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Simultaneous Production of Sphingolipids and Ethanol by *Kluyveromyces thermotolerans*

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ABSTRACT. *Kluyveromyces thermotolerans* strain NBRC 1674 was selected for the simultaneous production of sphingolipids and ethanol from beet molasses. The strain gradually synthesized ethanol with fermentation periods and attained a level slightly higher than that of the strain of *Saccharomyces cerevisiae* usually used for ethanol production. The sphingolipids accumulated in the cells were composed of almost equal amounts of free ceramides and glucosylceramides. The sphingoid bases and fatty acids of the two sphingolipids differed from each other and changed under aerobic and anaerobic growth conditions. Oxygen limitation may cause accumulation of sphinganine by inhibiting sphingolipid desaturases and enhance its proportion in both the sphingolipids.

Ceramide, which is composed of a sphingoid long-chain base with a 2-amino group amide linked to fatty acid, is a fundamental constituent of sphingolipids (Dickson and Lester 2002) and is organized in signaling domains on the cell surface of eukaryotic cells (van Meer *et al.* 2003). In yeasts and fungi, sphingolipids are shown to be related with the heat-shock response and cell differentiation (Dickson and Lester 2002; Warnecke and Heinz 2003).

There has been an increasing demand for ceramide preparation as a moisturizer to break the dry-skin cycle and maintain smooth skin (Loden 2003) or as a material for food supplements. Commercially available ceramide is extracted from plant tissues containing only 0.1–0.3 mg/g dry matter and is usually glucosylated, being referred to as glucosylceramide or cerebroside (Sugawara and Miyazawa 1999). We have previously surveyed the genera *Saccharomyces*, *Torulaspora*, *Zygosaccharomyces*, and *Kluyveromyces* and found glucosylceramides in *S. kluyveri*, *Z. cidri*, *Z. fermentati*, *K. lactis*, *K. thermotolerans*, and *K. waltii* (Takakuwa *et al.* 2002*a*).

Seventeen species accepted in the genus *Kluyveromyces* are divided into three distinct groups, and *K. thermotolerans* forms one of these groups with *K. waltii* and *Saccharomyces kluyveri* (Belloch *et al.* 2000; Malpertuy *et al.* 2000). *K. thermotolerans* is frequently found in grape juice and plays a significant role in the development of the characteristic flavor of wine (Holm Hansen *et al.* 2001; Mills *et al.* 2002). A commercial starter for wine making contains *K. thermotolerans* in addition to *S. cerevisiae* to give the desirable quality of wild fermentation under controlled conditions (Sommer *et al.* 2003). *K. thermotolerans* FRI 501 isolated from banana peel may have high glycolytic activity, as judged from its applicability for breadmaking by the frozen-dough method (Hino *et al.* 1987). These observations suggest that *K. thermotolerans* can be used for the simultaneous production of glucosylceramide and ethanol from beet molasses, an agricultural by-product from the sugar industry. Moreover, our preliminary survey of *K. thermotolerans* lipids has shown that the yeast accumulates not only glucosylceramide but also free ceramide, as in the case of mushrooms (Ohnishi *et al.* 1996).

The present paper reports the selection of a desirable strain from *K. thermotolerans* and the chemical composition of accumulated sphingolipids.

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MATERIALS AND METHODS

Yeast strains. Nine strains of *Kluyveromyces thermotolerans* were obtained from NBRC (*NITE Biological Resource Center*, Chiba, Japan) (Table I). *Saccharomyce cerevisiae* NBRC 0216 (deposited as Taiken 396) is a distillery strain used for the commercial production of ethanol.

Table I. Production of ethanol (g/L) and sphingolipids (free and glucosylated ceramides, μg/g dry matter) by *Kluyveromyces thermotolerans* NBRC strains[a]

Strain	Biomass g/L dry matter	Ethanol	Ceramides	
			free	glucosylated
0662	2.33 ± 0.07	78 ± 8	330 ± 60	720 ± 10
1050	2.28 ± 0.02	53 ± 8	330 ± 100	510 ± 160
1674	2.82 ± 0.33	101 ± 3	840 ± 30	500 ± 10
1779	2.06 ± 0.09	72 ± 1	570 ± 90	1400 ± 40
1780[b]	2.03 ± 0.05	36 ± 6	320 ± 40	490 ± 100
1985	2.86 ± 0.12	88 ± 8	510 ± 50	730 ± 70
10066	3.82 ± 0.10	103 ± 4	300 ± 70	370 ± 20
10067	3.83 ± 0.05	102 ± 2	240 ± 30	380 ± 30
10953	3.31 ± 0.19	102 ± 2	380 ± 80	390 ± 50

[a]Average values ± SD from 3 independent experiments.
[b]This strain has recently been reclassified and transferred to *Zygosaccharomyces* sp.

Culture. Yeast cells were grown aerobically in a seed medium containing (in %) polypeptone 2, glucose 2, yeast extract 1, at 25 °C for 1 d with shaking (3.67 Hz). Harvested cells (500 mg dry matter) were inoculated to 250 mL of fermentation medium with 20 % sugar in the form of beet molasses, 0.2 % diammonium sulfate, and 2 % corn steep liquor in a 500-mL Erlenmeyer flask and incubated anaerobically with standing for 6 d at 25 °C. Sugars were analyzed using a high-performance liquid chromatograph (CCP series; *Tosoh*, Japan) equipped with a packed column (Shodex KS-801; *Showa Denko*, Japan) and a refraction index monitor. Ethanol was analyzed using a gas chromatograph (GC-9A series; *Shimadzu*, Japan) equipped with a capillary column (TC-FFAP; *GL Science*, Japan).

Lipid analysis. Sphingolipids in the yeast cells were extracted and quantified according to Tanji *et al.* (2004*b*). Briefly, total lipids were extracted with chloroform–methanol and hydrolyzed in KOH–methanol. The alkali-stable lipids obtained as the organic phase were subjected to HPLC with an evaporative light-scattering detector to quantify both free ceramides and glucosylceramides. The amount of free ceramides was estimated as the glucosylceramide equivalent. Both sphingolipids were then isolated and purified to determine the compositions of their constituents (fatty acids, sphingoid bases, sugar) (Ohnishi *et al.* 1983).

RESULTS

Table I shows the yields of ethanol and sphingolipids (free ceramides and glucosylceramides) produced by the nine strains of *K. thermotolerans*. Some of the strains did not consume all of the sugar in the fermentation medium within 6 d and differed in the ethanol concentration. Considering the productivity of ethanol and sphingolipids, strain NBRC 1674 was the most suitable for the simultaneous production of sphingolipids and ethanol.

The strain NBRC 1674 was grown anaerobically and compared with *S. cerevisiae* strain NBRC 0216. The typical patterns of sugar consumption and ethanol formation are shown in Fig. 1. The biomass yields of the two strains were about 3 g/L dry matter and were relatively constant for further 2 d (*data not shown*). Strain NBRC 1674 synthesized ethanol and attained a level slightly higher than that of strain NBRC 0216. The content of free ceramides in strain NBRC 1674 increased but that of glucosylceramides remained unchanged; *S. cerevisiae* accumulated <0.3 mg/g of free ceramides and no glucosylceramides.

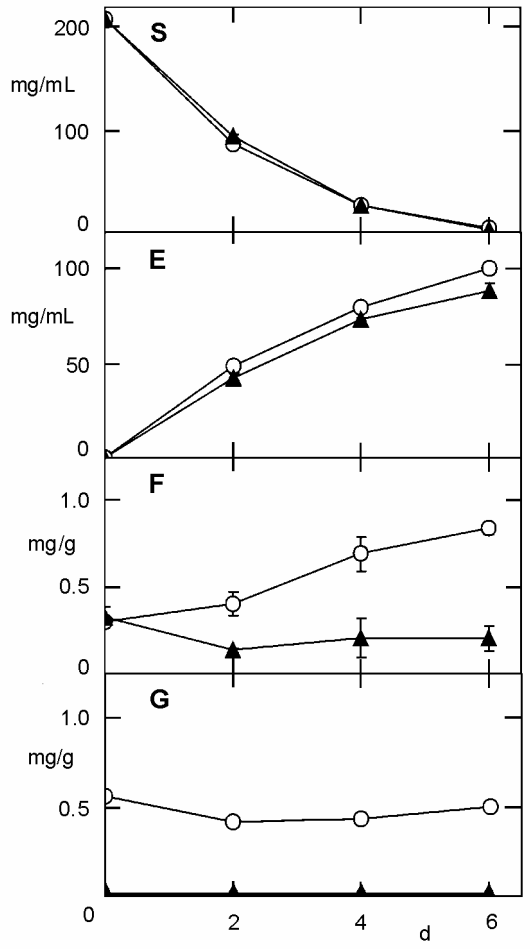

Fig. 1. Contents of metabolites (**S** – sugars, **E** – ethanol; both in mg/mL) in the media and intracellular sphingolipids (**F** – free ceramides, **G** – glycosylceramides; both in mg/g) during anaerobic fermentation of *Kluyveromyces thermotolerans* NBRC 1674 (*circles*) and *Saccharomyces cerevisiae* NBRC 0216 (*triangles*); average values ± SD from 3 independent experiments.

The composition of free ceramides and glucosylceramides from NBRC 1674 grown aerobically in the seed medium and anaerobically in the fermentation medium us shown in Table II. As for glucosylceramides, 18h:0 dominated in fatty acids, while the principal sphingoid bases in the cells grown aerobically and anaerobically were 9-Me d18:$2^{4t,8t}$ and d18:0, respectively. Free ceramides predominantly contained 26h:0 fatty acids except for 16h:0, which increased under anaerobic conditions. Opposite changes were observed in d18:0 and t18:0, but the sphingoid bases under the two sets of culture conditions retained low levels of 9-Me d18:$2^{4t,8t}$. The sugar moiety included was a single molecule of glucose.

DISCUSSION

Sphingolipids in the alkali-stable lipids prepared from most species of yeasts are usually glucosylated, while *K. thermotolerans* strains contained appreciable amounts of free ceramides (Table I), like those found in *Yarrowia lipolytica* (Rupčić and Marić 1998, 2004) with some differences. Yeast ceramides show a heterogeneous structure depending on the strain and culture conditions (Tanji *et al.* 2004*a*,*b*), but those of *Y. lipolytica* were more divergent than other species reported previously (Rupčić and Marić 1998; Rupčić *et al.* 1998). Over one-half of the sphingoid bases in free ceramides revealed trihydroxylated forms that were sparsely present in yeasts. This species lacked 9-Me d18:$2^{4t,8t}$, a typical sphingoid base of glucosylceramides from fungi and yeasts (Barreto-Bergter *et al.* 2004).

Fungal cells contain two distinct ceramide types destined for the syntheses of inositolphosphoceramides and glucosylceramides (Dickson and Lester 2002). Free ceramides from the cells of *K. thermotolerans* grown aerobically were similar to the former type, composed of t18:0 as a sphingoid base and 26h:0 as a fatty acid. During fermentation, the changes in the sphingoid bases were much more remarkable than those that occurred in the fatty acids. In this case, the amounts of free ceramides and glucosylceramides in the sphingoid base changed to d18:0. In higher organisms, conversions of d18:0 to d18:$2^{4t,8t}$ through d18:1^{4t} are catalyzed by Δ^4 and Δ^8 desaturases, which require NAD(P)H and O_2 (Sperling *et al.* 1998; Ternes *et al.* 2002), using ceramide and/or glucosylceramide as the substrate. Sphingolipid Δ^8 desaturases from *Saccharomyces*

kluyveri and *K. lactis* were revealed to have a heme-binding motif in cytochrome b_5 and three histidine boxes, as in plant desaturases (Takakuwa *et al.* 2002*b*). *K. thermotolerans* under anaerobic conditions may

Table II. Composition[a] of fatty acids (%) and sphingoid bases (%) in free ceramides and glucosylceramides from *Kluyveromyces thermotolerans* NBRC 1674 grown aerobically and anaerobically

Component[b]	Free ceramides		Glucosylceramides	
	aerobic	anaerobic	aerobic	anaerobic
Fatty acids				
16h:0	7.2 ± 0.9	43.5 ± 3.9	3.5 ± 0.5	3.6 ± 0.5
18h:0	32.0 ± 8.4	12.6 ± 1.5	93.7 ± 0.3	93.4 ± 0.7
22h:0	<0.1	<0.1	<0.1	<0.1
24h:0	2.3 ± 0.1	2.3 ± 0.3	0.1 ± 0	0.3 ± 0
25h:0	8.1 ± 1.1	3.8 ± 0.3	0.1 ± 0	0.2 ± 0
26h:0	50.4 ± 8.0	37.8 ± 4.7	2.6 ± 0.2	2.5 ± 0.1
Sphingoid bases				
t18:0	57.2 ± 5.6	34.1 ± 3.7	20.6 ± 5.9	14.8 ± 6.2
t20:0	30.2 ± 1.9	15.4 ± 0.9	13.5 ± 1.1	12.0 ± 0.5
d18:0	8.8 ± 2.0	50.4 ± 2.9	3.0 ± 0.2	62.6 ± 2.2
d18:1^{4t}	2.0 ± 1.5	0.1 ± 0.1	1.4 ± 0.1	0.6 ± 0.2
d18:$2^{4t,8t}$	1.5 ± 1.2	<0.1	21.9 ± 1.0	4.1 ± 0.4
9-Me d18:$2^{4t,8t}$	0.3 ± 0.4	<0.1	39.6 ± 5.6	5.9 ±3.2

[a]Average values ± SD from 3 independent experiments.
[b]Hydroxy fatty acids are abbreviated as *X*h:*Y*, where *X* and *Y* represent the number of carbon atoms and the number of double bonds, respectively; sphingoid bases: t18:0 – 4-hydroxysphinganine, t20:0 – 4-hydroxy-icosasphinganine, d18:0 – sphinganine, d18:1^{4t} – *trans*-4-sphingenine, d18:$2^{4t,8t}$ – *trans*-4,*trans*-8-sphinga-dienine, 9-Me d18:$2^{4t,8t}$ – 9-methyl-*trans*-4,*trans*-8-sphingadienine.

accumulate the two sphingolipids with d18:0 as their principal sphingoid base by the inhibition of desaturases. It is also possible that the composition of free ceramides changed in response to the environmental stress caused by ethanol (Šajbidor and Grego 1992). Although the chemical compositions changed depending on culture conditions, the ceramides accumulated in anaerobically grown cells may be used as a constituent in cosmetics or food supplements because various types of sphingolipid preparations from plants and fungi have been suggested to possess physiological functions (Aida *et al.* 2004, 2005). *K. thermotolerans* NBRC 1674 is suggested to be used for the simultaneous production of ethanol and sphingolipids. Our approach provides an available method to utilize an agricultural by-product, beet molasses.

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