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A biosynthetic pathway for hexanoic acid production in *Kluyveromyces marxianus*

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ABSTRACT

Hexanoic acid can be used for diverse industrial applications and is a precursor for fine chemistry. Although some natural microorganisms have been screened and evolved to produce hexanoic acid, the construction of an engineered biosynthetic pathway for producing hexanoic acid in yeast has not been reported. Here we constructed hexanoic acid pathways in *Kluyveromyces marxianus* by integrating 5 combinations of seven genes (*AtoB*, *BktB*, *Crt*, *Hbd*, *MCT1*, *Ter*, and *TES1*), by which random chromosomal sites of the strain are overwritten by the new genes from bacteria and yeast. One recombinant strain, H4A, which contained *AtoB*, *BktB*, *Crt*, *Hbd*, and *Ter*, produced 154 mg/L of hexanoic acid from galactose as the sole substrate. However, the hexanoic acid produced by the H4A strain was re-assimilated during the fermentation due to the reverse activity of *AtoB*, which condenses two acetyl-CoAs into a single acetoacetyl-CoA. This product instability could be overcome by the replacement of *AtoB* with a malonyl CoA-acyl carrier protein transacylase (*MCT1*) from *Saccharomyces cerevisiae*. Our results suggest that *Mct1* provides a slow but stable acetyl-CoA chain elongation pathway, whereas the *AtoB*-mediated route is fast but unstable. In conclusion, hexanoic acid was produced for the first time in yeast by the construction of chain elongation pathways comprising 5–7 genes in *K. marxianus*.

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1. Introduction

The biological production of chemicals or fuels from renewable bioresources is an attractive approach to a sustainable future. Renewable bioresources have been converted into alcohols, alkanes, and fatty acid ester by biological processing. Hexanoic acid (caproic acid), a six-carbon saturated fatty acid, is an oily light, colorless, or yellow liquid with an acrid odor. It has been used in diverse industrial applications such as perfumes, medicine, food additives, lubricating grease, tobacco flavor, rubber, and dyes. Hexanoic acid is also used as a precursor for the biosynthesis of hexyl esters, which can be converted to hexanol by esterification and hydrogenation reaction (Verser and Park, 2012).

Although hexanoic acid is often found in oils and animal fats, several natural anaerobic bacteria are also known to produce it. *Clostridium kluyveri* produced hexanoic acid from ethanol upon growing with methane-producing bacterium (Barker and Beck, 1942). The methanol-utilizing bacterium *Eubacterium limosum* produced acetate, butyrate, and hexanoic acid from methanol (Genthner et al., 1981). *Megasphaera elsdenii* produced n-butyric acid and hexanoic acid on various substrates such as glucose, maltose, and lactate (Marounek et al., 1989). Recently, hexanoic acid producing *Clostridium* sp. was screened and an efficient extractive process was established (Jeon et al., 2010, 2013). However, strict anaerobic fermentation is usually not preferred for industrial-scale productions since it requires a long fermentation time.

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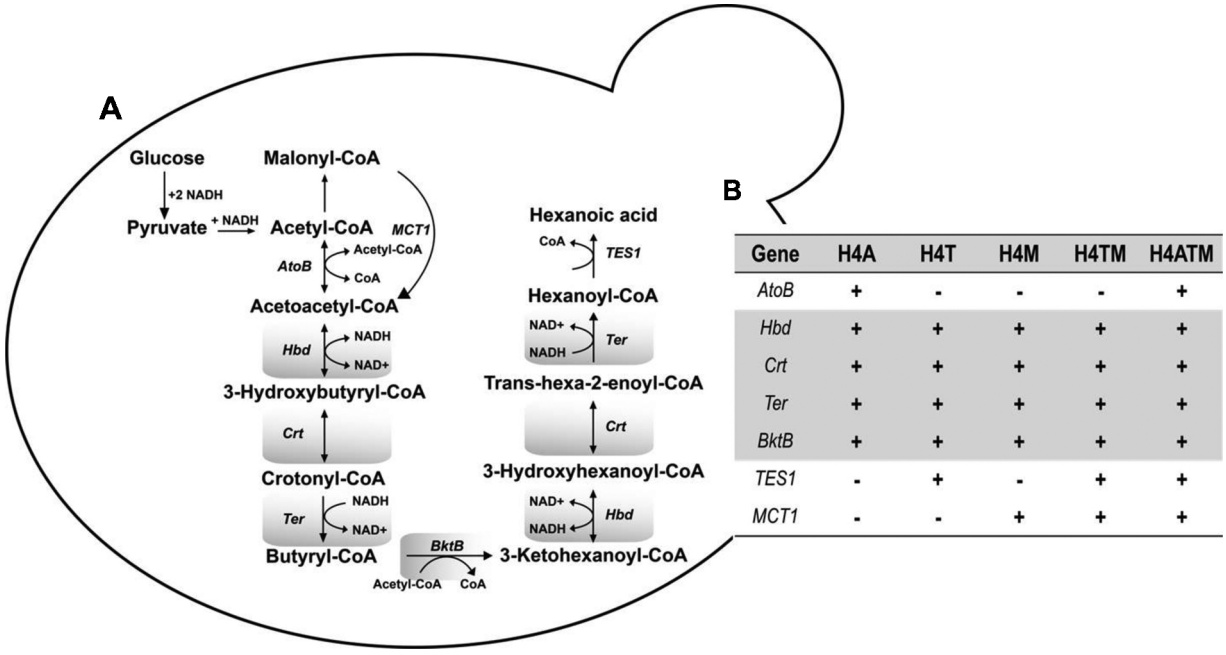

Gene	H4A	H4T	H4M	H4TM	H4ATM
AtoB	+	-	-	-	+
Hbd	+	+	+	+	+
Crt	+	+	+	+	+
Ter	+	+	+	+	+
BktB	+	+	+	+	+
TES1	-	+	-	+	+
MCT1	-	-	+	+	+

Fig. 1. Schematic of the hexanoic acid production in engineered *Kluyveromyces marxianus*. (A) Metabolic pathway constructed in this study. Shaded enzymes were commonly included in all 5 recombinant strains. (B) Recombinant strains constructed in this study.

Kluyveromyces marxianus is a yeast strain that is commonly used for the production of biofuel or valuable chemicals such as ethanol, glycerol, ethyl acetate, or lactic acid from a renewable resources (Lark et al., 1997; Loser et al., 2013; Plessas et al., 2008; Rapin et al., 1994). A thermotolerant yeast, *K. marxianus* has several advantages over mesophilic yeasts in practical aspects. For example, at high temperature, cooling cost can be reduced, enzymatic hydrolysis can be improved during simultaneous saccharification and fermentation (SSF), cell growth rate can be increased, and contamination issues can be minimized (Limtong et al., 2007). In addition, *K. marxianus* is capable of utilizing various substrates and is more acid-resistant than other yeast strains (Fonseca et al., 2008). Despite these advantages, the use of this strain for producing value-added products has been scarce, perhaps due to limited genetic information and engineering tools. To better utilize the advantages of this strain, the entire genome of *K. marxianus* KCTC 17555 was recently sequenced (Jeong et al., 2012) and various genetic engineering tools were developed (Lee et al., 2013; Pecota et al., 2007). Furthermore, an efficient gene transformation technique was developed that enabled the simultaneous integration of multiple genes into *K. marxianus* chromosomes (Heo et al., 2013).

In the present study, we constructed a synthetic pathways comprising 5–7 genes and integrated them into the chromosome of a *K. marxianus* strain for the production of hexanoic acid. We initially modified the 1-hexanol producing pathway which was is extended from the 1-butanol synthesis pathway (Dekishima et al., 2011; Machado et al., 2012; Zhang et al., 2008). However, hexanoic acid production was very unstable through this pathway. So we employed another chain elongation route for the stable hexanoic acid production. In this pathway, hexanoic acid synthesis starts from acetyl-CoA and is catalyzed by acetyl-CoA acetyltransferase (*AtoB*) from *Escherichia coli*, β-ketothiolase (*BktB*) from *Ralstonia eutropha*, 3-hydroxybutyryl-CoA dehydrogenase (*Hbd*) and crotonase (*Crt*) from *Clostridium acetobutylicum*, trans-enoyl-CoA reductase (*Ter*) from *Treponema denticola*, *MCT1* from *Saccharomyces cerevisiae*, and *TES1* from *K. marxianus* (Fig. 1). This report not only provides a novel chain elongation enzyme but also evaluates *K. marxianus* as the promising host for complicated metabolic engineering.

2. Materials and methods

2.1. Strains and plasmid constructions

All strains used in this study are described in Table 1. The KM5ΔURA3 strain, a *URA3*-deleted mutant of *K. marxianus* ATCC 17555, was used as the host strain for engineering (Jeong et al., 2012). The pJSKM316GPD vector was used for gene expression in *K. marxianus* under the control of the *GPD* promoter and the *CYC* terminator (Lee et al., 2013). Each DNA fragment (*AtoB* from *E. coli*, *BktB* from *R. eutropha*, *Hbd* and *Crt* from *C. acetobutylicum*, *Ter* from *T. denticola*, *MCT1* from *S. cerevisiae*, and *TES1* from *K. marxianus*) was amplified using the primers (Supplementary Table S1) and individually ligated into pJSKM316GPD. *E. coli* DH5 (*F–recA1 endA1 hsdR17* [*rK−mK+*] *supE44 thi-1 gyrA relA1*) (Invitrogen, Gaithersburg, MD, USA) was used for gene cloning and manipulation. Restriction enzymes and DNA-modifying enzymes were obtained from New England BioLabs (Beverly, MA, USA). Plasmid purification kits and other reagents were obtained from Promega (Madison, Wl, USA) and Elpis Biotech (Daejeon, Korea).

Table 1
Kluyveromyces marxianus strains used in this study.

Strains	Description/relevant genotype	Reference/source
KCTC17555 (KM5)	*K. marxianus var. marxianus* (KCTC 17555)	Jeong et al. (2012)
KM5ΔURA3	KCTC 17555 Δ*kmURA3*	Lee et al. (2013)
H4A	KM5::*AtoB, Crt, Hbd, Ter, BktB*	This study
H4TM	KM5::*MCT1, Crt, Hbd, Ter, BktB, TES1*	This study
H4M	KM5::*MCT1, Crt, Hbd, Ter, BktB*	This study
H4T	KM5::*Crt, Hbd, Ter, BktB, TES1*	This study
H4ATM	KM5::*AtoB, MCT1,Crt, Hbd, Ter, BktB, TES1*	This study

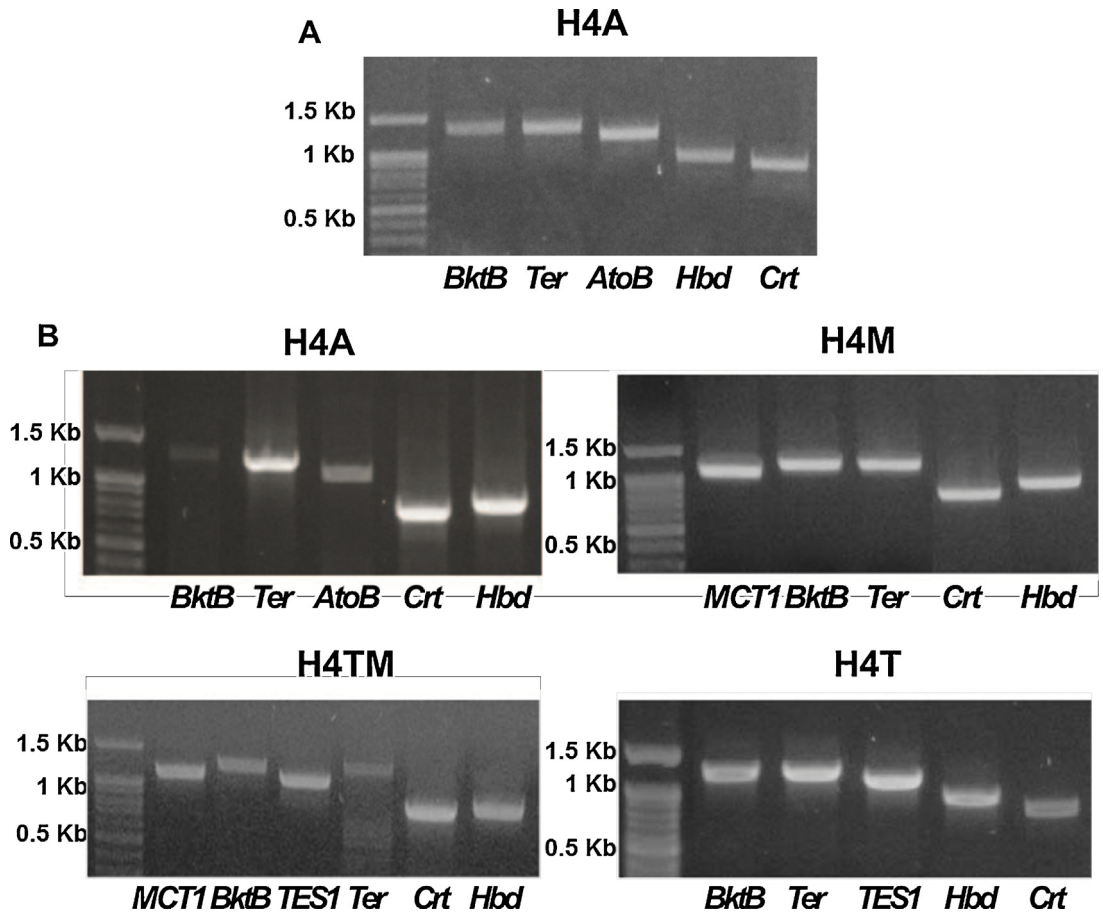

Fig. 2. Stable and functional integration of all genes. (A) The integrated genes were stably maintained. After three successive cultures of the H4A strain, the chromosome was isolated and the presence of all five genes (*AtoB*, *BktB*, *Crt*, *Hbd* and *Ter*) was confirmed by polymerase chain reaction (PCR). (B) All integrated genes were functionally expressed. After constructing four recombinant strains, the expression of the integrated genes were confirmed through real-time PCR of the mRNA extracted from each strain.

2.2. Constructions of hexanoic acid-producing K. marxianus strains

For the simultaneous integration of 5–7 genes, we followed the method described previously (Heo et al., 2013). Briefly, DNA fragments containing each of the genes (*AtoB*, *Crt*, *Hbd*, *Ter*, *BktB*, *MCT1*, or *TES1*) were amplified from pJS316GPD-derived vectors by polymerase chain reaction (PCR) using specific primers (*URA3* 5′-GCAGGAAACGAAGATAAATC and *CYC* 3′-GTGGCGGCCGCAACAGCTATGACCATGATTA). Each fragment spans the *URA3* gene, *GPD* promoter, the gene of interest and *CYC* terminator. After amplifications, the designated combinations of DNA mixtures were transformed into the *K. marxianus* KM5ΔURA3 strain by an electroporation method using a BioRad Gene Pulser (Bio-Rad Laboratories, Hercules, CA, USA). Transformants were selected on yeast synthetic complete (YSC) medium using a *URA3* auxotrophic marker. Amino acids and nucleotides were added when necessary.

2.3. Confirmations of multiple gene integrations and expression of each gene

Simultaneous multiple genes integration was confirmed by PCR using the primers specified (Table S1), and the functional expression of each gene was confirmed by reverse transcription PCR (RT-PCR). PCR was performed using HiPi DNA Polymerase Premix (Elpis Bio, Daejeon, Korea) with the specified primers (Table S1) according to manufacturer's instructions. Total RNA was extracted from each transformant using an Easy-RED™ Total RNA Extraction Kit (Intron Biotechnology, Daejeon, Korea) according to the manufacturer's instructions. Next, cDNA was synthesized from 1 μg of total RNA using a GoScript™ Reverse Transcription system (Promega) and used as a template for RT-PCR with the specified primers (Table S1).

2.4. Medium and fermentation conditions

E. coli was grown in Luria-Bertani medium at 37 °C and 50 μg/mL of ampicillin was added to the medium when required. *K. marxianus* strains were cultivated at 37 °C in YP medium (10 g/L yeast extract and 20 g/L Bacto peptone) with 20 g/L glucose or galactose. For the fermentation experiments, cells at the mid-exponential phase from YP medium containing glucose were harvested and inoculated after washing twice with sterilized water. All of the flask fermentation experiments were performed using 50 mL of YP medium containing 20 g/L of glucose or galactose in a 125 mL flask at 37 °C with an initial OD_{600} of approximately 0.05. All flask fermentation experiments were independently repeated twice.

2.5. Analytical methods

Cell growth was monitored at OD_{600} using an ultraviolet–visible spectrophotometer (Thermo Scientific, Madison, WI, USA). Glucose and galactose concentrations were determined by dinitrosalicylic acid methods (Miller, 1959). Hexanoic acid, butanol, and ethanol were quantified by gas chromatography on a YL GC 6100 system

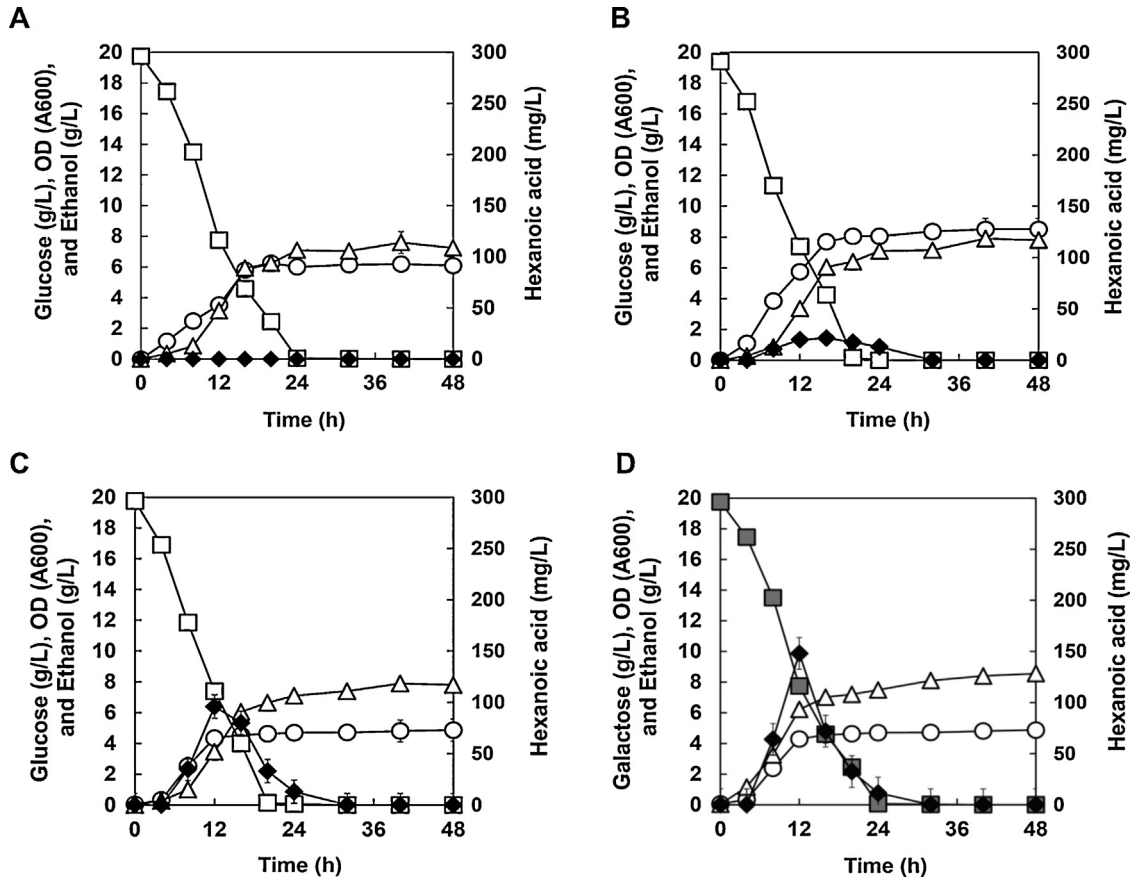

Fig. 3. Comparisons of hexanoic acid production by the engineered *K. marxianus* H4A strain under various sugar or aeration conditions. (A) The parental strain in high aerobic condition with 20 g/L glucose, (B) the H4A strain in high aerobic condition with 20 g/L glucose, (C) the H4A strain in micro-aerobic condition with 20 g/L glucose, and (D) the H4A strain in micro-aerobic condition with 20 g/L galactose. Symbols: glucose (□), galactose (■), OD (○), ethanol (△), hexanoic acid (♦). Full fermentation profiles including butyric acid and butanol are shown in Fig. S2.

equipped with a flame ionization detector (Younglin, Anyang, Korea) and DB-FFAP capillary column (Agilent, Santa Clara, CA, USA). Helium was used as a carrier gas. The injector temperature was 300 °C and the oven temperature was programed as follows: hold at 100 °C for 5 min, increase to 250 °C (10 °C/min), and hold at 250 °C for 1 min.

3. Results and discussion

3.1. Construction of a hexanoic acid-producing K. marxianus strain

To construct pathways for producing hexanoic acid (Fig. 1A), seven genes from various origins were cloned: acetyl-CoA acetyltransferase (*AtoB*) from *E. coli*, β-ketothiolase (*BktB*) from *R. eutropha*, 3-hydroxybutyryl-CoA dehydrogenase (*Hbd*), crotonase (*Crt*) from *C. acetobutylicum*, trans-enoyl-CoA reductase (*Ter*) from *T. denticola*, malonyl CoA-acyl carrier protein transacylase (*MCT1*) from *S. cerevisiae*, and acyl-CoA thioesterase (*TES1*) from *K. marxianus*. The seven sub-cloned genes were then transferred into the pJS316GPD vector, which constitutively expresses the cloned gene under the control of the strong GPD promoter (Lee et al., 2013).

To prepare the DNA fragments for transformation, the region encompassing the *URA3* selection marker, a *GPD* promoter, one of the seven structural genes, and a *CYC* terminator were amplified from pJS316GPD-derived vectors by PCR. The PCR-amplified DNA fragments were combined together for constructing 5 hexanoic acid producing pathways (H4A, H4T, H4M, H4TM and H4ATM) (Fig. 1B) and introduced into the KM5ΔURA3 strain according to the simultaneous multiple gene integration method (Heo et al., 2013). The resulting transformants were selected on YSC agar plate lacking uracil. The auxotrophic phenotype was confirmed again by cultivating on YSC agar media lacking uracil (Fig. S1).

Although the cells that uptake only a single gene can grow on the uracil-lacking media because all seven fragments contain the same *URA3* gene as a selection marker, about half of the colonies contained all of the transformed genes, perhaps due to the strong non-homologous end joining activity of *K. marxianus* (Heo et al., 2013). This strikingly high probability of simultaneous integration of 5–7 genes allows us to test the feasibility of employing this non-conventional yeast as the host of complicated metabolic engineering demanding many heterologous genes. The genomic DNA of H4A strain was isolated after three successive cultures and the preservation of the five integrated genes were confirmed by PCR (Fig. 2A). The integrated genes were not only stably maintained in the chromosome of *K. marxianus* but were also functionally expressed in the cell (Fig. 2B).

3.2. Hexanoic acid productions by the engineered K. marxianus H4A

First, we constructed the H4A strain according to the procedures described above. The pathway constructed in *K. marxianus* is essentially the same as those employed to produce 1-hexanol in *E. coli* (Dekishima et al., 2011) except that *adhE2* was not introduced into *K. marxianus*. The final conversion of hexanoic acid from

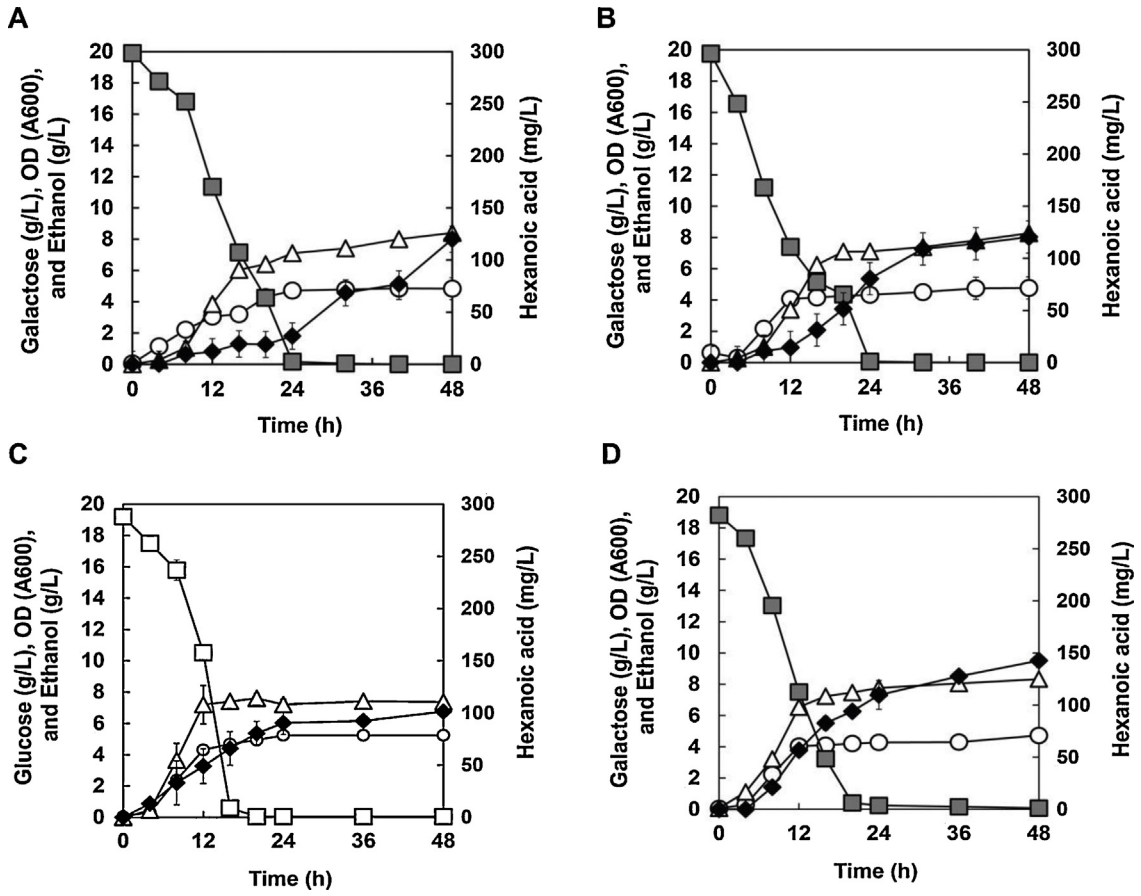

Fig. 4. Stable hexanoic acid production through replacing *AtoB* with *MCT1*. Fermentation profiles of the (A) H4T and (B) H4M strains in the presence of 20 g/L galactose. Fermentation profile of the H4TM strain in the presence of 20 g/L (C) glucose and (D) galactose. Symbols: glucose (□), galactose (■), OD (○), ethanol (△), hexanoic acid (⧫). Full fermentation profiles including butyric acid and butanol are shown in Fig. S3.

hexanoyl-CoA was not catalyzed by a recombinant enzyme but was mediated by the intrinsic cellular activity. The H4A strain contains *BktB*, *Crt*, *Hbd*, *Ter*, and *AtoB*. Two acetyl-CoAs are condensed to an acetoacetyl-CoA by *AtoB*. Fermentation experiments were performed in YP media containing 20 g/L glucose using the parental strain (KM5ΔURA) and the H4A strain. As expected, the H4A strain produced hexanoic acid (23 mg/L; Fig. 3B), whereas the parental strain did not (Fig. 3A). This result verified again that the genes for a biosynthetic pathway for hexanoic acid were integrated properly and expressed functionally in the engineered *K. marxianus* H4A strain. But, the hexanoic acid titer was low.

Next, the agitation speed of the shaking incubator was reduced from 250 rpm (Fig. 3B) to 100 rpm (Fig. 3C) to make the environment more fermentative (hereafter, micro-aerobic condition). Hexanoic acid production was dramatically increased from 23 mg/L to 101 mg/L by reducing the agitation speed. In addition, butyric acid and butanol production also slightly increased under the micro-aerobic condition (Fig. S2). The increased production of hexanoic acid under the micro-aerobic condition is not surprising because the synthetic pathway was modified from the 1-butanol pathway and the NADH can be efficiently regenerated under such a condition (Dekishima et al., 2011; Zhang et al., 2008).

To enhance hexanoic acid production by the H4A strain, the carbon source was changed from glucose to galactose, as yeast usually consumes glucose very rapidly and produces only ethanol without any side products. One of the advantages of *K. marxianus* is its broad substrate spectrum that does not demand further engineering to change substrate. Indeed, hexanoic acid production increased to 154 mg/L when galactose was used as the sole carbon source (Fig. 3D). Although ethanol production yield (0.42 g/g) was similar between sugars, switching a carbon source to galactose resulted in a significant increase of hexanoic acid production.

3.3. Stable hexanoic acid biosynthesis by MCT1

All fermentation profiles of the H4A strain showed sudden decreases in hexanoic acid concentration regardless of the fermentation conditions and substrates. We speculated that the high reversible enzymatic activity of *AtoB* might be one of the reasons because the condensation reaction of acetyl-CoA into acetoacetyl-CoA is not thermodynamically favorable (Lan and Liao, 2012). Thus, we replaced the *AtoB* gene with *MCT1* from *S. cerevisiae* which condenses an acetyl-CoA and a malonyl-CoA into an acetoacetyl-CoA. In addition, acyl-CoA thioesterase (*TES1*) from *K. marxianus* was overexpressed for the efficient conversion of hexanoyl-CoA to hexanoic acid. To this end, we constructed H4T (containing *Crt, Hbd, Ter, BktB*, and *TES1*) and H4M (containing *Crt, Hbd, Ter, BktB*, and *MCT1*) strains. Surprisingly, these new strains did not show such a sudden decrease in hexanoic acid concentration (Fig. 4A and B). Hexanoic acid concentration increased constantly and remained stable throughout the fermentation period. However, hexanoic acid production was severely slowed down especially when only *TES1* was introduced. This result suggests that AtoB's activity is responsible for the degradation of hexanoic acid and the malonyl CoA-acyl carrier protein transacylase mediates an efficient chain elongation pathway.

We also constructed H4TM and H4ATM strains. The newly constructed strain, H4TM, was then cultivated in YP medium containing glucose (Fig. 4C) or galactose (Fig. 4D). Hexanoic acid concentrations of 100 mg/L and 142 mg/L were produced using glucose and galactose, respectively, and represent 92–99% of the highest hexanoic acid concentration produced by the H4A strain in the same condition. However, the time period to reach the highest hexanoic concentration by the H4A strain was two times faster than those by the H4TM strain. This result suggests that the condensation of two acetyl-CoAs to an acetoacetyl-CoA mediated by the *AtoB* is very fast, perhaps when acetyl-CoA amounts are sufficient, but is easily and quickly reversed, resulting in severe product instability. In contrast, *MCT1* seems to slowly build up the acetoacetyl-CoA content using both acetyl-CoA and malonyl-CoA, and had very low reaction reversibility. It is notable that the introduction of recombinant *TES1* only slightly enhanced the rate of hexanoic acid production, perhaps due to the presence of endogenous *TES1*. Hexanoic acid produced by H4ATM strain containing all seven genes was also decreased during fermentation confirming that the decrease of hexanoic acid was due to the reversible activity of *AtoB* (Fig. S4). Thus, we conclude that *MCT1* is a better substitute of *AtoB* for chain elongation.

There were huge variations in hexanoic acid production between colonies of each strain. For example, 9 colonies (H01–H09) of the H4TM strain picked after transforming six genes variably produced 0–150 mg/L hexanoic acid (Fig. S5). Though some of the colonies were missing several genes (H08 and H09) the other colonies producing hexanoic acid contained all six genes. The same colony-dependent variations were also observed from other recombinant strains such as H4M and H4T (Fig. S5). We reason that the variation resulted from the differences in the position at which genes were integrated. Because random integration of genes should accompany the disruption of certain chromosomal regions overall metabolism of the cell can be affected by the genetic perturbation. As such, a careful screening of colonies seems to be necessary when genes are randomly integrated into the chromosome of the yeast. Interestingly, cell-to-cell variation of growth was hardly observed while the hexanoic acid production was strictly colony-dependent.

We also note that *AtoB* is not likely to directly degrade hexanoic acid because it is the starting enzyme for hexanoic acid synthesis. Rather, the concentration of hexanoic acid can be decreased during fermentation by the H4A strain if there is competition between hexanoic acid production and degradation pathways, and the degradation pathway overrides during stationary phase. We think such a situation may happen when a limiting intermediate is lacking. Thu, it is likely that *AtoB* causes the lack of acetoacetyl-CoA during fermentation due to its strong reverse activity, leading to predomination of degradation pathway. H4ATM strain which contained *MCT1* as well as *AtoB* also exhibited such a sudden disappearance of hexanoic acid, indicating that *AtoB*'s activity was the cause of hexanoic acid degradation.

4. Conclusions

We engineered *K. marxianus* for the biological production of hexanoic acid. By simultaneously integrating 5–7 genes into the chromosomes of *K. marxianus*, we constructed 5 recombinant strains. These strains differ by the enzyme that mediates acetoacetyl-CoA formation (*AtoB* or *MCT1*) and commonly share central portions of the pathway (*Crt, Hbd, Ter*, and *BktB*). *AtoB* enabled fast production of hexanoic acid, but the product was suddenly disappeared. Although the rate was relatively slow compared to that of the *AtoB*-mediated pathway, *MCT1* provided a stable alternative route to hexanoic acid production. Thus, malonyl-CoA-mediated chain elongation deserves further attention because its pathway stability could be obtained by compensating for the speed of the *AtoB*-mediated pathway. The successful demonstration of hexanoic acid production, which requires the transformation of as many as 5–7 genes, will accelerate the use of this versatile yeast for complicated metabolic engineering.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jbiotec.2014.04.010.

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