0273-2289/1559-0291 (Online)/06/133/0203/$30.00

Production of Ceramide With *Saccharomyces cerevisiae*

Kyu Hyuk Kwun,[1] Jung-heon Lee,*[,1] Kyung-ho Rho,[2] and Hyun-shik Yun[2]

[1]Department of Chemical Engineering, Chosun University, Kwangju 501-759, Korea, E-mail: leejh@chosun.ac.kr; and [2]Department of Chemical Engineering, Inha University, Inchon 402-751, Korea

Received February 3, 2004; Revised September 19, 2005; Accepted October 5, 2005

Abstract

The possibility of producing the biologically active material of the skin, ceramide, was studied using yeasts. The yeast strain that produced the most ceramide, *Saccharomyces cerevisiae* (KCCM 50515), was selected, and the optimal conditions for ceramide production were determined using shake-flask culture and batch fermentation. By measuring the production rate of ceramide at various pH values and temperatures, the optimal conditions for ceramide production were found to be pH 6.0 and 30°C. When heat shock was applied to the cells for 1 h by increasing the culture temperature from 30 to 40°C after cell growth, the amount of ceramide produced was increased 5.9-fold. A cell growth and ceramide production model was developed with Monod kinetics and the Leudecking-Piret model. It showed that ceramide production was increased when the cells were in the stationary phase.

Index Entries: Ceramide; heat shock; optimal condition; *Saccharomyces cerevisiae*.

Introduction

Human skin is an organ that protects the body from various types of stimulation and damage and controls the physiologic functions by promoting its metabolism. In addition to its biological functions, skin also has a significant effect on various aspects of the daily lives of people, such as their appearance and social exchanges *(1)*. Numerous studies have been conducted with a view to protecting skin from the effects of aging caused by both age and stress and to rejuvenate it, in order for people to benefit from

*Author to whom all correspondence and reprint requests should be addressed.

natural and healthy-looking skin. One of the methods that attempted to improve the skin was to supply the skin with the chemicals, such as ceramide, cholesterol, and fats, at concentrations below the minimum levels. Biologically active materials are chemicals that promote physiological functions and aid in the synthesis of proteins needed in the body that nourish skin and prevent aging *(2)*. When skin is provided with biologically active materials that it is lacking, it should function better and improve in appearance. Biologically active materials in the skin promote physiological functions and metabolism, so they are used extensively in the pharmaceutical and cosmetic industries, in order to prevent aging and keep skin elastic and healthy *(3,4)*.

The outermost layer of the skin, known as the cuticle, is composed mainly of ceramide, cholesterol, and fats. Thus, the epidermis plays an important role in protecting the skin, such as by preventing it from drying. Fats lacking in the skin owing to temporary skin damage are supplied and biosynthesized during cellular activity, so that the skin can recover to its normal state. However, this ability of the skin to recover decreases significantly when people reach middle age, because the ceramide content in the cuticle decreases with age *(5)*.

Ceramide has the effect of compensating for the side effects caused by α-hydroxy acids. Thus, attempts have been made to prescribe or use ceramide along with α-hydroxy acids. Currently, many complex synthetic materials containing retinol, vitamins, and other substances are being developed. According to a recent study, ceramide inhibits the enzyme collagenase from degrading collagen, which is present under the cuticle layer and maintains skin flexibility and tension. Studies are currently being conducted to examine the possibility of using the biologically active material ceramide as an antimicrobial, antibiotic, and anticancer agent *(6)*.

The production of ceramide from animal products has been significantly reduced since the discovery of prion disease. As a result, instead of producing it from animal products, an alternative method of producing ceramide using microorganisms has been developed. Yeasts are known to be appropriate for the production of ceramide, owing to their ability to grow rapidly, their nontoxicity, and their ability to be genetically engineered. It is known that ceramide is synthesized by yeasts, such as *Saccharomyces cerevisiae* and *Torulopsis* (*Candida*) *utilis (7)*. A ceramide production process using microorganisms needs to be developed for commercial application.

In the present work, the effect of pH and temperature on ceramide production was studied and the optimal pH and temperature conditions were determined. For the development of a production process, ceramide was produced with a batch fermentor.

Materials and Methods

Culture Conditions and Methods

The yeast strains used are those strains known to produce sphingolipid, including *S. cerevisiae* (KCCM 50515), *Pichia ciferrii*, and *Candida*

lipolytica. To maintain the activity of the strains, they were subcultured in culture plates during a 2-wk period and used as inocula while keeping them in a refrigerator at 4°C. YEPD medium containing 20 g/L of glucose, 20 g/L of bacto peptone, and 10 g/L of yeast extract was used. After placing 50 mL of the medium in a 250-mL Erlenmeyer flask, culturing was done at 30°C using a rotary shaker at 200 rpm for 48 h. For the development of the batch fermentation process, *S. cerevisiae* was cultured at 30°C, 300 rpm, and pH 6.0 using a 5-L fermentor (KoBiotech, Inchon, Korea).

Analysis of Ceramide

Five grams of yeast (wet weight) was mixed with 20 mL of a mixture of chloroform and methanol (1:2 [v/v]) and disrupted using a sonicator. The sample was filtered through a 0.2-μm RC filter (Sartorius) and purified by solvent evaporation. To remove the polar components and nonlipid contaminants, chloroform-methanol-water (8:4:3 [v/v/v]) solvent was added for the purpose of phase separation. Sphingolipid was purified from the harvested lipid using mild alkaline hydrolysis. After dissolving the dry lipid sample in methanol–carbon tetrachloride solution (5:1 [v/v]) and adding 0.2 *M* methanolic NaOH, the sample was hydrolyzed for 1 h at room temperature. The unsaturated lipid was extracted with chloroform after distilled water was added to the sample, and it was neutralized with 1 *M* acetic acid *(7)*. To obtain quantitative results on the production of ceramide using yeasts, high-performance liquid chromatography (HPLC) (Waters, Milford, MA) was used with a multisolvent delivery system. Yeast ceramide changes its structure according to the culture conditions. To obtain the lipid composition quickly and accurately, HPLC-evaporative light scattering detector (ELSD) was used *(8)*. ELSD is known to be more effective for the analysis of ceramide than HPLC-UV *(9)*. The drift temperature of ELSD was 65°C, and the gas flow rate was 1.6 mL/min. A mixture of chloroform and methanol was used as the mobile phase, and the flow rate was set to 1.0 mL/min. The HPLC column used was a Licroprep Si 60 (15 μm, 3.9 × 150 mm) *(10)*. Using two yeast strains, *S. cerevisiae* and *C. lipolytica*, which are known to produce the base material of ceramide, sphingolipid, the amount of ceramide produced and the change in composition of the ceramide were measured under different fermentation conditions, in order to determine the optimal conditions.

Results and Discussion

Effect of pH and Temperature on Production of Ceramide

To determine the optimal culture conditions for *S. cerevisiae*, an experiment was conducted by varying the temperature and pH. Cell culture was done in shake flasks by adjusting the pH to 4.0, 5.0, 6.0, 7.0, and 8.0 before sterilization and the temperature to 20, 25, 30, 35, and 40°C. Ceramide was produced using different initial conditions in which the initial pH was set to 4.0, 5.0, 6.0, 7.0, and 8.0. The peak area of the HPLC was calculated,

Table 1
Effect of Temperature and pH on Ceramide Production

Temperature (°C)	pH	Ceramide (mg/L)
20	5.0	1.35
20	6.0	0.77
20	7.0	0.38
30	5.0	1.41
30	6.0	1.46
30	7.0	0.83
40	5.0	1.01
40	6.0	0.62
40	7.0	0.64

in order to compare the amount of ceramide produced by culturing *S. cerevisiae*. The optimal pH values for producing ceramide at 30°C were 5.0 and 6.0, as shown in Table 1. The amount of ceramide produced at pH 5.0 was almost the same as that produced at pH 6.0. The ceramide content in the lipid was 3%.

The culture temperature for cell growth was varied from 20 to 40°C. At pH 7.0, the amount of ceramide produced at 30°C was twice as much as that produced at 35°C, showing the same optimal growth conditions for *S. cerevisiae* at 30°C. When the culture temperature was increased to 40°C, the amount of ceramide produced decreased significantly. Figure 1 shows the contour graph of the amount of ceramide produced with change in temperature and pH. The optimal pH for the production of ceramide was 5.5 and the optimal temperature was 30°C.

Factorial design was performed using the temperature as the *x*-axis and the pH as the *y*-axis, in order to determine the optimal conditions for producing ceramide according to the pH and temperature. Based on the results, shown in Fig. 1, the following equation was used to determine the variables:

$$Y = \alpha_0 + \sum_i \alpha_0 X_i + \sum_i \sum_j B_{ij} X_i X_j \qquad \text{(in which } i, j = 1, 2) \qquad (1)$$

in which X_1 is the temperature and X_2 is the pH. The amount of ceramide produced was expressed as a function of temperature and pH. The parameters were determined using nonlinear regression analysis:

$$Y = 3.40 \times 10^{-1} X_1 + 1.70 X_2 - 5.66 \times 10^{-3} X_1^2 - 0.142 X_2^2 - 8.76 \qquad (2)$$

Using the equation obtained from this study, the optimal pH and temperature were determined. The optimal pH was found to be 6.0 and the optimal temperature 30°C. These results were modeled to reflect the effects of pH and temperature on ceramide production. Simulation of the optimization technique maximizing cell growth and ceramide produc-

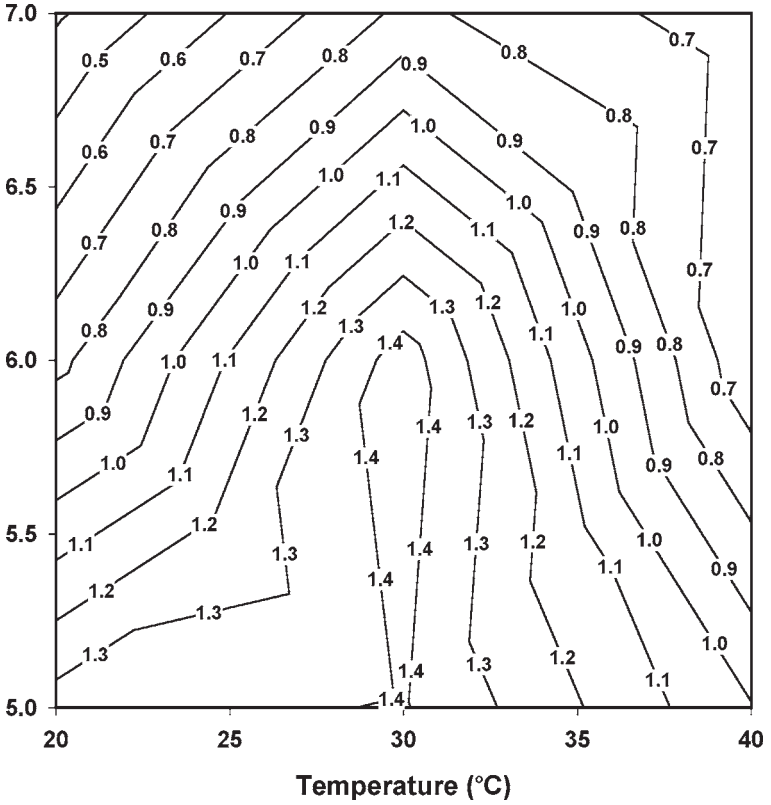

Fig. 1. Contour diagram of ceramide production at different pH values and temperatures (mg of ceramide/g of cell).

tion was possible when these results were combined with the effects on cell growth.

Effect of Heat Shock on Ceramide Production

When heat shock was applied at the time of ceramide production, the amount of ceramide produced increased, owing to the synthesis of heat-shock protein and the accumulation of trehalose *(6)*. A study was conducted using optimal heat-shock conditions for increasing ceramide production. After culturing, *S. cerevisiae* was incubated at 40°C. The effect of heat shock on ceramide production was investigated by incubating the cells at 40°C for different time periods. When organisms face the risk of unpredictable danger caused by harmful physical environments such as high temperature, they develop defense mechanisms in order to cope with danger. In this case, *S. cerevisiae* produces a great deal of ceramide in response to heat shock. When ceramide was extracted at each time period, the results showed that the amount of ceramide produced when the culture was done at 40°C for 1 h (Fig. 2B) was 5.9 times higher than that when the culture was done at 40°C for 30 min (Fig. 2A).

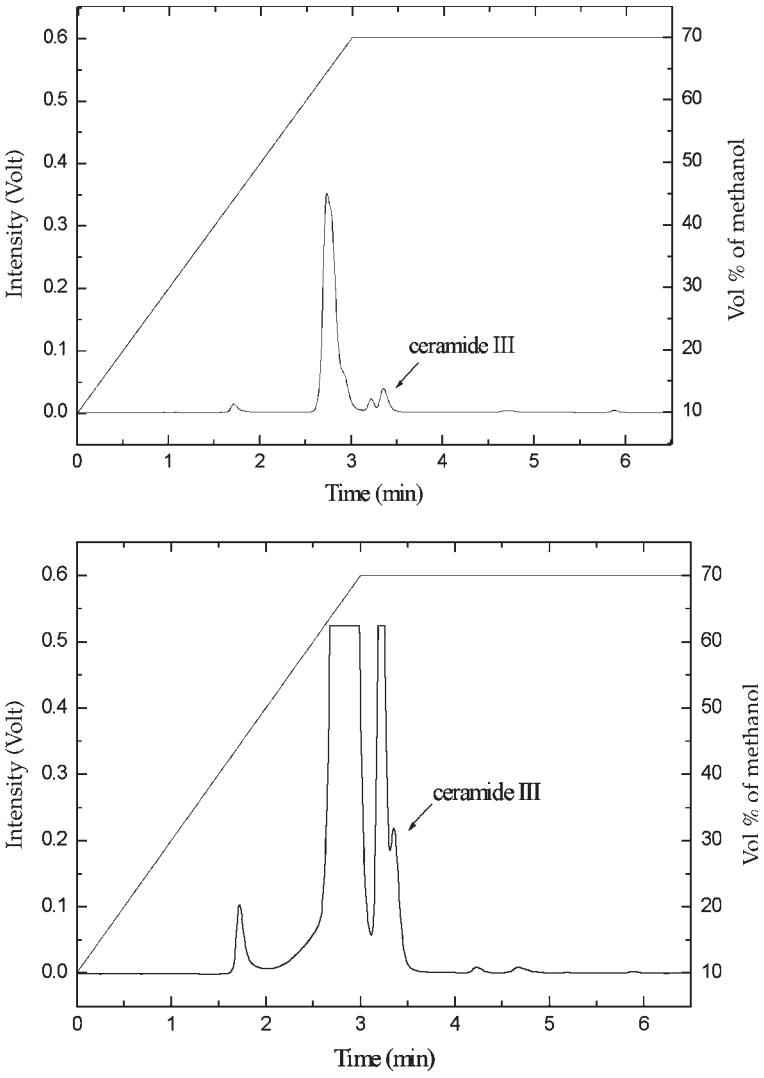

Fig. 2. Chromatogram of ceramide production while culture was stirred for **(Top)** 30 min after increasing temperature from 30 to 40°C and **(Bottom)** 1 h after increasing temperature from 30 to 40°C.

Ceramide Production With Batch Fermentation

To the best of our knowledge, no studies have previously been carried out on the production of ceramide with *S. cerevisiae* batch fermentation. In the work, the feasibility of ceramide production with batch fermentation was studied using the optimal conditions of pH 6.0 and 30°C. Cell mass and ceramide concentrations were measured and are represented in Fig. 3. The amount of ceramide produced was about 3.5% of the total lipids, and the majority of the ceramide was produced during the period after cell growth.

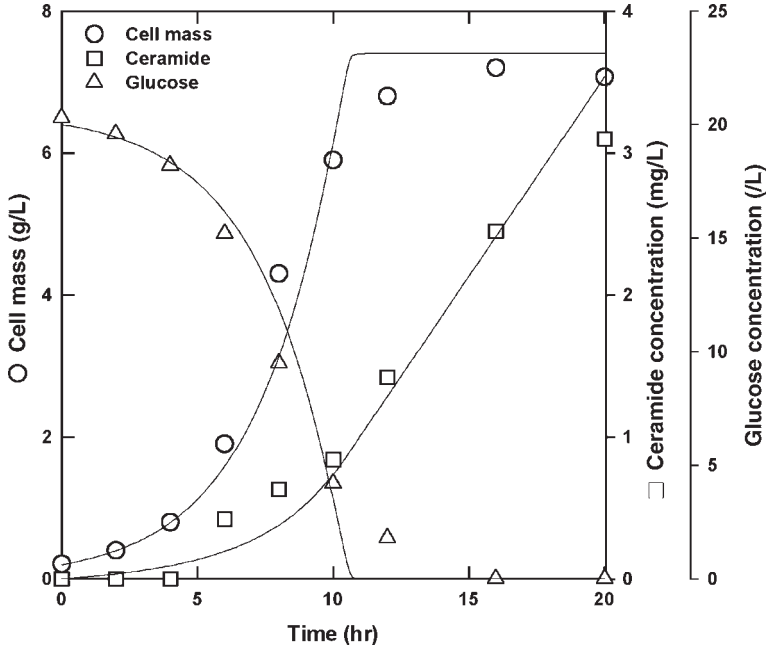

Fig. 3. Cell growth and ceramide production with batch fermentation (at 30°C and pH 6.0).

A cell growth and ceramide production model was developed based on this study. We assumed that the cell growth rate follows Monod kinetics and that the ceramide production rate follows the Leudecking-Piret model:

$$\frac{dX}{dt} = \frac{0.35 \cdot S}{0.3 + S} X \tag{3}$$

$$\frac{dS}{dt} = -\frac{1}{Y_{X/S}} \frac{0.35 \cdot S}{0.3 + S} X \tag{4}$$

$$\frac{dP}{dt} = 0.02 \cdot \frac{dX}{dt} + 0.038 \cdot X \tag{5}$$

in which X is the cell mass (g/L), S is the substrate concentration (g/L), and P is the product concentration (mg/L). The cell yield obtained from the substrate was 0.36. The constitutive production rate of ceramide was higher than that produced by the associated cell growth. Based on the results of the present study, the production of ceramide requires cell growth at high concentrations, the amount of lipid needs to be increased during heat shock, and the fermentation time needs to extended for the lipid synthesis following cell growth.

Conclusion

The optimal conditions for ceramide production were examined by selecting two different yeast strains. Among the different yeast strains, not many produce ceramide. After *S. cerevisiae* (KCCM 50515) and *C. lipolytica* were compared for their ability to produce ceramide, *S. cerevisiae* was selected and used for ceramide production with batch fermentation. The amount of ceramide produced was investigated as a function of pH, and it was found that the maximum amount of ceramide was produced between pH 5.0 and 6.0, because maximum yeast growth was observed at pH 5.5. As the pH increased up to pH 7.0, the amount of ceramide produced decreased.

During cell cultivation, heat shock was applied to the cells to increase the amount of ceramide produced. The amount of ceramide produced was increased by 5.9 times after heat shock was applied to the cells cultured at 30°C, by increasing the temperature to 40°C. We believe that this increase in the amount of ceramide produced probably resulted from the accumulation of trehalose, owing to the synthesis of heat-shock protein. Thus, the amount of ceramide produced could be drastically increased using this heat-shock treatment. Cell growth occurred rapidly during batch fermentation and, as in the case of other yeasts, the accumulation of ceramide occurred after cell growth. We therefore believe that ceramide production could be maximized by performing the fermentation with a limited supply of substrate.

Acknowledgment

This work was supported by research funds from Chosun University 2003.

References

1. Rupeic, J., Mesaric, M., and Maric, V. (1998), *Appl. Microbiol. Biotechnol.* **50,** 583–588.
2. Wertz, P. W. and van den Bergh, B. (1998), *Chem. Phys. Lipids* **91,** 85–96.
3. Dickson, R. C. and Lester, R. L. (1999), *Biochim. Biophys. Acta Biomembr.* **1426,** 347–357.
4. Skrzypek, M. S., Nagiec, M. M., Lester, R. L., and Dickson, R. C. (1998), *J. Biol. Chem.* **273,** 2829–2834.
5. Bouwstra, J. A., Gooris, G. S., Dubbelaar, F. E. R., Weerheim, A. M., Ijzerman, A. P., and Ponec, M. (1998), *J. Lipid Res.* **39,** 186–196.
6. Vicanova, J., Weerheim, A. M., Kempenaar, J. A., and Ponec, M. (1999), *Arch. Dermatol. Res.* **291,** 405–412.
7. Kim, S. K., Kang, D. H., Yun, H. S., and Row, K. H. (2001), *Theories Appl. Chem. Eng.* **7,** 773–776.
8. Wells, G. B., Dickson, R. C., and Lester, R. L. (1998), *J. Biol. Chem.* **273,** 7235–7243.
9. Norlen, L., Nicander, I., Lundsjo, A., Cronholm, T., and Forslind, B. (1998), *Arch. Dermatol. Res.* **290,** 508–516.
10. Zhou, J. Y., Chaminade, P., Gaudin, K., Prognon, P., and Baillet, A. (1999), *J. Chromatogr. A* **859,** 99–105.