J. Gen. Appl. Microbiol., **45**, 125–128 (1999)

Short Communication

Substrate specificities in triacylglycerol-secretion by the yeast, *Trichosporon* sp.

Yasuhiro Nojima, Masami Matsumoto,[1] Chie Kurose,[1] Hiroaki Matsuzaki,[1] Takushi Hatano,[1,*] Takashi Yagi,[2] and Sakuzo Fukui[1]

Department of Fermentation Technology, Faculty of Engineering, Hiroshima University, Higashi-Hiroshima 739–8527, Japan
[1]Department of Bioscience and Biotechnology, Faculty of Engineering, Fukuyama University, Fukuyama 729–0292, Japan
[2]Research and Development Center, Showa Sangyo Co., Ltd., Funabashi 273–0015, Japan

(Received January 6, 1999; Accepted July 2, 1999)

Key Words——chain length; fatty acid; secretion of triacylglycerol; *Trichosporon* sp.

Research and development on the microbial production of useful biolipids have been progressed with deep interest in a stable supply of the valuable oils by fermentation processes. Until now, the production of eicosapentaenoic acid (Shimizu et al., 1988), γ-linolenic acid (Hansson and Dostalek, 1988), and docosahexaenoic acid (Li and Ward, 1994), using various oleaginous fungi, has been reported. However, like other oleaginous plants or animals, processes for cell disruption and oil extraction were still essential to recover the products because they were entirely accumulated in the cell. We have challenged development of the excretive production of triacylglycerols (TGs) by microorganisms, such as yeasts and fungi, for realization of low-cost lipid fermentation.

We previously, isolated the yeast *Trichosporon* sp. strain SH45Y and bred its mutant strain E2, which extracellularly accumulates TGs containing palmitoleic acid in the ethyl palmitate medium (Yagi et al., 1994). Palmitoleic acid has the biological function of preventing apoplexy (Yamada et al., 1989). For an increased production of palmitoleic TGs from ethyl palmitate, we have further obtained the strain E228 (Fukui and Yagi, 1994) by the screening, using an unsaturated fatty acid-auxotrophic yeast strain as a tester (Yagi et al., 1994), after mutagenesis on E2. Furthermore, we have bred the novel mutant strain of *Trichosporon* sp., L-12, which excretes TGs from glucose as the substrate for growth (Nojima et al., 1995). In this communication, we studied substrate specificities in the excretive production of TGs by the mutant strains of yeast *Trichosporon* sp.

At first, we examined the production of TGs by the strain E228 cultivated in the liquid medium (Yagi et al., 1994) containing 1% free fatty acids or fatty acid-ethyl esters. Cultivation was performed at 28°C with shaking (120 strokes/min). After a 4-day incubation, products accumulated in the intra- and extracellular space were extracted and analyzed by TLC and HPLC (Nojima et al., 1995; Yagi et al., 1994). Table 1 shows the growth of cells and the extracellular production of TGs on various substrates. Cells were grown on long-chain free fatty acids (C_{14}–C_{22}), but not on middle chain fatty acids (C_8–C_{12}). Because free fatty acids are hardly soluble in water, we exchanged them for their ethyl esters, which are more emulsive than free acids. The cell growth was recovered on ethyl caprate (C_{10}) and ethyl laurate (C_{12}), though not on caprylate (C_8). Extracellular accumulation of TGs was accompanied with cell growth when fatty acids or its esters having a chain length of up to 16 carbon-atoms were used as the substrates. Over the length of 18 carbons, however, oleic acid and its ester could be used only as the substrates for TG-excretion. Cells might be just able to grow and excrete TGs on oleate ($C_{18:1}$) because this substrate is more stable in the oxidation conditions during cultivation than linoleate ($C_{18:2}$). The melt-

* Address reprint requests to: Dr. Takushi Hatano, Department of Bioscience and Biotechnology, Faculty of Engineering, Fukuyama University, Fukuyama 729–0292, Japan.

ing temperatures of arachidate ($C_{20:0}$) and behenate ($C_{22:0}$) are much higher than the cultivation temperature, so it was thought that cells could hardly use these substrates. Amounts of the extracellular TGs have not corresponded with cell growth.

The constitution of fatty acids in TGs secreted by the yeast grown in fatty acid-ethyl esters was shown in Table 2. In almost all products, the numbers of carbon-atoms of major constituent fatty acid were coincident with that of the substrate. When ethyl palmitate was used as the substrate, palmitic acid and palmitoleic acid were accumulated in the TGs. The amount of palmitoleic acid was about half of palmitic acid. A small amount of palmitoleic TGs was also produced from ethyl palmitate; however, further desaturation and chain elongation of the constituents were not observed on this substrate. From these results, it was considered that this yeast could take up the fatty acids or ethyl esters, which have 12 to 16 carbon-atoms as the growth substrates; then it could deposit the excess of input as TGs in the extracellular space. In this process, the conversion of saturated fatty acid to an unsaturated one specifically occurs for palmitic acid (C_{16}).

Table 1. Cell growth and triacylglycerol (TG)-secretion on various substrates.[a]

Substrate			Growth[b]	TG-secretion[c]
Caprylate	($C_{8:0}$)	Free	−	−
		Ethyl ester	−	−
Caprate	($C_{10:0}$)	Free	−	−
		Ethyl ester	±	+
Laurate	($C_{12:0}$)	Free	−	−
		Ethyl ester	++	++
Myristate	($C_{14:0}$)	Free	+	++
		Ethyl ester	++	++
Palmitate	($C_{16:0}$)	Free	±	±
		Ethyl ester	+	++
Palmitoleate	($C_{16:1}$)	Free	±	±
		Ethyl ester	±	±
Stearate	($C_{18:0}$)	Free	±	−
		Ethyl ester	±	−
Oleate	($C_{18:1}$)	Free	±	+
		Ethyl ester	±	±
Linoleate	($C_{18:2}$)	Free	±	−
		Ethyl ester	±	−
Arachidate	($C_{20:0}$)	Free	±	−
		Ethyl ester	±	−
Behenate	($C_{22:0}$)	Free	±	−
		Ethyl ester	±	−

[a] The strain E228 was used.
[b] −, no; ±, poor; +, moderate; ++, well.
[c] −, no; ±, slightly; +, fairly; ++, well.

It is generally known that the middle chain fatty acids are poisonous for many organisms. In this experiment, growth of this microorganism was not inhibited by the fatty acids having 10 to 14 carbon-atoms. We have already reported the microbial transformation of fatty acids to methylalkylketones (*n*-alkane-2-ones), useful chemicals as biofuel, flavor, solvent, and biological signals, using the fungi and yeasts (Yagi et al., 1992, 1991). In these reports, middle chain (C_8–C_{14}) fatty acids were easily converted to *n*-alkane-2-ones, but the fatty acids containing more than 16 carbons were hardly transformed by all the microorganisms examined. From these observations, we speculated that the microorganisms would avoid the toxicity of middle chain (C_8–C_{14}) fatty acids through their conversion to nonpoisonous compounds such as *n*-alkane-2-ones. In regard to extracellular production of TGs, therefore, we would propose the following consideration: The conversion of the fatty acids to TGs and excretive TG-accumulation is a convenient mechanism to avoid the toxicity of middle chain fatty acids in the yeast *Trichosporon* sp.

The production of TGs in the medium containing fatty acid-ethyl esters (C_{12}–C_{16}, 1%) or glucose (1%) was monitored for one week (Figs. 1, 2). The measurement of glucose in the medium was performed by HPLC (Nojima et al., 1995). The substrates were con-

Table 2. Extracellular accumulation of triacylglycerol (TG) on fatty acid-ethyl esters by the strain E228.

Substrate		Substrate consumption (mg)	Extracellular TG (mg/100ml)	Constituent fatty acids (mg/100 ml)							
				$C_{10:0}$	$C_{12:0}$	$C_{14:0}$	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$
Ethyl caprate	($C_{10:0}$)	386.4	62.2	39.8	ND	ND	3.4	ND	4.4	9.9	1.9
Ethyl laurate	($C_{12:0}$)	751.8	191.9	ND	30.6	ND	20.6	2.6	19.1	82.8	13.7
Ethyl myristate	($C_{14:0}$)	501.3	192.9	ND	ND	134.6	1.2	ND	ND	20.5	9.9
Ethyl palmitate	($C_{16:0}$)	380.1	167.6	ND	ND	ND	90.6	38.6	1.4	11.1	5.1
Ethyl palmitoleate	($C_{16:1}$)	84.8	24.4	ND	ND	ND	0.5	20.9	ND	1.6	0.3
Ethyl oleate	($C_{18:1}$)	183.7	4.5	ND	ND	ND	1.0	ND	ND	2.1	1.2

Cells were cultivated at 28°C for 96 h in the medium containing 1% fatty acid-ethyl esters.
Inoculation: 2×10^7 cells.
ND: Not detectable.
Abbreviations: $C_{10:0}$, $C_{12:0}$, $C_{14:0}$, $C_{16:0}$, $C_{16:1}$, $C_{18:0}$, $C_{18:1}$, and $C_{18:2}$ indicate capric, lauric, myristic, palmitic, palmitoleic, stearic, oleic, and linoleic acid, respectively.

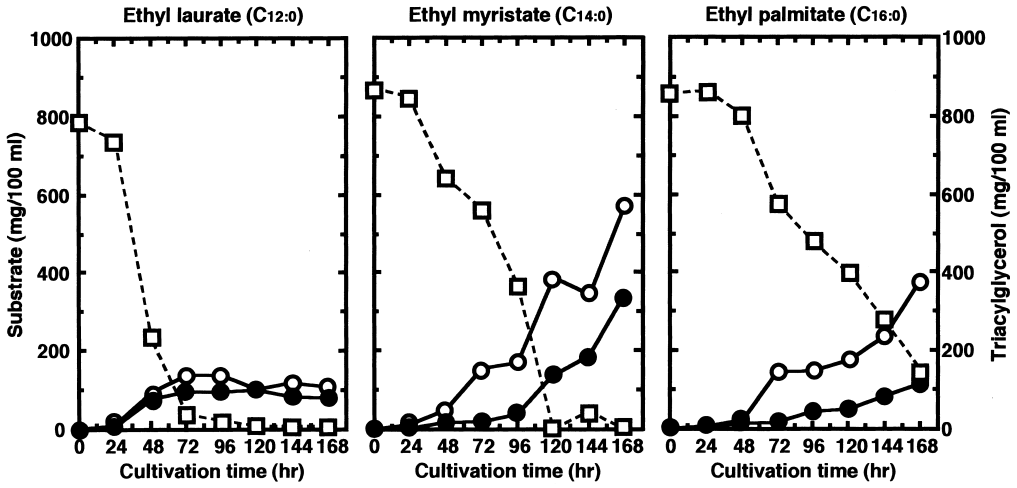

Fig. 1. Time course of triacylglycerol (TG) production on fatty acid-ethyl esters by the strain E228.
Cells were cultivated at 28°C in the medium (100 ml) containing 1% of each substrate. ○, extracellular TGs; ●, intracellular TGs; □, substrate.

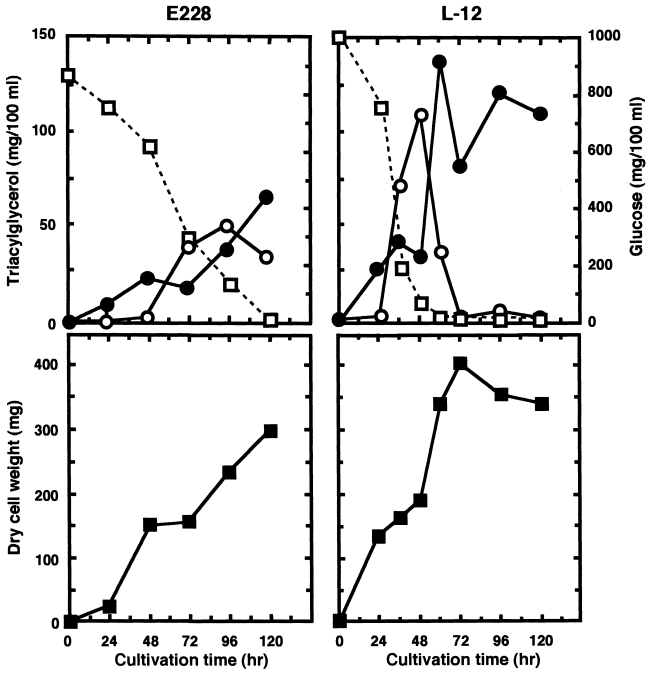

Fig. 2. Time course of triacylglycerol (TG) production on glucose by the strain E228 and L-12.
Cells were cultivated at 28°C in the medium (100 ml) containing 1% of glucose. Cell growth was also monitored as the dry cell weight (■). ○, extracellular TGs; ●, intracellular TGs; □, substrate.

sumed, and the products (TGs) were accumulated both in the intra- and extracellular spaces. The amounts of TGs were increased as long as each substrate was remaining in the medium. After consumption of the substrate, the modes of TG-accumulation were dissimilar in the fatty acid medium and in the glucose medium. On ethyl laurate, the level of both intra- and extracellular TGs reached a plateau, and that of TGs formed on glucose was drastically changed. Subsequent to a use up of glucose, the amounts of extracellular TGs immediately decreased with an increase of the intracellular TGs (Fig. 2). This phenomenon was more remarkable in the strain L-12 than in the E228. The accumulation of extra- and intracellular TGs has not arrested the cell growth of both strains. The middle chain fatty acids were not detectable in the constituents of TGs formed on glucose (Nojima et al., 1995). The activities of lipase, also, were hardly detected in either medium. These results indicate that the extracellular TGs accumulated in the glucose medium were able to be taken up once more and were usable as the energy sources by the cells, although the TGs converted from ethyl laurate (Fig. 1, Table 1) would not be used anymore because of the toxicity of its constituent lauric acid (Hatton and Kinderlerer, 1991). The decrease in the amounts of excreted TGs on other fatty acid-ethyl esters (C_{14} and C_{16}) could not be checked in the term of this experiment. Recently we have observed that the level of extracellular TGs does not reduce on the substrates of fatty acids (1%), as long as they remained over 0.2% in the medium.

In this study, the highest conversion rate (about 60%, TGs excreted to substrate consumed) for TG-secretion was obtained in ethyl myristate medium after a one-week cultivation (Fig. 1). So myristic acid was considered to be most effective for the secretion of TGs by the *Trichosporon*; however, this substrate is more expensive. Further research is necessary for the establishment of low-cost excretive fermentation of TGs, which are available for the biofuels, using this yeast system.

We wish to express our sincere gratefulness to T. Miyakawa (Hiroshima University) for meaningful discussion and helpful advice. We also thank T. Suetsugu, N. Hata, and H. Saeki (Fukuyama University) for their technical assistance. This work was supported in part by a Grant-in-Aid for scientific research (Nos. 05556017 and 06660123) from the Ministry of Education, Science, Sports, and Culture, Japan.

References

Fukui, S. and Yagi, T. (1994) Japan Patent, No. 6-245759.

Hansson, L. and Dostalek, M. (1988) Effect of culture condition on mycelial growth and production of γ-linolenic acid by the fungus *Mortierella ramanniana*. *Appl. Microbiol. Biotechnol.*, **28**, 240–246.

Hatton, P. V. and Kinderlerer, J. L. (1991) Toxicity of medium chain fatty acids to *Penicillium crustosum* Thom and their detoxification to methyl ketones. *J. Appl. Bacteriol.*, **70**, 401–407.

Li, Z. Y. and Ward, O. P. (1994) Production of docosahexaenoic acid by *Thraustochytrium roseum*. *J. Ind. Microbiol.*, **13**, 238–241.

Nojima, Y., Yagi, T., Miyakawa, T., Matsuzaki, H., Hatano, T., and Fukui, S. (1995) Extracellular formation of triglycerides from glucose by a mutant strain of *Trichosporon*. *J. Ferment. Bioeng.*, **80**, 88–90.

Shimizu, S., Shinmen, Y., Kawashima, H., Akimoto, K., and Yamada, H. (1988) Fungal mycelia as a novel source of eicosapentaenoic acid. Activation of enzyme(s) involved in eicosapentaenoic acid production at low temperature. *Biochem. Biophys. Res. Commun.*, **150**, 335–341.

Yagi, T., Hatano, T., Fukui, F., and Fukui, S. (1992) Subterminal hydroxylation of ketoalkanes by fungi. *J. Ferment. Bioeng.*, **74**, 218–221.

Yagi, T., Hatano, A., Nakanishi, T., Hatano, T., and Fukui, S. (1994) Extracellular production of palmitoleic triglycerides by a yeast, *Trichosporon*. *J. Ferment. Bioeng.*, **77**, 164–168.

Yagi, T., Kawaguchi, M., Hatano, T., Hatano, A., Nakanishi, T., Fukui, F., and Fukui, S. (1991) Formation of *n*-alkane-2-ones and *n*-alkane-2-ols from triglycerides by a black yeast, *Aureobasidium*. *J. Ferment. Bioeng.*, **71**, 93–99.

Yamada, H., Shimizu, S., Shinmen, Y., Kawashima, H., and Akimoto, K. (1989) Biotechnological process for production of polyunsaturated fatty acids. *J. Disp. Sci. Technol.*, **10**, 561–579.