http://www.pjbs.org

PJBS

ISSN 1028-8880

Pakistan
Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 12 (13): 986-990, 2009
ISSN 1028-8880

Growth Temperatures and Various Concentrations of Ricinoleic Acid Affect Fatty Acid Composition in Two Strains of *Hansenula polymorpha*

Wanida Rakpuang
Department of Biology, Faculty of Science, Mahasarakham University,
Maha Sarakham 44150, Thailand

Abstract: The fatty acid composition of two strains (wild-type and M2 mutant cells of *Hansenula polymorpha* strain CBS 1976) were studied at different growth temperatures and various concentrations of ricinoleic acid. Two strains of yeast cultured on YEPD medium containing 1, 2, 3 and 8 mM of ricinoleic acid at 25, 30, 37 and 45°C. Lipids were extracted from the yeast culture and the fatty acids esterified with BF_3-MeOH. Gas chromatography analysis of total lipids showed that C16:1 ($\Delta7$), which has been synthesized in low concentration by WT strain, was found to increase in the M2 mutant. The biotransformation of C16:1 ($\Delta7$) found in M2 indicated the presence of dehydroxylation and β-oxidation systems. An increase in the growth temperature from 25 to 45°C resulted in a decrease in the total unsaturated fatty acids of C16:1, C18:1, C18:2 and C18:3 from 44.0 to 22.1% and 65.0 to 49.3% of the total fatty acids in M2 and wild-type strains, respectively. The differential production of unsaturated fatty acids, especially C16:1, indicated that regulation of unsaturated fatty acid levels, is an important control point in membrane composition in the adaptation of *H. polymorpha* M2 to diet and temperature.

Key words: Fatty acid auxotrophic mutant, *Hansenula polymorpha*, ricinoleic acid, temperature

INTRODUCTION

Temperature has a profound influence on the fatty acid composition in many organisms (Farrell and Rose, 1967; Brown and Rose, 1969; Harris and James, 1969; Sato *et al*., 1979; Konova *et al*., 2009). It is known that yeasts, like other microorganisms, alter their fatty acid composition in response to different growth temperatures. These temperature-induced alterations in lipid composition have been interpreted as the adaptation of yeast cells in order to optimize membrane structure and function to different growth temperatures. In some yeast species, lowering the temperature at which yeasts were grown resulted in increased levels of the Unsaturated Fatty Acid (UFA) content (Suutari *et al*., 1997; Wongsumpanchai, 2005).

The effect of supplementation of substrate to the growth medium of microorganisms results in a modification of the lipid composition (Papanikolaou and Agglis, 2003; Lin, 2006; Papanikolaou *et al*., 2006; Easterling *et al*., 2009). The proportion of gamma-linolenic acid (GLA) was affected by media composition in *H. polymorpha* (Laoteng *et al*., 2005). Supplementing the M2 mutant of *H. polymorpha* 's growth medium with palmitoleic acid (C16:1-$\Delta9$) results in increased expression of the $\Delta12$ fatty acid desaturase and in the synthesis of the unusual UFA of C16:2 ($\Delta9$,12-cis-hexadecadienoic acid). The realization of the pathway of fatty acid synthesis and the role of fatty acids on biological processes has led to the development of the fatty acid elongation defective mutant which isolated from the ethyl methanesulfonate (EMS) treated *H. polymorpha.* Substantial information concerning substrate specificity and regulation of desaturase activity in *H. polymorpha* have been obtained by biochemical and genetic analyses of mutants defective in some specific steps of desaturation (Anamnart *et al*., 1998). However, it is still poorly understand in its fatty acid biosynthesis mechanisms in *H. polymorpha*.

The objectives of this study were to examine the cellular manipulation of fatty acid production in response to the simultaneous changes in growth temperature and availability of fatty acid supplements in wild-type and M2 mutant of *H. polymorpha* CBS 1976.

MATERIALS AND METHODS

Strains, media and culture conditions: From July 2007 to September 2008, the auxotrophic strain *leu1-1* of *H. polymorpha* derived from CBS 1976 (NCYC 495) (Gleeson and Sudbery, 1988), donated by Prof. M.A. Gleeson (University of Sheffield, UK), was used as parental strain and compared of relative fatty acid composition to M2 mutant of *H. polymorpha*. The unsaturated fatty acid (UFA) auxotrophic mutant, M2, of *H. polymorpha* which require UFAs for growth was obtained after EMS mutagenesis of a parental strain *leu1-1*, supplied by Dr. S. Anamnart (University of Chulalongkorn, Thailand) (Anamnart *et al*., 1998). Strains were grown in a YEPD medium containing 1% yeast extract, 2% bactopeptone and 2% glucose, supplemented with four concentrations of ricinoleic acid (C18:1 OH), contained 1, 2, 3 and 8 mM at four temperatures (25, 30, 37 and 45°C). Fatty acid C18:1 OH emulsifying in 1% Triton X-100 was supplemented in the YEPD media at various concentrations. The yeast cells were suspended at the concentration of 2×10^4 cells in 5 mL YEPD supplemented with different concentrations of C18:1 OH at noted growth temperatures and then were cultured to the late logarithmic phase on a reciprocal shaker (120 rpm). Cell densities were determined by spectrophotometry with absorbance at 660 nm.

Lipid extraction: Following incubation, total volume of each of the fatty acid mixtures was extracted according to the method of Lepage and Roy (1984). Total fatty acids, including both free and incorporated into different cellular compounds, were extracted from whole cells by direct saponification with 10% KOH in methanol. Lipids were saponified in a 80°C water bath for 2 h and immediately cooled to room temperature. After the extraction of unsaponified materials from the mixture using petroleum ether, the aqueous phase was acidified with 6 N HCl and fatty acids were recovered by diethyl ether extraction. The recovered fatty acids were converted to methyl esters by BF_3-catalyzed methanolysis (Morrison and Smith, 1964).

Fatty acid analysis: Samples of Fatty Acid Methyl Esters (FAMEs) were analyzed by gas chromatography on a Shimadzu 17A gas chromatograph using a DB-WAX capillary column (30×0.25 mm i.d.×0.25 μm in film thickness). Instrument conditions were as follows: injector temperature 250°C, detector temperature 250°C. The oven temperature was programmed as follows: 20 min at 200°C, 10°C $\min^{-1}$ and held at this latter temperature for 10 min. Identification of FAMEs was by comparison of retention times to those of known FAME standards (NuCheck, Inc., Elysian, MN). The fatty acid compositions were expressed as percentages in total fatty acids (Carroll, 1961). Relative amounts of fatty acids were calculated from their chromatographic peak areas. All analysis were performed in three replications and three fatty acid analyses were done for each replication. The statistically significant differences in fatty acid contents between the conditions are indicated by asterisks.

RESULTS AND DISCUSSION

The growth profiles of WT and M2 mutant of *H. polymorpha* in liquid media containing various concentrations of C18:1 OH and different growth temperatures were further analysed by spectrophotometry with absorbance at 660 nm. (data not shown). The growth of M2 mutant on media supplemented with C18:1 OH were also less than the growth of WT strain under in any conditions. The defection in some specific steps of desaturation could be proposed as the simplest explanation for this phenomenon (Anamnart *et al*., 1998). Moreover, the highest growth of the M2 were detected in the optimal condition of 3 mM C18:1 OH supplementation at 37°C. The compositions of the FAMEs from WT strain and M2 mutant of *H. polymorpha* cultivated on YEPD supplemented with various concentrations of C18:1 OH and different growth temperatures are shown in Table 1 and 2, respectively. When yeast cells were fed the fatty acid C18:1 OH into the culture media in various concentrations, no harmful effects on the growth of WT and M2 strains were observed. Fatty acid supplied to yeast cells in the growth media were found to be

Table 1: Relative fatty acid composition of parental strain (WT) and M2 mutant of *H. polymorpha* grown in YEPD broth supplemented with various concentrations of C18:1 OH (A, 1mM; B, 2 mM; C, 3 mM; D, 8 mM) at 37°C (logarithmic phase, 24 h)

	Composition in total fatty acids (%)[a]							
	Strain WT				Strain M2			
Fatty acid	A	B	C	D	A	B	C	D
14:0	1.4±0.3	2.5±0.3	1.7±0.2	<1	<1	1.3±0.1	1.2±0.2	<1
16:0	5.8±0.2	7.2±0.3	7.4±0.3	4.3±0.2	18.7±0.9	13.2±0.6	16.8±0.8	16.5±0.8
16:1(Δ7)	<1	1.1±0.1	1.7±0.3	<1	6.3±0.3	10.8±0.5	15.6±0.7	4.2±0.2
18:0	12.5±0.6	13.2±0.6	9.1±0.8	8.2±0.4	12.6±0.6	14.7±0.7	17.5±0.4	19.4±0.9
18:1(Δ9)	30.6±0.9	26.3±1.3	29.1±0.8	18.9±0.9	25.5±1.2	16.3±0.3	10.9±1.0	4.7±0.2
18:1 OH	9.6±0.4	15.0±0.7	20.8±0.4	48.1±1.4	12.9±0.6	17.8±0.8	21.1±1.5	50.2±1.1
18:2(Δ9,12)	36.5±1.2	32.4±1.1	28.1±0.9	17.9±0.9	17.7±0.8	16.5±0.8	11.9±1.0	3.1±0.1
18:3(Δ9,12,15)	2.6±0.1	1.8±0.2	1.4±0.2	1.1±0.1	5.3±0.2	8.9±0.4	4.5±0.2	1.2±0.2
Other	<1	<1	<1	<1	<1	<1	<1	<1

[a]Values represent the averages of three independent experiments. The fatty acid composition of each experiment was analyzed triplicate

Table 2: Relative fatty acid composition of parental strain (WT) and M2 mutant of *H. polymorpha* grown in YEPD broth supplemented with 3 mM of C18:1 OH at different growth temperatures. (logarithmic phase, 24 h)

	Composition in total fatty acids (%)[a]							
	Strain WT				Strain M2			
Fatty acid	25°C	30°C	37°C	45°C	25°C	30°C	37°C	45°C
14:0	<1	1.3±0.1	1.7±0.2	<1	1.1±0.1	2.1±0.1	1.2±0.2	3.2±0.1
16:0	5.7±0.2	10.2±0.5	7.4±0.3	15.3±0.7	15.8±0.7	16.3±0.8	16.8±0.8	29.2±1.4
16:1(Δ7)	<1	1.3±0.2	1.7±0.3	<1	8.1±0.4	10.5±0.5	15.6±0.7	4.5±0.2
18:0	7.4±0.3	6.6±0.3	9.1±0.8	11.7±0.5	16.3±0.8	16.4±0.8	17.5±0.4	26.6±1.3
18:1(Δ9)	34.6±1.2	28.2±1.4	29.1±0.8	24.5±0.6	24.4±1.2	19.7±0.9	10.9±1.0	14.9±0.7
18:1 OH	19.9±1.0	20.1±0.7	20.8±0.4	22.4±0.9	22.5±1.1	21.8±1.0	21.1±1.5	18.4±0.9
18:2(Δ9,12)	28.2±0.5	30.6±1.1	28.1±0.9	23.8±1.1	8.4±0.4	8.7±0.4	11.9±1.0	2.2±0.1
18:3(Δ9,12,15)	1.7±0.1	1.1±0.0	1.4±0.2	<1	3.1±0.1	4.1±0.2	4.5±0.2	<1
Other	<1	<1	<1	<1	<1	<1	<1	<1

[a]Values represent the averages of three independent experiments. The fatty acid composition of each experiment was analyzed triplicate

preferentially incorporated into the cells during the incubation. It is clear from the data that the exogenous fatty acid become a major fraction of the fatty acids into the cells. Major fatty acid constituents of the WT strain investigated are the normal stearic (C18:0), oleic (C18:1-Δ9) and linoleic (C18:2-Δ9,12) acids, in cells grown in media supplemented with various concentrations of substrate at 37°C. The amounts of myristic (C14:0), palmitic (C16:0), palmitoleic (C16:1-Δ7) and α-linolenic (C18:3-Δ9,12,15) acids are low (Table 1). C16:1(Δ7) was never detected in the WT cells grown on C18:1 OH-free media and minor amounts (<1-1.7% of total fatty acids, Table 1) were detected during grown on YEPD media supplemented with C18:1 OH.

Total fatty acids of M2 cells grown in media supplemented with various concentrations of substrate at 37°C showed similar substrate-induced changes in their fatty acid compositions (Table 1). The major fatty acids were C16:0, C16:1(Δ7), C18:0, C18:1(Δ9) and C18:2(Δ9,12). The highest proportion of C16:1(Δ7) was observed in media supplemented with 3 mM of C18:1 OH at 37°C. The greater concentrations of endogenous C16:0, C16:1(Δ7), C18:0 and C18:3(Δ9,12,15) are present in M2 cells cultured at 37°C with C18:1 OH supplement, while in WT cells, the greater concentrations of endogenous fatty acids are C18:1(Δ9) and C18:2(Δ9,12) (Table 1, Fig. 1). The results obtained (Table 1) seem to indicate that addition of fatty acid to the growth medium of WT and M2 cells of *H. polymorpha* resulted in dramatic alteration in fatty acid profiles. The majority of the published data on the effects of substrate on the lipid composition of fungi have been primarily concerned with the fatty acid composition (Anamnart *et al*., 1998; Papanikolaou and Aggelis, 2003; McDonough and Roth, 2004; Laoteng *et al*., 2005; Fakas *et al*., 2007). However, the expression profiles were different depending on the type of exogenous fatty acids supplementing the cultures. The supplemented fatty acid became a predominant species of the fatty acid profile. It was noted with interest that the relative proportion of endogenous C16:1(Δ7) is the main focus of response. This response may come from regulation of expression and activity of dehydroxylase and β-oxidase. It is possible that the -OH might be cleaved from a step wise conversion of C18:1 OH to C18:1 via fatty acid dehydroxylation and C18:1(Δ9) was converted to C16:1(Δ7) by β-oxidation, the fatty acid chain shortening.

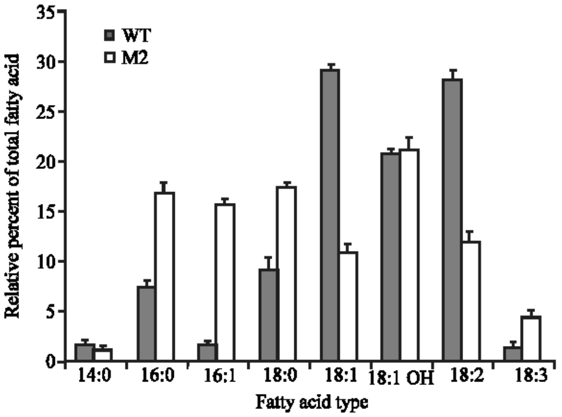

Fig. 1: Fatty acid composition of Wild-Type (WT) and M2 mutant of *H. polymorpha* grown in YEPD broth supplemented with 3 mM of C18:1 OH at 37°C for 24 h

The changes in fatty acid profile that occurred when the culture was exposed to supplemented substrate implied that there was a modification of the activity of enzymes involved in fatty acid synthesis, elongation, desaturation, dehydroxylation and β-oxidation. The data obtained from the analysis of the presence of C16:1(Δ7) showed that the highest level of C16:1(Δ7) content of the culture grown in 3 mM of C18:1 OH. This results may be explained by the assumption that the feedback regulatory mechanism could be proposed. Thus, it might be presumed that low (1 and 2 mM) or high (8 mM) concentrations of C18:1 OH also repress the biosynthetic pathway of C16:1(Δ7), whether at the level of gene expression or at the level of enzyme activity. There have been a few reports on the repression of enzyme activity, which is responsible for the formation of fatty acids when cultivated under various concentrations of supplemented substrate (Choi *et al*., 1996; Anamnart *et al*., 1998).

The effect of temperature on fatty acid proportion was observated in a medium with C18:1 OH, Table 2 shows the relative fatty acid composition of WT and M2 grown at 25, 30, 37 and 45°C. An increase in growth temperature from 25 to 45°C caused a decrease in the UFA content of M2 and WT cells (Table 2). Since UFAs are known to have lower melting points than the corresponding saturated fatty acids, it is suggested that this decreased fatty acid unsaturation with increasing growth temperature serves to maintain the optimal membrane fluidity for the cellular activities at higher temperatures. This finding is similar to that from a previous report by other investigators (Veerkamp, 1971; Wijeyaratne *et al*., 1986). In M2, the highest proportion of C16:1(Δ7) was observed at 37°C, whereas minor amounts of C16:1(Δ7) was found in WT cells grown at different temperatures (Table 2). Cells of M2 grown at 37°C contained very high proportions of C16:0, C16:1(Δ7), C18:0 and C18:3, while large proportions of C18:1(Δ9) and C18:2(Δ9,12) were found in WT (Table 2, Fig. 1). The majority of the published data on the effects of growth temperature on the fatty acid composition of yeasts have been primarily concerned with the fatty acid composition (McDonough and Roth, 2004; Wongsumpanchai, 2005).

In conclusion, the results of the present study showed that the plasticity of WT and M2 cells of *H. polymorpha* requirements for specific fatty acid species and the adaptation of the cells to exogenous fatty acids and growth temperature. The highest amount of C16:1(Δ7) was detected in the optimal condition of 3 mM C18:1 OH supplementation at 37°C, indicating the presence of dehydroxylase and β-oxidase activity in the culture of M2. However, the enzyme activity was low and further investigation on improving dehydroxylase and β-oxidase activity, including optimizing C16:1(Δ7) production conditions is needed. This most likely results from a modification of expression and/or activity of the specific enzyme. The determination of fatty acid composition in different conditions provides insight into the understanding of metabolic pathways in this yeast.

ACKNOWLEDGMENT

The author is grateful to Mahasarakham University, Thailand, for financial support with funds from the annual income budget of 2008. I am indebted to Assoc. Prof. A. Petsom and Dr. S. Anamnart of the Institute of Biotechnology and Genetic Engineering, Chulalongkorn, Thailand, for providing standard fatty acid methyl esters for the use of the gas chromatograph analysis.

REFERENCES

Anamnart, S., I. Tolstorukov, Y. Kaneko and S. Harashima, 1998. Fatty acid desaturation in methylotrophic yeast *Hansenula polymorpha* strain CBS 1976 and unsaturated fatty acid auxotrophic mutants. J. Ferment. Bioeng., 85: 476-482.

Brown, C.M. and A.H. Rose, 1969. Fatty acid composition of *Candida utilis* as affected by growth temperature and dissolved-oxygen tension. J. Bacteriol., 99: 371-378.

Carroll, K.K., 1961. Quantitative estimation of peak areas in gas-liquid chromatography. Nature, 191: 377-378.

Choi, J.Y., J. Stukey, S.Y. Hwang and C.E. Martin, 1996. Regulatory elements that control transcription activation and unsaturated fatty acid-mediated repression of the *Saccharomyces cerevisiae OLE1* gene. J. Biol. Chem., 71: 3581-3589.

Easterling, E.R., W.T. French, R. Hernandez and M. Licha, 2009. The effect of glycerol as a sole and secondary substrate on the growth and fatty acid composition of *Rhodotorula glutinis*. Bioresour. Technol., 100: 356-361.

Fakas, S., M. Galiotou-Panayotou, S. Papanikolaou, M. Komaitis and G. Agglis, 2007. Compositional shifts in lipid fractions during lipid turnover in *Cunninghamella echinulata*. Enz. Microb. Technol., 40: 1321-1327.

Farrell, J. and A.H. Rose, 1967. Temperature effects on microorganisms. Annu. Rev. Microbiol., 21: 101-120.

Gleeson, M.A. and P.E. Sudbery, 1988. Genetic analysis in the methylotrophic yeast *Hansenula polymorpha*. Yeast, 4: 293-303.

Harris, P. and A.T. James, 1969. The effect of low temperatures on fatty acid biosynthesis in plants. Biochem. J., 112: 325-330.

Konova, I.V., IA.E. Sergeeva, L.A. Galanina, G.A. Kochkina, N.E. Ivanushkina and S.M. Ozerskaia, 2009. Lipid synthesis by *Geomyces pannorum* under the impact of stress factors. Mikrobiologiia., 78: 52-58.

Laoteng, K., R. Ruenwai, M. Tanticharoen and S. Cheevadhanarak, 2005. Genetic modification of essential fatty acids biosynthesis in *Hansenula polymorpha*. FEMS Microbiol. Lett., 245: 169-178.

Lepage, G. and C.C. Roy, 1984. Improved recovery of fatty acid through direct transesterification without prior extraction or purification. J. Lipid Res., 25: 1391-1396.

Lin, T.Y., 2006. Conjugated linoleic acid production by cells and enzyme extract of *Lactobacillus delbrueckii* ssp. bulgaricus with additions of different fatty acids. Food Chem., 94: 437-441.

McDonough, V.M. and T.M. Roth, 2004. Growth temperature affects accumulation of exogenous fatty acids and fatty acid composition in *Schizosaccharomyces pombe*. Antonie Van Leeuwenhoek, 86: 349-354.

Morrison, W.R. and L.M. Smith, 1964. Preparation of fatty acid methyl esters and dimethylacetals from lipids with boron fluoride-metanol. J. Lipid Res., 3: 600-608.

Papanikolaou, S. and G. Aggelis, 2003. Selective uptake of fatty acids by the yeast *Yarrowia lipolytica*. Eur. J. Lipid Sci. Technol., 105: 651-655.

Papanikolaou, S., M. Galiotou-Panayotou, I. Chevalot, M. Komaitis, I. Marc and G. Aggelis, 2006. Influence of glucose and saturated free-fatty acid mixtures on citric acid and lipid production by *Yarrowia lipolytica*. Curr. Microbiol., 52: 134-142.

Sato, N., N. Murata, Y. Miura and N. Ueta, 1979. Effect of growth temperature on lipid and fatty acid compositions in the blue-green algae, *Anabaena variabilis* and *Anacystis nidulans*. Biochim. Biophys. Acta., 572: 19-28.

Suutari, M., A. Rintamaki and S. Laakso, 1997. Membrane phospholipids in temperature adaptation of *Canida utilis*: Alterations in fatty acid chain length and unsaturation. J. Lipid Res., 38: 790-794.

Veerkamp, J.H., 1971. Fatty acid composition of *Bifidobacterium* and *Lactobacillus* strains. J. Bacteriol., 108: 861-867.

Wijeyaratne, S.C., K. Ohta, S. Chavarnich, V. Mahamontri, N. Nilubol and S. Hayashida, 1986. Lipid composition of thermotolerant yeast, *Hansenula polymorpha*. Agric. Biol. Chem., 50: 827-832.

Wongsumpanchai, W., 2005. Effect of temperatures and various concentrations of palmitoleic acid for the production of hexadecadienoic acid from unsaturated fatty acid auxotrophic mutant of *Hansenula polymorpha*. J. Sci. Technol. MSU., 24: 7-12.