction of oxalic acid using immobilized mycelia of *A. niger* under submerged conditions was previously reported [16]. However, the possible high-yield production of oxalic acid by overexpression of the OAH gene in *A. niger* has not been reported thus far.

In this study, we sought to generate oxalic acid hyperproducers by constructing *oahA*-overexpressing strains using citric acid-producing *A. niger* WU-2223L as a host. We succeeded in generating oxalic acid hyperproducers by overexpressing this single gene, i.e., *oahA*, in citric acid-producing *A. niger*.

Materials and methods

Strains and cultivation conditions

Aspergillus niger WU-2223L [7–13, 27], which has already been deposited to NITE (http://www.nite.go.jp/), a hyperproducer of citric acid, and LND-1 [12], i.e., a *niaD* mutant derived from strain WU-2223L, were used. Strain LND-1 shows no growth on plate of Czapek-Dox (CD) medium as a minimal synthetic medium, described later, due to defect of nitrate utilization. LND-1 was used as a host and transformed using the plasmid pPANOAH-1 containing *niaD* gene, as described later.

Conidia of these strains were suspended in 60 ml of a synthetic medium to give a final concentration of 5.0×10^6 conidia/ml and cultivated aerobically with shaking (120 rpm) in shake flasks (500 ml) at 30 °C. The citric acid production test medium (SLZ30 medium) contained the following (per liter of distilled water): glucose, 30 g; $(NH_4)_2SO_4$, 3 g; K_2HPO_4, 1 g; KH_2PO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $MnSO_4 \cdot 5H_2O$, 0.014 g; $FeCl_3 \cdot 6H_2O$, 0.01 g; and $ZnCl_2$, 0.075 mg. The initial pH was adjusted to 3.0. Unless otherwise indicated, 2 % (v/v) methanol was added to promote citric acid production. To determine the oxalic acid production capacity, we used OAP30 medium, composed of SLZ30 medium supplemented with

1 M 2-[*N*-morpholino] ethanesulfonic acid (MES). Before cultivation, the initial pH was adjusted to 7.0 by NaOH, and the pH of OAP30 medium was maintained above 5.8 during the entire cultivation period. To isolate and maintain *A. niger* transformants, we used CD medium, which contained the following (per liter of distilled water): glucose, 30 g; $NaNO_3$, 2 g; K_2HPO_4, 1 g; $MgSO_4{\cdot}7H_2O$, 0.5 g; KCl, 0.5 g; and $FeSO_4{\cdot}7H_2O$, 0.01 g. Additionally, 0.7 M KCl was added as an osmostabilizer for protoplasts. The initial pH was adjusted to 6.0, and, if necessary, 10 g/l agar BA-10 (Ina Food Industry, Nagano, Japan) was supplemented to prepare solidified CD agar plates.

DNA and RNA isolation

After cultivation for appropriate intervals, mycelia were harvested using a glass microfiber filter GF/A and then qualitative filter paper No. 4 (Whatman, Brentford, UK); harvested mycelia were washed twice with pure water on the filter paper. Washed mycelia were frozen in liquid nitrogen and ground into fine powder with a mortar and pestle. Chromosomal DNA was isolated from powdered mycelia using ISOPLANT II (Nippon Gene, Tokyo, Japan) in accordance with the manufacturer's manual. Total RNA was isolated from the mycelial powder by the acid guanidinium-phenol-chloroform method, as described previously [7, 9–11].

Cloning of oxaloacetate hydrolase gene (oahA)

To amplify the gene encoding *oahA* by PCR, chromosomal DNA of *A. niger* WU-2223L was used as the template. Specific primers were designed in accordance with the sequence of the gene encoding OAH reported for *A. niger* N400 (GenBank Accession No. CAD99195). To amplify *oahA*, the Pr1 primer (5′-ATG AAA GTT GAT ACC CCC GAT T-3′) and Pr2 primer (5′-TTA AAC ACC ATT AGC AAA CC-3′) were used. The cDNA fragment of *oahA* was also amplified by reverse-transcription PCR using Pr1 and Pr2 primers including start and stop codons. Reverse-transcription PCR was performed using ReverTra-Plus- (Toyobo, Osaka, Japan) in accordance with the manufacturer's recommended protocols. The flanking region of *oahA* in *A. niger* WU-2223L was amplified as follows. Chromosomal DNA of *A. niger* WU-2223L was digested with *Pst* I (Nippon Gene), and the digested DNA fragment was self-ligated using T4 DNA ligase (Takara Bio, Otsu, Japan) at 16 °C for 16 h. Subsequently, the self-ligated DNA was used as a template for inverse PCR using the Pr3 primer (5′-AAT CAG CAC GAT GTC GCT GC-3′) and Pr4 primer (5′-CGG CTA CGA CGA GTG CAT TC-3′). The resulting 2,546-bp fragment was cloned, sequenced, and used as *oahA* and its flanking regions.

Plasmid construction

We constructed the plasmids pDOAHPTR-1 and pNANOAH-1 for *oahA* disruption and overexpression, respectively, as follows. The genomic DNA fragment of *oahA* and its flanking region (2,546 bp) was digested with *Hinc*II and inserted into the *Hinc*II site of the plasmid pUC19 (Nippon Gene) to construct the plasmid pOAH. Subsequently, pOAH was digested with *Nru*I to delete the 286-bp partial region of *oahA*, and the pyrithiamine resistance gene *ptrA*, amplified from pPTRI (Takara Bio), was inserted into the *Nru*I site of *oahA* to construct the plasmid pDOAHPTR-1. For construction of the plasmid pNANOAH-1, the Pr5 primer (5′-GCG TCG ACA TGA AAG TTG ATA CCC CCG A-3′) and Pr6 primer (5′-GCG CGC ATG CTT AAA CAC CAT TAG CAA ACC-3′), containing *Sal*I and *Sph*I sites, respectively, were used for amplification of the genomic DNA fragment of *oahA*. The amplified fragment of *oahA* was inserted into the *Sal*I and *Sph*I sites of the overexpression plasmid for aspergilli, i.e., pNAN8142 [17], to construct the plasmid pNANOAH-1.

Transformation of *A. niger*

The transformation of *A. niger* WU-2223L and its derivative LND-1 was performed by the protoplast-PEG method, as described previously [12].

Southern and Northern blot analyses

Southern and Northern blot analyses were performed as described previously [11]. The chromosomal DNA fragment encoding *oahA* (1,263 bp), amplified by PCR using primers Pr5 and Pr6, was used as the probe for Southern blot analysis. For Northern blot analysis, the cDNA fragment of *oahA*, amplified by PCR using primers Pr1 and Pr2, was also labeled by random priming with digoxigenin-dUTP (Roche Diagnostics, Basel, Switzerland) and used as the *oahA*-specific probe. The cDNA fragments of the genes encoding CAK48221 and CAK 42659 homolog proteins, which were amplified by PCR using primers Pr7 (5′-CTC GAG AAC CCC GAT TCC-3′), Pr8 (5′-TCG CGC ATC GCT GCA ACG GC-3′), Pr9 (5′-GGT TGT CAC GCT GAA CGA A-3′), and Pr10 (5′-ACG AAC CGC CGA GTA GAC-3′), were also labeled. The cDNA fragment encoding the cytoskeleton protein actin, i.e., *act1*, from *A. niger* WU-2223L was labeled and used as an internal standard. Northern blot analysis was carried out with a Scion Image system (Scion, Frederick, MD, USA).

Preparation of cell-free extracts and enzymatic analysis

Cell-free extracts of *A. niger* strains were prepared as follows and used for the enzymatic analysis of OAH and GDH. Mycelia were harvested using qualitative filter paper No. 4 and washed twice with pure water on the filter. Washed mycelia were frozen in liquid nitrogen and ground into fine powder with a mortar and pestle. The mycelial powder was suspended in 20 mM Tris–HCl buffer (pH 8.0) containing 0.1 mM $MnCl_2$, 50 mM 2-mercaptoethanol, and 7 % (w/v) sucrose. Residual mycelia were removed by centrifugation (15,000 × *g*, 4 °C, 20 min) to prepare cell-free extracts. Protein concentrations in these cell-free extracts were determined using a Coomassie protein assay reagent (Pierce Co., Ltd., IL, USA) with bovine serum albumin as the standard. OAH activity was measured in accordance with the method of Lenz et al. [15], and GDH activity was measured in accordance with the method of Tokimatsu et al. [26] using a U-3010 spectrophotometer (Hitachi, Tokyo, Japan).

Determination of glucose, citric acid, and oxalic acid

The culture filtrate was recovered by filtering the culture broth through a glass microfiber filter GF/A and qualitative filter paper No. 4. Citric acid and oxalic acid in the culture filtrate were determined using an HPLC LC-20 system (Shimadzu, Kyoto, Japan) equipped with a Shimpack SCR-101H column (Shimadzu). For quantification, the refractive index detector RID-10A and the photodiode array detector SPD-M20A at 210 nm (Shimadzu) were used; 4 mM $HClO_4$ was used as the mobile phase. Residual mycelia on the filter paper were dried, and mycelial dry weight was determined as described previously [8].

Results

Cloning and nucleotide sequence analysis of oahA

The chromosomal DNA fragment of *oahA* and its flanking region (2,546 bp) was cloned from *A. niger* WU-2223L by PCR. The cDNA of *oahA* (1,026 bp) was also cloned from *A. niger* WU-2223L by reverse-transcription PCR. The nucleotide sequences of chromosomal *oahA* and cDNA were determined and analyzed (GenBank Accession No. AB723988). The *oahA* gene of *A. niger* WU-2223L occupied the 1,263-bp region containing two introns (105 and 132 bp) and encodes a protein consisting of 341 amino acids. In the upstream region of *oahA*, some putative transcriptional factors, including TATA boxes (−25 and −147 bp) and binding sites of PacC (−292 and −371 bp) and CreA (−339 bp), are located. Southern blot analysis confirmed that there was only one copy of *oahA* on the *A. niger* WU-2223L chromosome. The nucleotide sequence of *oahA* derived from *A. niger* WU-2223L showed 96, 99, and 98 % homology to those of annotated OAH genes reported for *A. niger* N400 (GenBank Accession No. CAD99195), CBS 513.88 [20], and ATCC 1015 (http://genome.jgi-psf.org/) [1], respectively. The upstream region from the start codon to −621 bp of *oahA* showed 87 % homology at the nucleotide level to that of *A. niger* N400, and the binding site of the pH-responsive regulatory factor PacC existed in the *oah* genes of both strains.

Construction of *A. niger* transformant strains

The *oahA* disruptants derived from *A. niger* WU-2223L were constructed using pDOAHPTR-1 by the protoplast-PEG method [12]. Three hundred transformants were isolated on CD medium containing 100 μg/ml pyrithiamine as

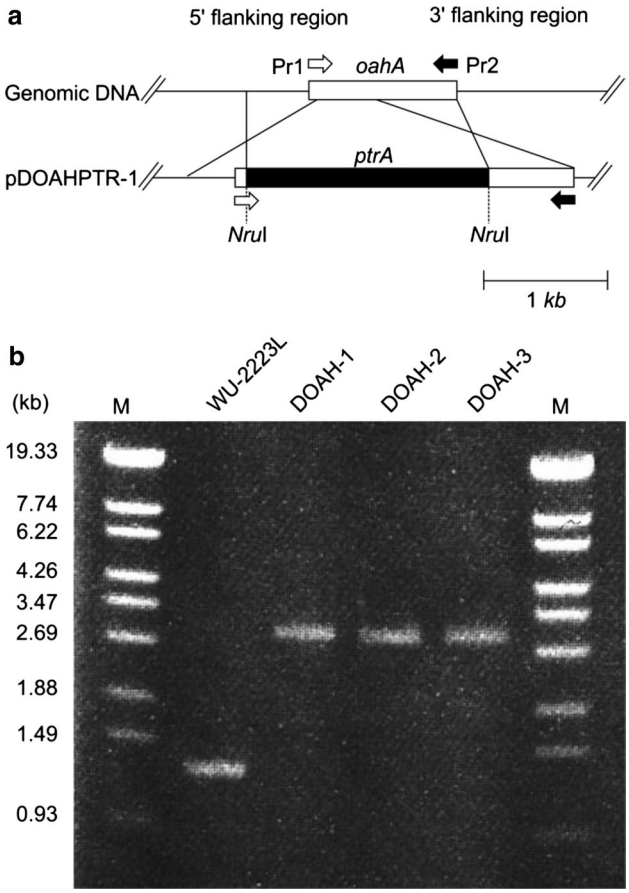

Fig. 2 Disruption of *oahA* in *A. niger* WU-2223L. **a** Schematic representation of the disruption of *oahA* through homologous recombination by transformation with pDOAHPTR-1. **b** Genomic PCR products for confirmation of *oahA* disruption using the primers indicated in (**a**). Note that the band patterns of the *oahA* disruptants (DOAH-1, DOAH-2, and DOAH-3) are different from those of WU-2223L, reflecting the insertion of *ptrA* into *oahA*. *Lane M*, λ/*Sty*I digest

the selection marker. The disruption of *oahA* was confirmed by PCR using Pr1 and Pr2 primers in three of the 300 transformants (Fig. 2). Among these three transformants, one strain termed DOAH-1 was used as the representative disruptant in subsequent analyses.

Several *oahA*-overexpressing strains derived from *A. niger* LND-1, a *niaD* mutant derived from WU-2223L, were also constructed using pNANOAH-1. Thirty transformants showing nitrate utilization were isolated on CD medium. Southern blot analysis using *oahA* as the probe allowed for selection and isolation of three strains in which only a single copy of pNANOAH-1 was introduced into each chromosome (data not shown). Among these three strains, one strain termed EOAH-1 was selected and used as the representative *oahA*-overexpressing strain, as will be described later.

Transcription of oahA and activities of OAH and GDH

Northern blot analysis was performed for WU-2223L, DOAH-1, and EOAH-1 in order to analyze the mRNA levels of *oahA*. As shown in Fig. 3a, in EOAH-1 cultivated in OAP30 medium, the mRNA level of *oahA* was 3.04 times greater than that in WU-2223L. In contrast, transcription of *oahA* completely disappeared in DOAH-1 cultivated in both media (Fig. 3a, b). When cultivated in SLZ30 (pH 3.0) medium, the transcription of *oahA* in *A. niger* WU-2223L was not detected (Fig. 3b).

Next, the specific activities of OAH and GDH were measured using cell-free extracts of WU-2223L, DOAH-1, and EOAH-1 cultivated in SLZ30 or OAP30 medium for 7 days. As shown in Table 1, no specific activity of OAH was detected in DOAH-1 due to disruption of *oahA*. Under cultivation conditions with OAP30 medium, the maximum specific activity of OAH in EOAH-1 was 2.47-fold greater (1.84×10^4 U/mg) than that of WU-2223L (7.44×10^3 U/mg). No GDH activity was detected in either strain cultivated in OAP30 medium (Table 1). These results indicate that oxalic acid is produced by OAH, but not GDH, in the strains WU-2223L and EOAH-1.

Transcription of *oahA* homolog genes

Complete genome sequence analysis of *A. niger* CBS 513.88 revealed the presence of *oahA* homolog genes encoding OAH-like proteins (GenBank Accession Nos.

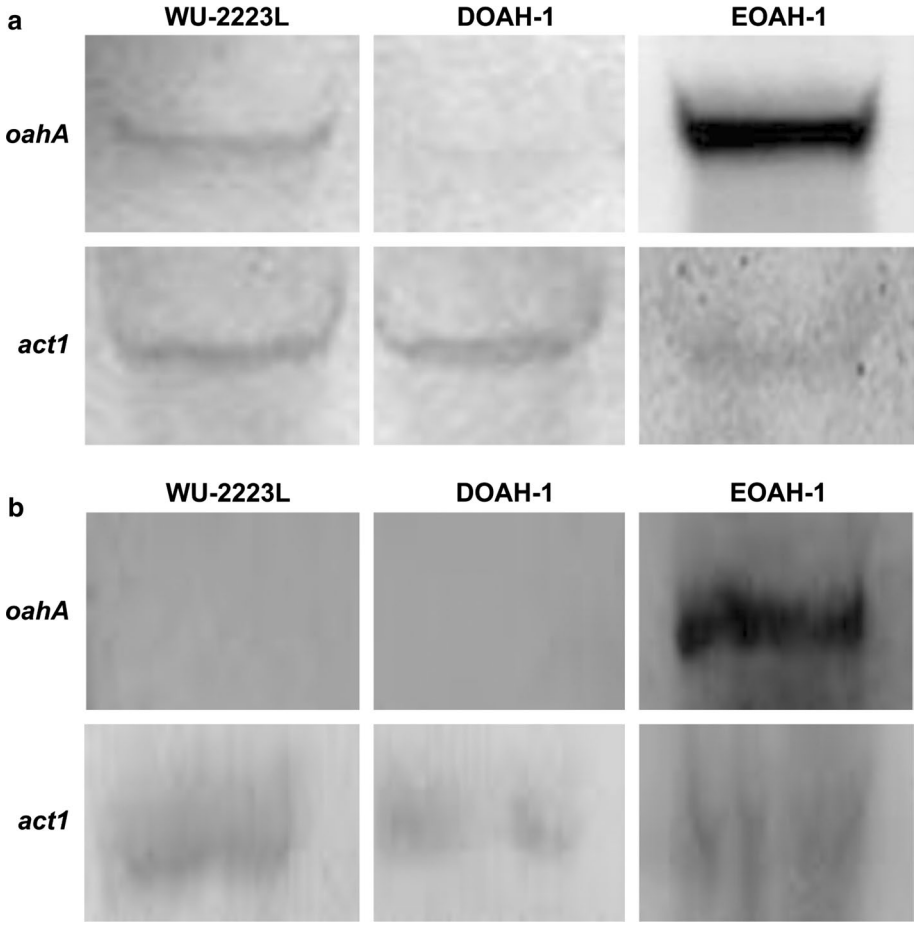

Fig. 3 Northern blot analysis of mRNAs from the mycelia obtained by 7-day cultivation under the conditions for oxalic acid production (**a**) and citric acid production (**b**). The OAH gene (*oahA*) and actin gene (*act1*) were used as specific probes. **a** Relative mRNA levels (*oahA*/*act1*) are shown. Data were quantified as 1.12 (1.00), 0, and 3.41 (3.04) for WU-2223L, DOAH-1, and EOAH-1, respectively, where the levels relative to that of WU-2223L are given in *parentheses*. **b** Relative *oahA* mRNA level for EOAH-1 (3.02). In WU-2223L and DOAH-1, *oahA* mRNA was not detected

Table 1 OAH and GDH activities of *A. niger* strains

Strain	Specific activity			
	OAH		GDH	
	U/mg protein	Relative	U/mg protein	Relative
WU-2223L	7.44×10^3	1.00	0	–
DOAH-1	0	0	0	–
EOAH-1	1.84×10^4	2.47	0	–

The OAH activity of mycelia obtained by 7-day cultivation in OAP30 medium was measured. One unit (U) is defined as the amount of enzyme that hydrolyzes 1 μmol of oxaloacetic acid per minute

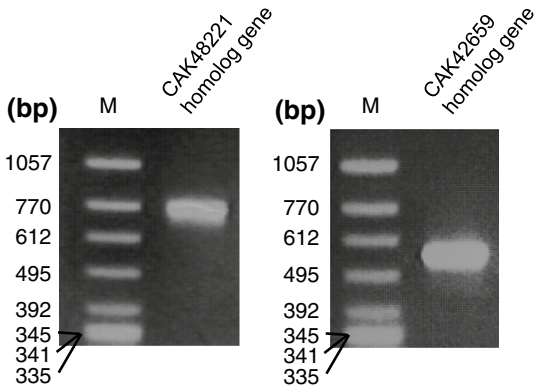

Fig. 4 Genomic PCR products of *A. niger* WU-2223L using specific primers targeting the genes encoding CAK48221 and CAK 42659 homolog proteins. *Lane M*, ϕX174/*Hin*cII digest

CAK48221, CAK42659). CAK48221 and CAK42659 have the same domains (phosphoenolpyruvate mutase and isocitrate lyase domains) as *oahA* and show 60 and 52 % homologies at the amino acid residue level to *oahA*, respectively. We confirmed the presence of these open reading frames (ORFs) on the chromosome of *A. niger* WU-2223L by PCR using the specific primers Pr7/Pr8 and Pr9/Pr10 (Fig. 4). Sequence analysis revealed that these PCR amplification products showed high homologies to the corresponding regions of CAK48221 and CAK42659 (data not shown). However, no transcripts from these ORFs were detected by Northern blot analysis in WU-2223L or DOAH-1 cultivated in OAP30 medium (data not shown), indicating that no OAH-like protein was produced by *A. niger* WU-2223L or DOAH-1 under the cultivation conditions tested.

Oxalic acid production capacity by transformants

To examine the oxalic acid production capacity of these strains, DOAH-1 and EOAH-1 were cultivated in OAP30 medium for 12 days. As shown in Fig. 5, the amount of oxalic acid produced by EOAH-1 increased from the 3rd day to the 9th day of cultivation and reached 28.9 g/l on the

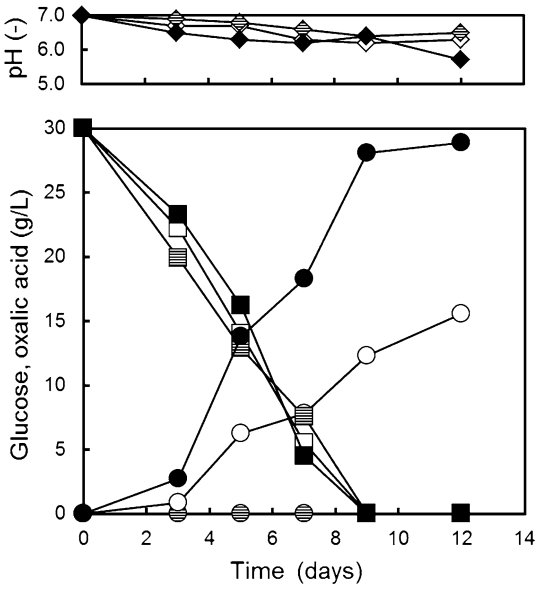

Fig. 5 Time courses of oxalic acid production in OAP30 medium. Symbols: *empty square*, glucose consumption by *A. niger* WU-2223L; *shaded square*, glucose consumption by DOAH-1; *closed square*, glucose consumption by EOAH-1; *empty circle*, oxalic acid production by *A. niger* WU-2223L; *shaded circle*, oxalic acid production by DOAH-1; *closed circle*, oxalic acid production by EOAH-1; *empty diamond*, pH of medium for *A. niger* WU-2223L; *shaded diamond*, pH of medium for DOAH-1; *closed diamond*, pH of medium for EOAH-1

12th day. Finally, the yield for EOAH-1 based on glucose consumed was 96.3 % (w/w), which was 1.85-fold greater than that for WU-2223L. However, during the entire 12-day cultivation period, no oxalic acid was detected in the culture filtrate of DOAH-1, regardless of glucose consumption. Interestingly, during the cultivation period, the maximum amount of citric acid produced by DOAH-1 (3.03 g/l) was somewhat higher than those of EOHA-1 (1.60 g/l) and WU-2223L (1.90 g/l), and no other organic acid containing acetic acid was detected by HPLC analysis of the culture filtrate (data not shown). In addition, to examine the oxalic acid production capacity in detail, oxalic acid production tests were carried out under various conditions, including conditions with a high concentration of glucose (120 g/l) or 0.3 g/l yeast extract for mycelial growth. At each cultivation day, the amount of oxalic acid produced by EOAH-1 exceeded that by *A. niger* WU-2223L (data not shown). Additionally, no oxalic acid was detected in the culture filtrate of DOAH-1 for all samples tested.

Discussion

In this study, we sought to investigate the production of oxalic acid, which is used as a chelator, detergent, and

tanning agent, using *A. niger* strains as hosts. By overexpressing the *oahA* gene in *A. niger* WU-2223L, we succeeded in developing a hyperproducer of oxalic acid. Thus, our data have important implications in the industrial production of oxalic acid.

Cloning of the 2,546-bp fragment including the *oahA* gene and its flanking region from *A. niger* WU-2223L revealed the presence of putative binding sites of PacC and CreA in the upstream region of *oahA*. These motifs were also found in the *oahA* genes of *A. niger* N400 (GenBank Accession No. CAD99195) and *A. niger* CBS513.88 (GenBank Accession No. XP_001402473). Therefore, it seems likely that the transcription of the *oah* gene in *A. niger* is regulated by pH or the carbon source. Indeed, the transcription level of *oahA* in *A. niger* WU-2223L cultivated in OAP30 medium (pH 7.0) was greater than that in SLZ30 medium (pH 3.0). Generally, for oxalic acid production by *A. niger*, the initial pH of cultivation conditions must be around 7.0 [13, 15, 18, 27]. Therefore, in this study, to evaluate oxalic acid production by *A. niger* strains, OAP30 medium with an initial pH of 7.0 was used; this medium was a modified SLZ30 medium prepared by supplementation with 1 M MES as a buffering substance to adjust the pH of the culture broth to 5.8–7.0.

The mRNA transcripts of the two *oahA* homolog genes were not detected by Northern blot or RT-PCR analyses in *A. niger* WU-2223L (data not shown). Moreover, as shown in Fig. 3, only *oahA* mRNA transcript was detected by Northern blot analysis. Furthermore, OAH activity completely disappeared with the disruption of a single gene, i.e., *oahA* (Table 1). These results clearly indicated that *oahA* was the only gene encoding OAH, consistent with the reports of Pedersen et al. [23] and Ruijter et al. [24].

The amount of citric acid produced by the *oahA* disruptant DOAH-1 in OAP30 medium (3.03 g/l) increased in comparison to that produced by WU-2223L (1.90 g/l), similar to the results observed for *A. niger* N400 [24]. Since oxaloacetic acid is a precursor of citric acid synthesized by citrate synthase in the mitochondria [13], citric acid production may be increased in response to defects in OAH in the cytosol due to accumulation of oxaloacetic acid.

The amount of oxalic acid produced by the *oahA*-overexpressing strain EOAH-1 in OAP30 medium increased compared with that produced by WU-2223L during the whole cultivation period and reached 1.85-fold that produced by WU-2223L (28.9 g/l) on the 12th day; the final yield based on glucose consumed was 96.3 % (w/w). This increase in oxalic acid production was caused by the high activity of OAH throughout the cultivation period. Levels of microbial oxalic acid production have been examined by several researchers [16, 19]. In the oxalic acid-producing basidiomycete *F. palustris*, the amount of oxalic acid produced from 10.5 g/l glucose was 7.0 g/l for a 13-day cultivation [19]. For immobilized *A. niger* NCIM 548, the maximum amount of oxalic acid produced was 20.6 g/l [16]. In this study, the amount of oxalic acid produced by EOAH-1 reached 28.9 g/l, the highest value reported to date.

Generally, in eukaryotic cells, OAH converts oxaloacetic acid into oxalic acid and acetic acid in cytosol. However, in WU-2223L and EOAH-1, the *oahA*-overexpressing strain, no acetic acid was detected in the culture filtrate or extracts from mycelia as the intracellular fraction (data not shown). Therefore, we concluded that acetic acid generated by the hydrolysis of oxaloacetic acid by OAH is rapidly converted to other metabolite(s), such as acetyl-CoA, and recycled for metabolism and that only oxalic acid is secreted into the culture broth. Acetic acid produced from oxaloacetic acid by OAH appeared to be recycled and converted to oxalic acid, and the following equation (Eq. 1) could be derived for the overall conversion of glucose ($C_6H_{12}O_6$) to oxalic acid [$(COOH)_2$] in *A. niger*.

$$C_6H_{12}O_6 + \frac{9}{2}O_2 \rightarrow 3\,(COOH)_2 + 3\,H_2O \quad (1)$$

Equation (1) shows that 1 mol of glucose (180 g) is converted to 3 mol of oxalic acid (270 g). Therefore, the maximum theoretical yield of oxalic acid, based on Eq. (1) is 150 % (w/w), and the oxalic acid yield of EOAH-1 in this study reached 64.2 % of the maximum theoretical yield.

In summary, we succeeded in generating an oxalic acid hyperproducer, EOAH-1, by metabolic engineering through overexpression of a single gene, *oahA*, in citric acid-producing *A. niger* WU-2223L. Although a bioprocess for oxalic acid production has not been designed to date, oxalic acid hyperproducers such as EOAH-1 will be useful for oxalic acid production from renewable raw materials.

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