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Selection of lactic yeast producing glucosylceramide from cheese whey

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Abstract

From 2,150 isolates from raw milk and milk products, yeast strains were surveyed to produce glucosylceramide from cheese whey. Most of the 54 strains that had accumulated a detectable amount of glucosylceramide were identified as *Kluyveromyces lactis* var. *lactis*. The cells of *K. lactis* var. *lactis* strain M-11 derived from domestic raw milk accumulated glucosylceramide 2.5-fold higher than *K. lactis* var. *lactis* NBRC 1267, the reference strain selected from the culture collections. Strain M-16 of *K. lactis* var. *lactis* derived from the same origin was found to synthesize a considerable amount of steryl glucoside in addition to glucosylceramide. Sequence analysis of ribosomal DNA intergenic spacer 2 regions revealed that strains M-11 and M-16 were diverged from a type strain of *K. lactis* var. *lactis* in the same species.

Key words: Glucosylceramide; steryl glucoside; cheese whey; *Kluyveromyces lactis* var. *lactis*

1. Introduction

Dairy farms are located on large grasslands in the cool climate of Eastern and Northern Hokkaido, the northernmost island of Japan. The annual production of raw milk in Hokkaido amounts to 3.9 million tons, corresponding to 47% of the overall domestic production. A gradual increase in the recent market for natural cheese requires 350,000 tons of raw milk in Japan, predominantly from Hokkaido. During the production of natural cheese, the whey containing 5% lactose, 1.9% protein and fats, 0.9% salt and a small amount of vitamins emerges from clotted milk as a by-product (Ghaly and Kamal, 2004). In Europe and North America, about half of the whey is used as animal feed, as a food ingredient or for microbial fermentation and as the material for ethanol fermentation (González-Siso, 1996). The remainder places high levels of biological and chemical oxygen demands on sewage treatment plants even after the protein concentrate has been separated by ultrafiltration (Ghaly and Kamal, 2004). These problems are common to the cheese industry in Japan and demand further methods for the economic utilization of whey.

Ceramides are the predominant lipid of lamellae in the stratum corneum responsible for the epidermal permeability barrier (Hamanaka et al., 2002) and paid attention in Japan as a material for food supplements since oral intake has shown to improve skin symptoms and reduced allergic responses (Miyanishi et al., 2005). Commercially available ceramide is extracted from plant tissues including rice bran, wheat germ, konjac tuber and mushrooms, and is usually glucosylated, being referred to as glucosylceramide or cerebroside. In 2005, domestic market for ceramide preparation was 5.8 tons as a crude extract of <5% purities, which corresponded to 11.4 million U.S. dollars, and is increasing by 20% annually as application to usual food materials has expanded. These situations have encouraged us to propose various alternative sources of glucosylceramide from agricultural by-products to respond to growing demands with low costs.

Among 31 samples of crop tissues and by-products from their processing, apple pulp was shown to include the highest amount of glucosylceramide (Takakuwa et al., 2005b). Yeasts synthesizing glucosylceramide are *Saccharomyces kluyveri*, *Zygosaccharomyces cidri*, *Z. fermentati*, *K. lactis*, *K. thermotolerans*, *K. waltii* (Takakuwa et al., 2002), *Debaryomyces hansenii* (Takakuwa et al., 2005a) and *Candida lipolytica* (Rupčić and Marić, 2004). Some strains of *S. kluyveri* and *K. thermotolerans* can be used for the production of glucosylceramide from beet molasses (Tamura et al., 2006; Tamura et al., 2005). However, these strains cannot grow in cheese whey due to a defect in the function of lactose fermentation. In the present study, yeast strains accumulating high amounts of glucosylceramide were surveyed in the isolates from raw milk and milk products.

2. Methods

2.1. Yeast strains and culture

Each portion of 21 samples from raw milk, yogurt and cheese was inoculated to 10% (w/v) skim milk and incubated for two days. The culture broth was diluted and spread on potato dextrose agar. After incubation for several days, 2,150 yeast-like colonies were isolated for subsequent examinations. Other strains classified as *Kluveromyces lactis* var. *lactis* were obtained from NBRC (NITE Biological Research Center, Chiba, Japan). Strain NBRC 1267 was shown to accumulate higher amounts of glucosylceramide than the other strains, NBRC 0433, NBRC 0648, NBRC 1090^{T} and NBRC 1903 and was, thus, used as a reference. Yeast cells were routinely grown in the medium composed of 2.0% lactose as whey powder, 1.0% corn steep liquor, 0.5% ammonium sulfate, 0.075% KH_2PO_4 and 0.075% $MgSO_4$ (pH 5.5).

2.2. Extraction, purification and analysis

Purified preparations (98%<) of glucosylceramide and steryl glucoside derived from soybean were purchased from Matreya LLC (Pleasant Gap, PA). Glucosylceramide and steryl glucoside

were extracted and quantified according to a method described elsewhere (Takakuwa et al., 2005b). Briefly, alkali-stable lipids were extracted from 0.2 g of the lyophilized cells by the extraction with chloroform-methanol and successive hydrolysis in KOH-methanol and dissolved in 0.2 ml of chloroform:methanol (2:1, v/v). For the analysis of alkaline-stable lipids, 5 μl of the extract was subjected to thin layer chromatography (TLC) development by chloroform: methanl: acetic acid: water (20:3.5:2.3:0.7, v/v). The orcinol-sulfuric acid reagent visualized the spots, which were determined by comparing their densities to those of authentic standards with a Lane & Spot Analyzer (Atto Co., Tokyo).

2.3. Analysis of the DNA sequence

The internal transcribed spacer (ITS) and intergenic spacer 2 (IGS2) of the rRNA genes were amplified by PCR as described elsewhere (Naumova et al., 2004; Oda et al., 1997). The DNA fragments were sequenced on both strands with an automated DNA sequencer (Applied Biosystems Model 310). A phylogenetic tree was constructed using the CLUSTAL W and TreeView programs (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). The nucleotide sequences for IGS2 have been assigned EMBL/GenBank/DDBJ accession numbers AB266620 to AB266630.

3. Results and discussion

Among the 2,150 isolates tested, 480 strains grew on an agar medium composed of cheese whey as the sole carbon source. The original raw milk and milk products seemed to contain an appreciable number of yeasts depending on the monosaccharides produced from lactose by the action of other microorganisms. When each colony on the agar medium was subjected to TLC analysis, 54 strains showed clear spots derived from glucosylceramide. From the sequence analysis of ITS, these strains were classified into three groups. The species and number of strains were: *Kluyveromyces lactis* var. *lactis*, 41; *Debaryomyces hansenii*, 9 and *Candida*

intermedia, 4. *D. hansenii* is a salt-tolerant yeast and was shown to form glucosylceramide (Takakuwa et al., 2005a). All of the 54 strains were individually cultured on 40 ml of a liquid medium for comparison with the reference strain (Fig. 1). Four strains identified as *K. lactis* var. *lactis* M-6, M-8, M-11 and M-16, isolated from domestic raw milk, accumulated relatively high amounts of glucosylceramide (>0.40 mg g^{-1}) and that of M-11 (0.71 mg g^{-1}) attained 2.5-fold that of strain NBRC 1267 (0.28 mg g^{-1}). The biomass of this strain was not so high, but the yield of glucosylceramide per culture medium by strain M-11 (0.10 mg ml^{-1}) was greater than that of strain NBRC 1267 (0.06 mg ml^{-1}).

The results of the TLC analysis were interesting. Surprisingly, strain M-16 produced a compound corresponding to steryl glucoside that is usually below the detection limit among cells grown under normal conditions (Fig. 2). In the methanol-utilizing yeast *Pichia pastoris*, stress, such as heat shock or a high concentration of ethanol, increased the content of steryl glucoside (Sakaki et al., 2001). A steryl glucosyltransferase gene from this yeast was also shown to be involved in the vacuole-dependent selective degradation of peroxisomes (Oku et al., 2003). These findings indicate that the synthesis of steryl glucoside is strictly regulated in response to changes in the environment. The accumulation of steryl glucoside in the cells of strain M-16 may be explained by its derepressed synthesis, but the mechanism is still unknown.

For further taxonomical analysis of strains M-6, M-8, M-11 and M-16, the IGS2 sequences, which appear to be a powerful technique to differentiate between phylogenetically intimately related species (Sugita et al., 2002), were determined. Some divergences were observed in the four strains of *K. lactis* var. *lactis*, but included in differences of individual strains as judged from the position of *K. lactis* var. *drosophilarum* as the outgroup (Fig. 3).

Cheese whey and beet molasses usually emerge in agricultural region under the cool climate in the world. Recent reports on utilization of cheese whey are concerned with lactose removal (Aktas et al., 2006), single cell protein production (Schultz et al., 2006) and ethanol

fermentation (Kourkoutas et al., 2002) by *K. marxianus* but not with valuable products from the yeast cells. The present study indicates that glucosylceramide can be produced from cheese whey in addition to our previous finding for beet molasses (Tamura et al., 2006; Tamura et al., 2005). The two production methods will compensate each other and contribute stable supply of glucosylceramide for growing markets.

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Figure legends

Fig. 1. Distribution of glucosylceramide contents in yeast strains isolated from raw milk and milk products. Fifty-four strains were identified as *Kluyveromyces lactis* var. *lactis* (shaded bar), *Debaryomyces hansenii* (closed bar) and *Candida intermedia* (open bar) by the sequence analysis of ITS. An arrow indicates the level in the reference strain NBRC 1267.

Fig. 2. TLC analysis of alkali-stable lipids of the four strains isolated from domestic raw milk and the five NBRC strains of *K. lactis* var. *lactis*. Strain NBRC 1673, which cannot grow in a medium composed of cheese whey, was not included in this experiment. The lane at both ends represents the authentic standards of glucosylceramide and steryl glucoside at 5 μg.

Fig. 3. Phylogenetic tree constructed by the neighbor-joining method from IGS2 sequences of *K. lactis* var. *lactis* strains. *K. lactis* var. *drosophilarum* NBRC 1012 was used as the outgroup. The bar indicates one estimated substation per 100 nucleotide positions. Bootstrap values were calculated from 1,000 trees.

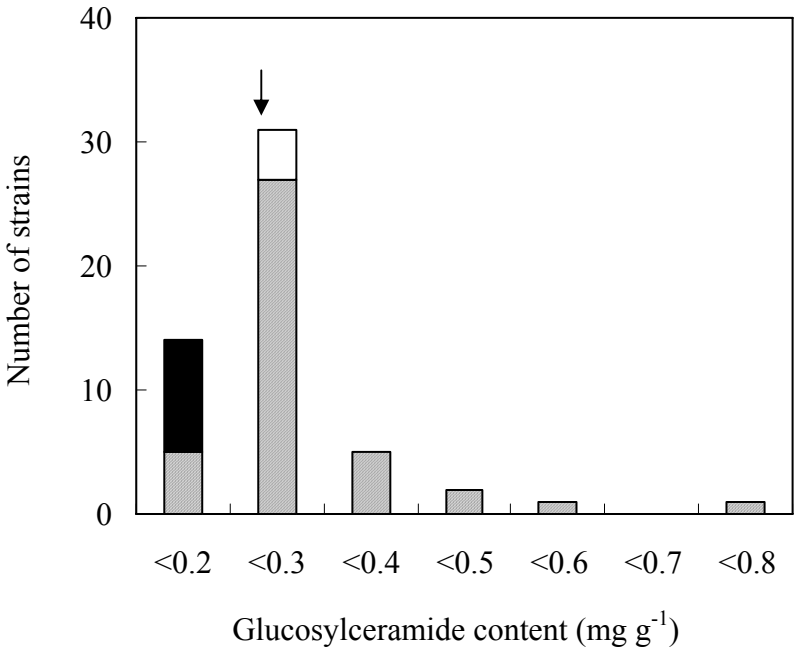
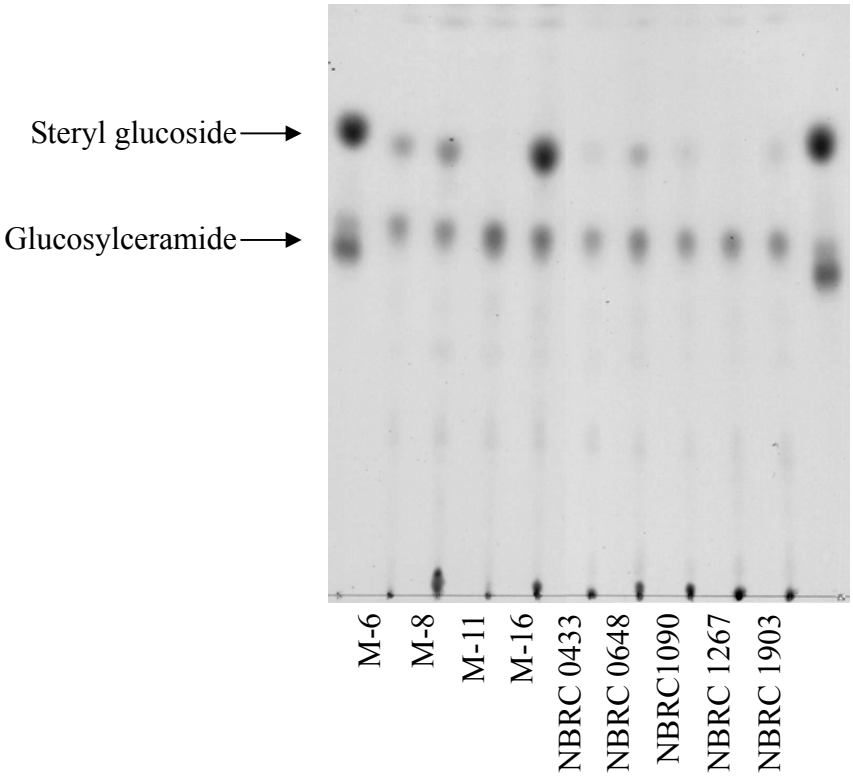


Fig. 1. Sugai *et al.*



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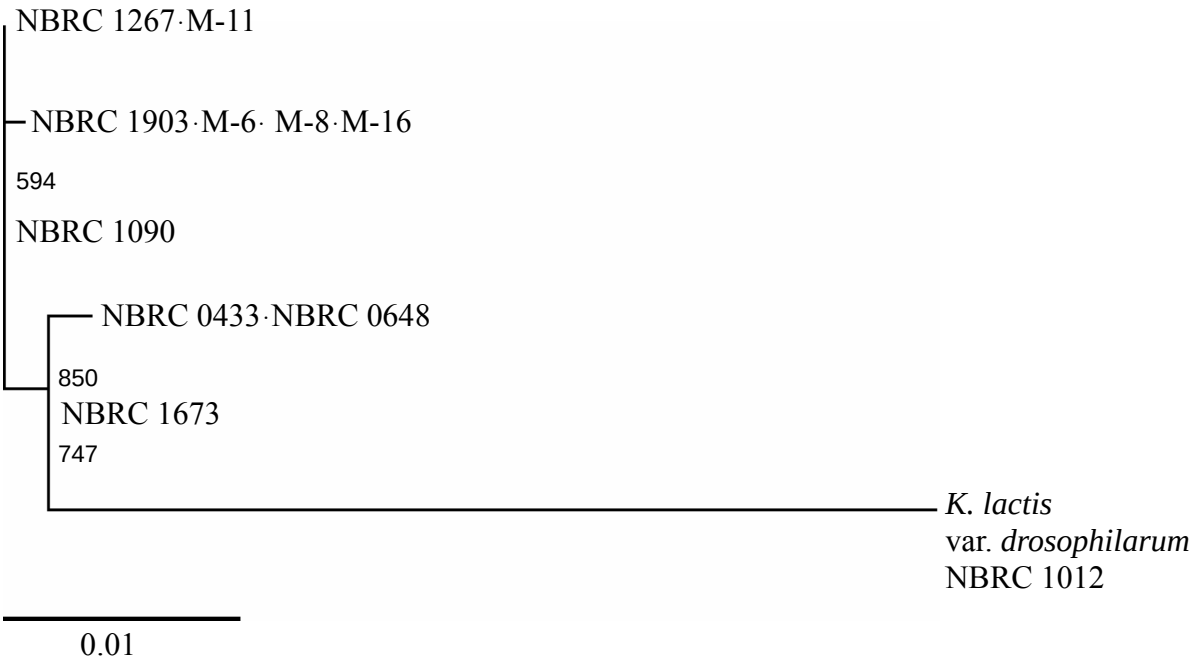

Fig. 3. Sugai *et al.*