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Alkane Utilization, the Expression and Function of Cytochrome P450 in Sophorolipid Synthesis in *Starmerella bombicola* CGMCC 1576

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Research Article

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ABSTRACT

Several alkanes were respectively added to four fermentation media to investigate the effects of alkanes on sophorolipids (SLs) production and expression levels of cytochrome P450 (P450) genes in *Starmerella bombicola* CGMCC 1576. The production of total SLs produced from n-tetradecane, n-hexadecane and n-octadecane was respectively 14.07 g l^{-1}, 31.73 g l^{-1} and 27.2 g l^{-1}. More lactonic SLs were produced from n-tetradecane and n-hexadecane while more acidic SLs were produced from n-octadecane. Furthermore, six P450 genes were found from *S. bombicola* by genome sequencing. CYP52-M1 gene was inducibly expressed by all used n-alkanes except that n-decane was not expressed without glucose. CYP52-E1 gene was inducibly expressed by some specific alkanes in various C/N ratios. In the media without glucose, CYP52-E2 gene was inducibly expressed by all used n-alkanes except n-octane and CYP52-N1 gene was inducibly expressed by n-octadecane. CYP52-N2 and CYP-X genes did not play the same important roles on alkane utilization as other P450 genes. Varying degrees of alkane utilization can guide the purposeful production of total, lactonic or acidic SLs, and the six P450 genes were expressed at different levels by alkanes and played different roles on alkane utilization.

INTRODUCTION

Sophorolipids (SLs) are a kind of biosurfactants mainly produced by several species of non-pathogenic yeasts [1,2]. Different SLs have different surface and biological activities. Generally, lactonic SLs exhibit stronger biological activity [3] while acidic SLs have better ability of foam formation and solubility [4]. Because of their good biodegradability, well biocompatibility, lower toxicity, good antitumor and highest yield among all biosurfactants, SLs have great application prospect in many sectors such as petroleum, environmental protection, cosmetic, and detergent and medicine industries [5].

Cytochrome P450s (P450s), belonging to a large family of monooxygenase [6], can oxidize fatty acids and alkanes to hydroxyl fatty acids and carboxylic acids [7,8] and were considered to be first-step speed-limiting enzymes in SL synthesis pathway [9]. Two P450 genes of Candida tropicalis (CYP52A13 and CYP52A17) have been reported to be responsible for the utilization of fatty acids and alkanes [10]. Two P450 genes from Candida apicola were cloned and named as CYP52E1 and CYP52E2 [11]. After that, three different P450 genes from Candida bombicola were cloned and named as CYP52M1, CYP52E3 and CYP52N1 [12]. A sophorolipid-producing yeast, Wickerhamiella domercqiae var. sophorolipid (now named as *Starmerella bombicola*) isolated by our lab was proved to be able to grow on some alkanes and utilize alkanes to produce sophorolipid, which demonstrated the probable relationship between alkane utilization and sophorolipid production. Therefore, it is necessary to discover more P450

genes in the genome of SL-producing strains. It is also important to investigate the effects of different alkanes on the expression levels of P450 genes and the synthesis of sophorolipids. The aim of the work was to investigate the effects of alkanes on SL production, P450 genes expression and the roles of P450 genes on alkane utilization in *Starmerella bombicola*.

MATERIALS AND METHODS

The production and composition analysis of sophorolipids (SLs)

Starmerella bombicola CGMCC 1576 (*S. bombicola*), a high SL-producing yeast strain, was isolated from sewage [13]. The production of SLs was measured by Anthrone method [14]. The SLs were analyzed by HPLC-MS using Venusil MP-C18, 4.6 μm columns (250 mm × 4.6 mm, Agela Technologies Inc.) monitored with UV detector at 207 nm. The total flow rate was 1 mL min^{-1} and the injection volume was 15 μL. The mobile phase was acetonitrile/water in which acetonitrile concentration was programmed from 40% to 90% (v/v) in 45 min. MS analysis was performed on an API4000 mass spectrometer (Applied Biosystems) and its ion source was ESI.

Real-time fluorescent quantitative PCR measurements

The mRNAs **(Supplementary Figure 1)** were extracted using Trizol and mRNA levels of target genes were determined using real-time fluorescent quantitative PCR (RT-qPCR). **Supplementary Table 1 and Supplementary Table 2** were the primers used for RT-qPCR and **Supplementary Table 3** was standard curves of RT-qPCR.

RESULTS

Sophorolipid (SL) production in the media containing different alkanes

Table 1 showed that the total SLs production decreased in the order of n-hexadecane (C16), n-octadecane (C18), n-tetradecane (C14), n-dodecane (C12), n-decane (C10) and n-octane (C8). Interestingly, the total SLs produced from C18 were second to that produced from C16 while the ratio of lactonic SLs to total SLs from C18 was the smallest. C8, C10, and C12 made no contributions to the production of SLs, whereas, the production of total SLs produced from C14, C16, and C18 was respectively 14.07 g l^{-1}, 31.73 g l^{-1} and 27.2 g l^{-1}, which much higher than that without alkanes (3.72 g l^{-1}).

Table 1. The production of sophorolipids (SLs) after 168 hrs' cultivation in fermentation media (G+) containing different alkanes or oleic acid (OA). The fermentation media (G+) contained 80 g glucose l^{-1}, 3 g yeast extract l^{-1}, 1 g KH_2PO_4 l^{-1}, 1 g $Na_2HPO_4 \cdot 12\ H_2O$ l^{-1}, 0.5 g $MgSO_4 \cdot 7H_2O$ l^{-1}, 30 mL alkanes or OA l-1. Alkanes included n-octane (C8), n-decane (C10), n-dodecane (C12), n-tetradecane (C14), n-hexadecane (C16) and n-octadecane (C18).

	G^+	(G^+) + C8	(G^+) + C10	(G^+) + C12	(G^+) + C14	(G^+) + C16	(G^+) + C18	(G^+) + OA
Total SLs g L^{-1}	3.72 ± 0.53	2.19 ± 0.62	2.83 ± 0.79	4.44 ± 0.70	14.07 ± 1.98	31.73 ± 3.01	27.2 ± 3.09	57.84 ± 2.55
Lactonic SLs g L^{-1}	2.92 ± 0.54	1.73 ± 0.67	1.95 ± 0.93	3.45 ± 0.92	6.43 ± 0.99	18.77 ± 1.84	2.25 ± 0.63	35.74 ± 3.29
The ratio*	78.5%	79.4%	68.9%	77.7%	45.7%	59.2%	8.3%	61.8%

*The ratio of lactonic SLs to total SLs, Data represented the means and standard deviations of three measurements.

The composition analysis of SLs produced in the media with different alkanes

Figure 1 and **Supplementary Table 4** showed that a same peak of SLs from C8, C10, C12, C14 and C16 appeared at 35-37.2 min and was inferred as Di-acetylated lactonic SL with a C18:0 fatty acid (C18:0, 2Ace, Lac SL) according to its molecular weight (MW) of 690 [15]. The peaks from C16 and C18 were a little different. The peaks of SLs that occured at 31.6-32.4 min and 32.4-33.8 min from C16 were inferred as the same molecule of mono-acetylated lactonic SL (C18:1, 1Ace, Lac SL) according to their MW of 646 [15], were significantly higher than that from C8, C10 and C14. Almost no SLs molecules from C18 occurred at 18-40 min. The SL peak from C18 at 8.8-9.3 min was as high as that from oleic acid and was not detected in the SLs mixture obtained from other alkanes, and they were identified as the same SL molecule (C18:1, 2Ace, Acid SL) according to their MW of 706 [15].

Nomination of the six cytochrome P450 (P450) genes

Six P450 genes were found from *Starmerella bombicola* by genome sequencing. According to genome annotation and Nelson classification system [16], the six P450 genes of *S. bombicola* were respectively named CYP52-M1, CYP52-N1, CYP52-N2, CYP52-E1, CYP52-E2 and CYP-X (CYP52-X gene was annotated as cytochrome P450 but not a specified P450 family and then was named CYP52-X). The GenBank accession numbers for the studied six genes are as follows: CYP52-M1, KM492812; CYP52-N1, KM492813; CYP52-N2, KM492814; CYP52-E1, KM492816; CYP52-E2, KM492817; CYP-X, KM492819.

Expression levels of six P450 genes in the media with or without glucose

As shown in **Figure 2a**, CYP52-M1 and CYP52-E1 genes were highly expressed. CYP52-M1 gene could be highly expressed by all used n-alkanes except n-decane. When n-decane was used as the hydrophobic carbon source, the transcriptional level of the CYP52-M1 gene decreased almost 20-40 folds. The CYP52-E1 gene was highly expressed by all used n-alkanes except

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n-octane. When n-octane was used as the hydrophobic carbon source, the transcriptional level of the CYP52-E1 gene decreased about 25 folds. Moreover, CYP52-M1 gene was also highly expressed, of which the transcriptional level was at least 15 folds more than with others in the fermentation media without alkanes but with glucose or both of glucose and oleic acid. Other P450 genes showed relatively low expression in the presence of glucose.

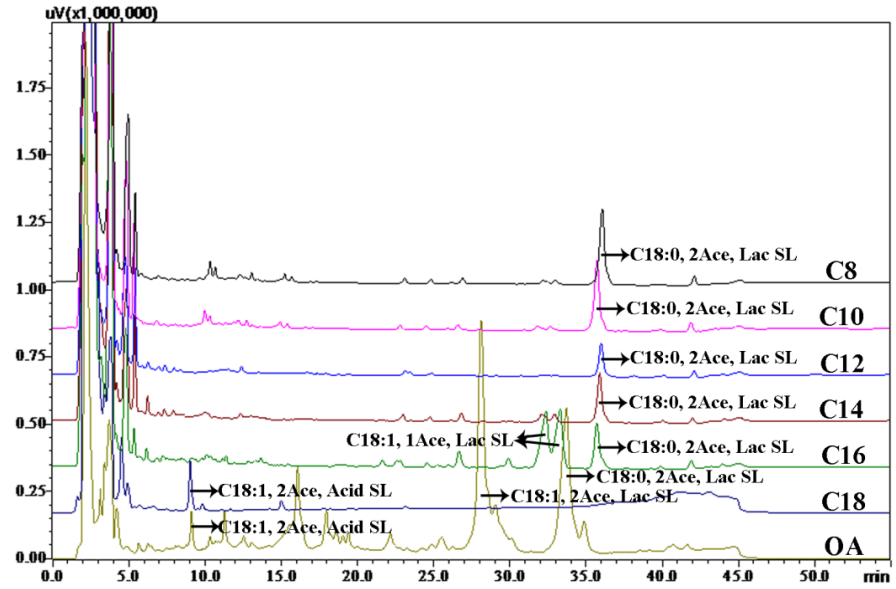

Figure 1. HPLC-MS analysis of compositions of sophorolipids (SLs) produced in fermentation media with different alkanes or oleic acid (OA). The fermentation media (G+) contained 80 g glucose l-1, 3 g yeast extract l^{-1}, 1 g KH_2PO_4 l^{-1}, 1 g $Na_2HPO_4 \cdot 12$ H2O l^{-1}, 0.5 g $MgSO_4 \cdot 7H_2O$ l^{-1}, 30 mL alkanes or OA l^{-1}. Alkanes included n-octane (C8), n-decane (C10), n-dodecane (C12), n-tetradecane (C14), n-hexadecane (C16) and n-octadecane (C18). Ace: acetylated. Lac: lactonic. Acid: acidic.

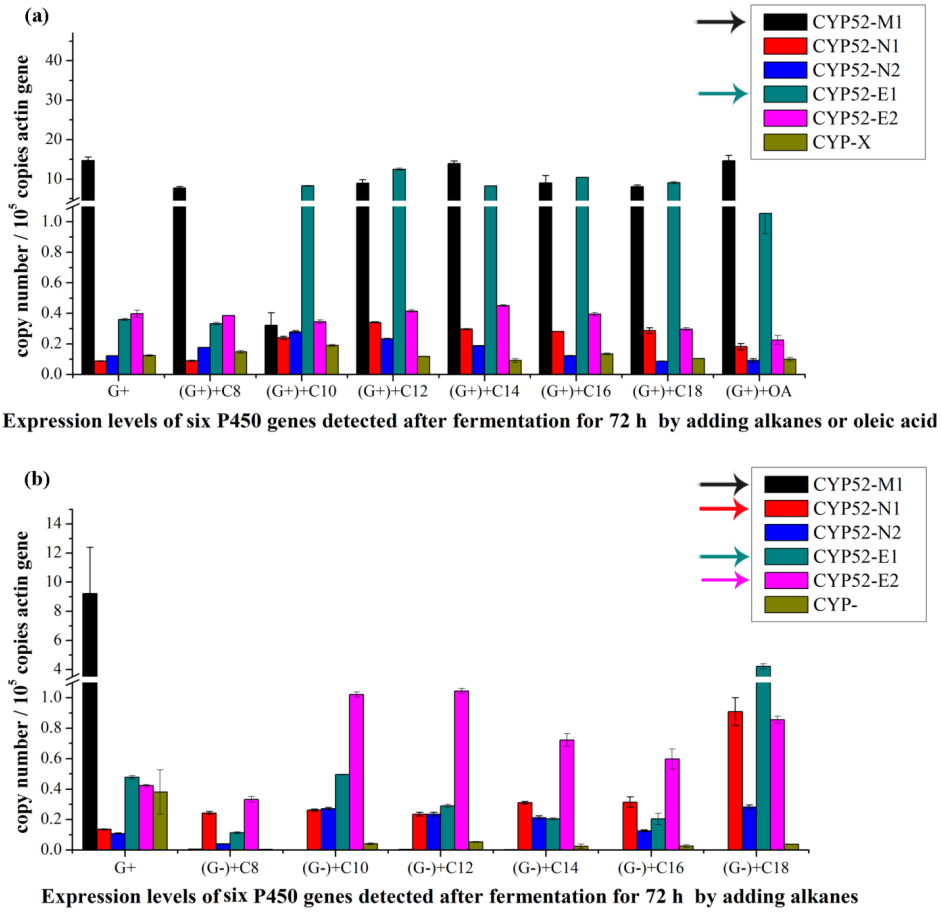

Figure 2. Expression levels of six cytochrome P450 (P450) genes after 72 hrs' cultivation in a) fermentation media (G^+) by adding different alkanes or oleic acid (OA) and b) the fermentation media without glucose (G^-) but with different alkanes. The fermentation media without glucose (G^-) contained 3 g yeast extract l^{-1}, 1 g KH_2PO_4 l^{-1}, 1 g $Na_2HPO_4 \cdot 12$ H_2O l^{-1}, 0.5 g $MgSO_4 \cdot 7H_2O$ l^{-1}, 30 mL alkanes or OA l^{-1}. Data represented the mean of three independent experiments and the error bars showed the standard error of the mean.

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As shown in **Figure 2b**, CYP52-M1, CYP52-E1, CYP52-E2 and CYP52-N1 genes were highly expressed. CYP52-M1 gene was only highly expressed in the media with glucose, and was rarely expressed in the media with n-alkanes. CYP52-E2 gene exhibited relatively high expression level in the media without glucose but with all used n-alkanes except n-octane. Especially when oleic acid was used as the carbon source, the copy number of CYP52-E2 increased almost 10 folds. CYP52-E1 and CYP52-N1 genes were only highly expressed in the presence of n-octadecane. CYP52-N2 and CYP-X genes showed low expression in the absence of glucose.

Expression levels of six P450 genes after starvation treatment

As shown in **Figure 3a and Figure 3b**, the expression level of each P450 gene under glucose starvation condition showed no significant differences from that under carbon and nitrogen sources starvation conditions and the expression levels of all genes except CYP52-E1 gene after starvation treatment were quite low. Very interesting, CYP52-E1 gene was only highly expressed in the presence of n-decane, the copy number of this gene increased at least 10 folds compared with that under other fermentation conditions.

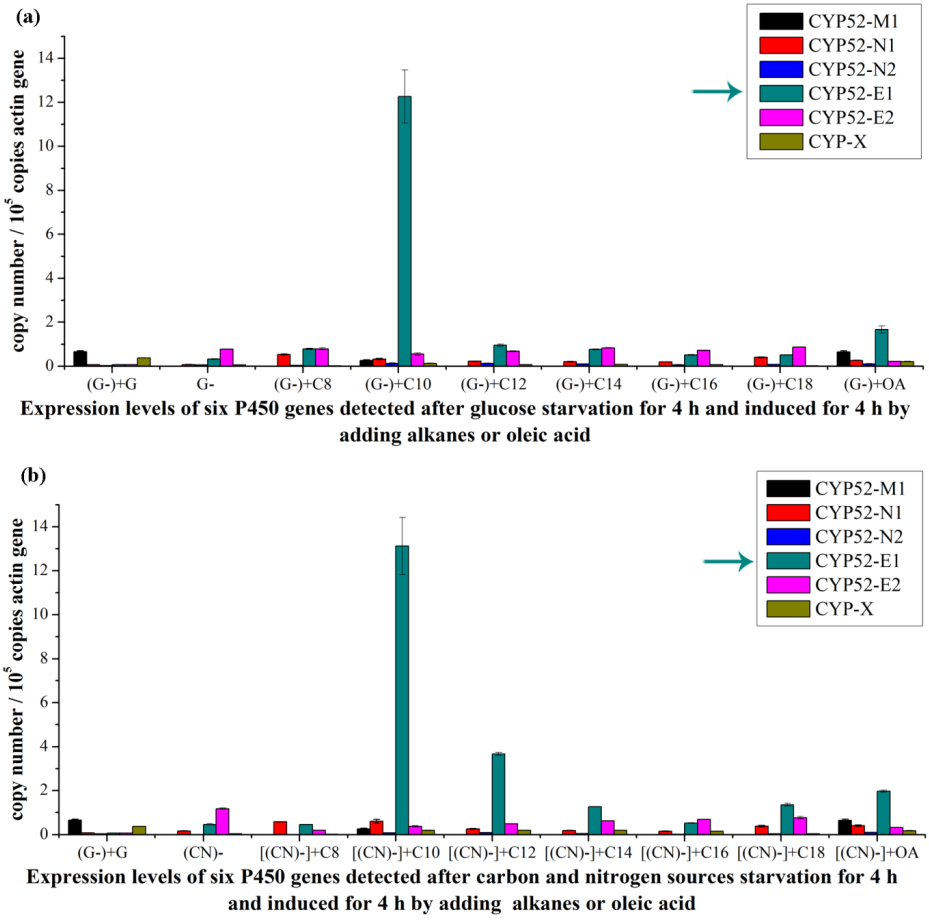

Figure 3. Expression levels of six P450 genes after 48 hrs' cultivation in fermentation media followed by 4 hrs' a) glucose starvation (G-) or b) carbon and nitrogen sources starvation [(CN)-] cultivation and then 4 hrs' induction by adding different alkanes or oleic acid (OA). G- media contained 3 g yeast extract l^{-1}, 1 g KH_2PO_4 l^{-1}, 1 g $Na_2HPO_4 \cdot 12\ H_2O$ l^{-1}, 0.5 g $MgSO_4 \cdot 7H_2O$ l^{-1}. (CN)- media contained 1 g KH_2PO_4 l^{-1}, 1 g $Na_2HPO_4 \cdot 12\ H_2O$ l^{-1}, 0.5 g $MgSO_4 \cdot 7H_2O$ l^{-1}. Alkanes or OA were added 30 mL to the media. Data represented the mean of three independent experiments and the error bars showed the standard error of the mean.

DISCUSSION

Alkanes were utilized in varying degrees by *Starmerella bombicola*. The overwhelming majority of sophorolipids (SLs) produced by *S. bombicola* were sixteen or eighteen carbon [2,15,17], which could explain why n-hexadecane and n-octadecane were utilized best by *S. bombicola*. HPLC analysis of SLs confirmed that the peaks occurred before 18 min were acidic SLs and the peaks occurred after 18 min were lactonic SLs [2,15,17]. Combined with our HPLC-MS data, more lactonic SLs were produced from n-tetradecane and n-hexadecane and more acidic SLs were produced from n-octadecane.

The six cytochrome P450 (P450) genes in *S. bombicola* were expressed at different levels and played different roles on alkane utilization. CYP52-M1 gene was inducibly expressed by all used n-alkanes except n-decane but was not expressed without glucose. Roelants proved the regulatory effect of glucose on the expression of cyp52M1 gene in Candida bombicola and the cyp52M1 gene was significantly downregulated in conditions of low glucose concentration [18]. And the CYP52M1 enzyme in Candida bombicola very likely took part in sophorolipid formation [12]. CYP52-E1 gene was inducibly expressed by all used n-alkanes

except n-octane **(Figure 2a)**, was inducibly expressed by n-octadecane without glucose **(Figure 2b)** and was specifically inducibly expressed by n-decane after starvation treatment **(Figure 3).** Thus, CYP52-E1 gene was inducibly expressed by some specific alkanes in various C/N ratios. In the media without glucose, CYP52-E2 gene was inducibly expressed by all used n-alkanes except n-octane and CYP52-N1 gene was inducibly expressed by n-octadecane. The transcriptional level of these two genes was closely associated with the n-alkanes in the fermentation medium. So we speculated that CYP52-E2 and CYP52-N1 played a distinct role on alkane utilization in the *Starmerella bombicola* CGMCC 1576 yeast. CYP52E3 and CYP52N1 genes in Candida bombicola were believed to take part in the metabolization of alkanes [12]. CYP52-N2 and CYP-X genes did not play the same important roles on alkane utilization as CYP52-M1, CYP52-E1, CYP52-E2 and CYP52-N1 genes.

CONCLUSION

The production of total sophorolipids (SLs) produced from n-tetradecane, n-hexadecane and n-octadecane was respectively 14.07 g l^{-1}, 31.73 g l^{-1} and 27.2 g l^{-1}. More lactonic SLs were produced from n-tetradecane and n-hexadecane while more acidic SLs were produced from n-octadecane. These results can guide the purposeful production of SLs. Six cytochrome P450 (P450) genes including CYP52-M1, CYP52-N1, CYP52-N2, CYP52-E1, CYP52-E2 and CYP-X were found from *Starmerella bombicola* by genome sequencing. These genes were expressed at different levels by alkanes and played different roles on alkane utilization. But the performing details of this pathway are not clear. According to these results, we can do some genetic modifications on the SLs-producer to obtain the mutants with the excellent capacity of using n-alkane.

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