Identification and characterization of a novel Δ^{12}-fatty acid desaturase gene from *Rhizopus arrhizus*

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Abstract **Based on the sequence information of Δ^{12}-fatty acid desaturase genes (from *Mucor circinelloides*, *Mortierella alpina*, *Mucor rouxii* and *Aspergillus nidulans*), which were involved in the conversion from C18:1 to C18:2, a cDNA sequence putatively encoding a Δ^{12}-fatty acid desaturase was isolated from *Rhizopus arrhizus* using the combination of reverse transcription-polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE) methods. Sequence analysis indicated that it had an open reading frame (ORF) of 1170 bp, coding for 389 amino acid residues of 45 kDa, p*I* of the deduced protein was 7.01. The deduced amino acid sequence of this cloned cDNA showed high identity to those filamentous fungal Δ^{12}-desaturases mentioned above, including three conserved histidine-rich motifs and two hydrophobic domains. Functional identification was done heterologously in *Saccharomyces cerevisiae* strain INVScI. The result demonstrated that the deduced amino acid sequence exhibited Δ^{12}-fatty acid desaturase activity, sugguesting that this gene encoded for a membrane-bound desaturase, Δ^{12}-fatty acid desaturase.**

Keywords: Δ^{12}-Fatty acid desaturase gene; γ-Linolenic acid; *Rhizopus arrhizus*; *Saccharomyces cerevisiae*

1. Introduction

Polyunsaturated fatty acids (PUFAs) play important roles as structural components of membrane lipids and storage lipids in eukaryotic cells [1–3]. The length of the chain and the extent of the saturation contribute to the biological properties particularly the membrane fluidity and permeability. They also serve as signaling molecules in response to environmental stresses such as low temperature, salt stress and pathogenic intrusion in the cells [4–6]. Apart from these, it has been increasingly clear that PUFAs play important roles in the control of adipogenesis, cholesterol regulation and immune response [7–9]. Very recently, the potential roles of PUFAs in reducing the heart disease and in improving the vision sensitivity and reading ability have been verified [10–13]. Therefore, the nutritional and pharmaceutical roles of PUFAs have driven people to explore more suitable resources and to study the mechanisms involving their biosynthesis pathways in order to meet the growing market for this product and to explore more unknown functions of the product. Currently, the essential fatty acids such as linoleic acid (LA) and linolenic acid which can not be synthesized by human and are the precursor of other PUFAs can be obtained from various sources such as fish oil, animal tissues, algal cells [14]. For practical uses, *Mortierella alpina*, belonging to the zygomycetes, has been investigated by Yamada et al. [15] and has been used to produce some C-20 PUFAs (e.g. dihomo-linolenic acid, arachidonic acid and eicosapentaenoic acid). But, the widely used strain has its innate short-backs such as miscellaneous products which make the subsequent purification steps uneconomical, labor and time-consuming.

Rhizopus arrhizus is phylogenetically related to *Mucor* sp. and also synthesize PUFAs only up to 18-carbon with γ isomer of linolenic acid (GLA) [16], which make the subsequent purification relatively easy. Thus, *R. arrhizus* is also a promising producer of GLA. Although the main reason of this feature is likely due to the lack of elongase which add two carbon units to the chain and the chain is subsequently desaturated by other fatty acid desaturases. The selectivity of the Δ^6-fatty acid desaturase for the substrate and other reasons may also be considered. Therefore, it is necessary to explore every details of the mechanism of fatty acid biosynthesis and desaturation. In this short report, we describe the isolation and characterization of the Δ^{12}-fatty acid desaturase gene from *R. arrhizus*. The function was determined using *S. cerevisiae* as a heterologous host.

2. Materials and methods

2.1. Organisms and growth conditions

Rhizopus arrhizus strain NK030037 was grown at 28 °C for 2 days in liquid medium containing 2% glucose, 1% bacto-yeast extract, 0.2% KH_2PO_4 and 0.1% $MgSO_4$, with pH adjust to 6.0. *S. cerevisae* strain **INVScI** was used as recipient strain in transformation experiments and was grown at 30 °C in complex medium containing 1% bacto-yeast extract, 2% bacto-peptone and 2% glucose. *Escherichia coli* strain DH5α was grown at 37 °C in Luria–Bertani medium (LB) supplemented with 100 mg/l of ampicillin.

2.2. Enzymes and chemicals

Restriction endonucleases and other DNA-modifying enzymes were obtained from TaKaRa Bio. Dalian. China Co. Ltd.

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Abbreviations: GLA, γ-linolenic acid; LA, linoleic acid; PUFAs, polyunsaturated fatty acids; FAME, fatty acid methyl esters

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2.3. Total RNA preparation

Mycelia were harvested by filtration and washed with phosphate-buffer. The extraction of total RNA was done according to the method of chomczynski and sacchi [17] based on guanidinium thiocyanate method and stored at −70 °C for future use.

2.4. DNA manipulation

All recombinant DNA procedures were done according to standard methods [18]. DNA sequencing was done by the dideoxy chain termination method [19], using T7 sequence, and version 2.0 DNA sequencing kit.

2.5. PCR-based cloning of partial Δ^{12}-desaturase gene

PCR was used for partial Δ^{12}-desaturase DNA amplification. Genome DNA was prepared as previously described [20]. Generally speaking, Δ^{12}-fatty acid desaturase gene exists only in plant and fungi, no homolog of the Δ^{12}-fatty acid desaturase gene has been cloned from animals until now. These homologs have two common characteristics: three conserved histidine-rich motifs and two hydrophobic domains [21]. The conserved motifs were usually used to design degenerate primers to clone these homologs. In this case, the degenerate sense primer and antisense primer were 5′-CA(TC)GA(AG)TG(TC)GG(I)-CA(TC)CA(CAG)-3′ (fully degenerate to the conserved amino acid sequence, HECGHQ) and 5′-(AG)TG(AG)TGIGCIAC(GA)TGIGT-3′ (fully degenerate to the amino sequence,THVAHH) respectively, where I indicates inosine or isoleucine. PCR was performed on a Biometra T-gradient thermal cycler using a program of 94 °C 1 min, 50 °C 1 min, 72 °C 2 min for 30 cycles, followed by extension for 10 min at 72 °C.

2.6. Cloning of the full-length cDNA

Total RNA was then used in RT-PCR to amplify the sequence of the 5′ and 3′ end of the cDNA. Nucleotide sequence of 5′-end of the Δ^{12}-fatty acid desaturase cDNA were amplified by the method of SMART rapid amplification of cDNA ends (RACE) 5′-RACE System (BD-Clontech, palo Alto, CA, USA), followed by the manufacture's instructions and using two gene specific primers (GSP1: 5′-CAT-CAGCTTGAGGGTCTTTGTCGCG-3′ and GSP2: 5′-TCGCAG-GATGGGTTGAAGTGAGAGG-3′), which were designed from the nucleotide sequence of the previously cloned genomic DNA fragment. Amplification of 3′-end was performed by 3′-RACE method as described by Innis et al. [22]. The primers used for 3′-RACE were oligo(dT)17 adaptor primer (for first cDNA synthesis), a 5′-CTGCTCTTACCGTTGACCG-3′ (forward GSP primer) and a adaptor primer 5′-CTGATCTAGAGGTACCGGATCCATAT-3′ (reverse primer), followed by the manufacture's instructions. Based on the sequence information of 5′ and 3′ end, two gene-specific primers were designed to amplify the full-length cDNA by the method mentioned in BD Kit. All PCR fragments were subcloned into pGEM-T vector (Promaga, Madison, WI) and transformed into *E. coli* DH5a. Subsequently, nucleotide sequence were determined (TaKaRa Bio, Dalian, China). Analysis of the sequences was done with DNAMAN software (version 4.0, Lynnon BioSoft, Quebec). The resultant cDNA sequence and the deduced amino acid sequence have been submitted into the GenBank database and assigned the Accession No. AY639877.

2.7. Plasmid construction and yeast transformation

The ORF of Δ^{12}-fatty acid desaturase from *R. arrhizus* was amplified by RT-PCR using two specific primers, 5′-CAC*GGTACC*-**ATG**GCAACCAAGAGAAATATCAGT-3′ and 5′-GGT*GAATTCA*-**TTA**TTTTTGTAAAACACAACATC-3′ which corresponded to the nucleotide sequence of start and stop codon (in boldface) of the Δ^{12}-fatty acid desaturase gene (RAD12), respectively. The 5′-end of both primers contained a *Kpn*1 and a *Eco*R1 sites, respectively, that were underlined to facilitate subsequent manipulation. The PCR product was digested and subcloned into the pYES2.0 (invitrogen, Beijing, China) to generate a plasmid designated pYRAD12. *S. cerevisiae* was transformed with pYRAD12 and pYES2.0 using the lithium acetate method [23]. Transformants were selected by plating on complex synthetic minimal medium agar lacking uracil (SC-Ura) and grown at 30 °C for 2–3 days.

2.8. Induction of Δ^{12}-fattyacid desaturase gene expression

The putative Δ^{12}-desaturase gene was heterologously expressed in yeast, which was induced under transcriptional control of the GAL1 promoter. Yeast cultures were grown to logarithmic phage at 30 °C in synthetic minimal medium containing 2% galactose, 0.67% yeast nitrogen. Subsequently, cells were harvested by centrifugation followed by washing the cells in sterile water for three times. The cells were dried and ground into a fine powder for determination of fatty acid composition by gas chromatography and for gas chromatography–mass spectrometry (GC-MS) analysis.

2.9. Fatty acid analysis

Total fatty acid was extracted from the cells by treating 100 mg yeast powder with 5 ml 5% KOH in methanol for saponification at 70 °C for 5 h. pH of the product was adjusted to 2.0 with HCl(6 N) before the fatty acid was methyl-esterified with 4 ml 14% boron trifluoride in methanol at 70 °C for 1.5 h. Then, fatty acid methyl esters (FAME) were solubilized with hexane after addition of saturated sodium chloride solution. FAME were analyzed by gas chromatography (GC; GC-9A, Shimadzu, Kyoto, Japan) and identified by the comparison of their peaks with that of standards (Sigma). Heptadecanoic acid (C17:0) methyl ester (Sigma) was use as internal standard for quantitative analysis of fatty acids. Qualitative analysis of FAME was performed by GC-MS using a HP G1800A GCD system (Hewlett–Packard, Palo Alto, CA, USA). Both analyses were carried out with the same polar capillary column (HP, 5.30 mU, 0.25 mm internal diameter, 0.25 mm internal 1 m thickness). The mass spectrum of a new peak was compared with that of the standard for identification of fatty acid.

3. Results and discussion

3.1. Isolation of R. arrhizus *Δ^{12}-fatty acid desaturase gene*

Although several Δ^{12}-fatty acid desaturase genes have been isolated from fungi [24–27], the distinguishing feature of *R. arrhizus* in PUFA metablism intrigues us to clone the Δ^{12}-fatty acid desaturase genes. Two conserved amino acid sequence found in *M. circinelloide*, *M. alpina*, *M. rouxii*, and *A. nidulans* were used as basis for the design of two degenerate primers for PCR. A fragment of a proximate 730 bp was generated from genomic DNA of *R. arrhizus* using the degenerate primers that annealed to sequences in conserved histidine-rich box 1 and 3 of fungal Δ^{12}-fatty acid desaturases genes. This fragment contained a putative intron of 46 bp when the OFR was translated into its 228 amino acids product. The product has 48.23% and 37.37% identity to the *M. circinelloides* and *M. rouxii*, respectively, which indicated that a partial putative Δ^{12}-fatty acid desaturase gene was isolated from the *R. arrhizus*. The fragment was then used to design gene specific primers for cloning the full-length Δ^{12}-fatty acid desaturase. A 567 bp fragment of 5′-RACE and a 328 bp fragment of 3′-RACE were amplified and sequences were determined. The nucleotides of both products shared identical sequence overlap on the flanking region of the cloned 5′- and 3′-end of the partial DNA fragment, suggesting that these fragments are portions of the same gene. Sequence analysis indicated that the cloned full-length cDNA contains an open reading frame of 1170 bp encoding for 389 amino acid residues with an estimated molecular mass of 45 kDa. The coding region was flanked by a 64 bp 5′ untranslated region of the mRNA along with a full 81 bp 3′ untranslated region with the characteristic of a putative polyadenylation site, AATAAA, located at 20 bp upstream of the poly(A) tail.

Table 1
List of fungi and membrane Δ^{12}-fatty acid desaturase amino acid sequences analyzed in the sequence comparison section

Species	Gene name	GenBank accession number	Sequence homology with *R. arrhizus* 12-fatty acid desaturase gene (identity/similarity)	References
M. circinelloides	MCD12	AB052087	78%/86%	Directed submission
M. alpina	MAD12	AF110509	53%/68%	Sakuradani et al. (1999)
M. rouxii	MRD12	AF161219	67%/78%	Supapon et al. (1999)
S. kluyveri	SKD12	AB115968	38%/55%	Watanabe et al. (2004)
A. nidulans	AND12	AF262955	45%/60%	Calvo et al. (2001)

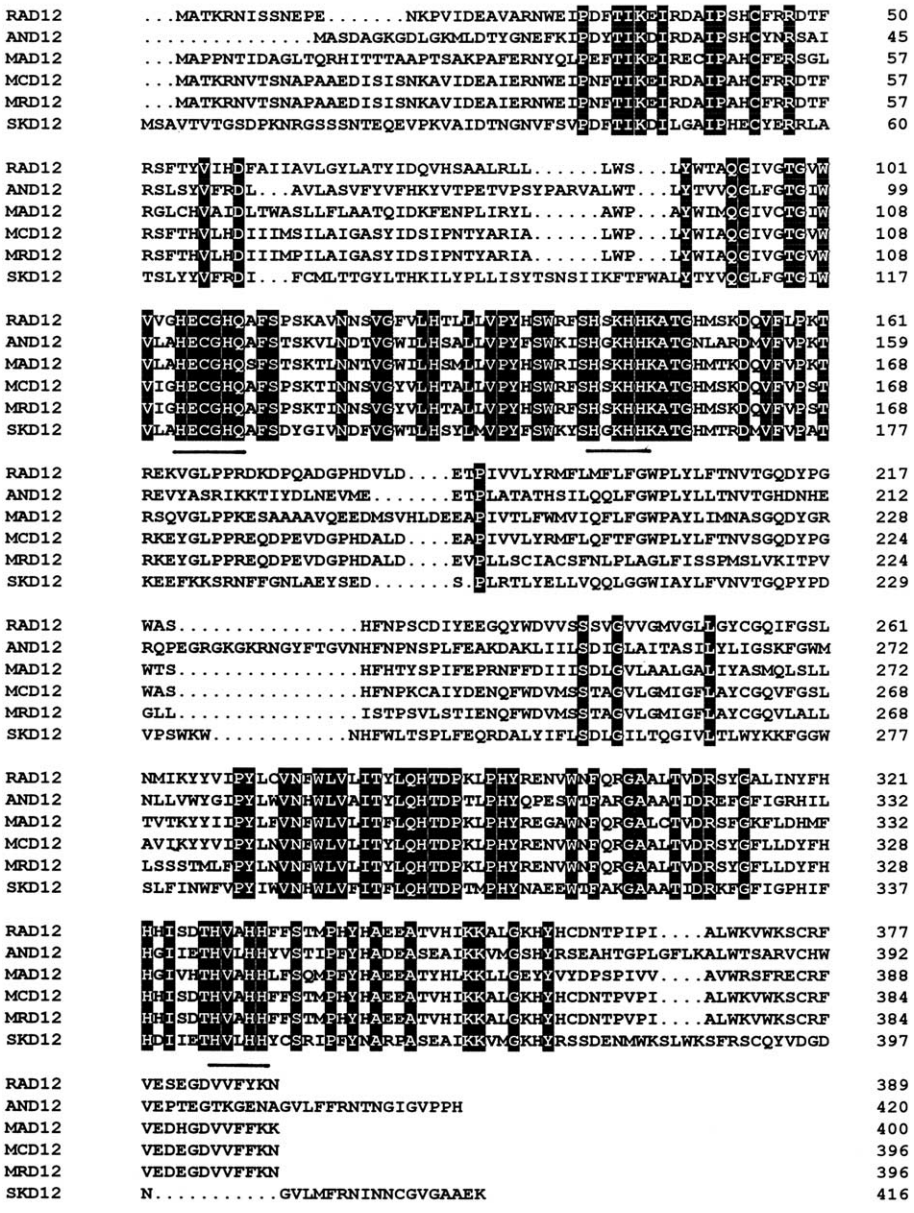

Fig. 1. Sequence alignment of deduced amino acids of the *R. arrhizus* Δ^{12}-fatty acid desaturase (RAD12) with the Δ^{12}-fatty acid desaturase of *M. circinelloides* (MCD12), *A. nidulans* (AND12), *M. rouxii* (MRD12), *M. alpina* (MAD12) and *S. kluyveri* (SKD12). Black background indicates identity of amino acid residues. The three conserved histidine-rich motifs are indicated by bars.

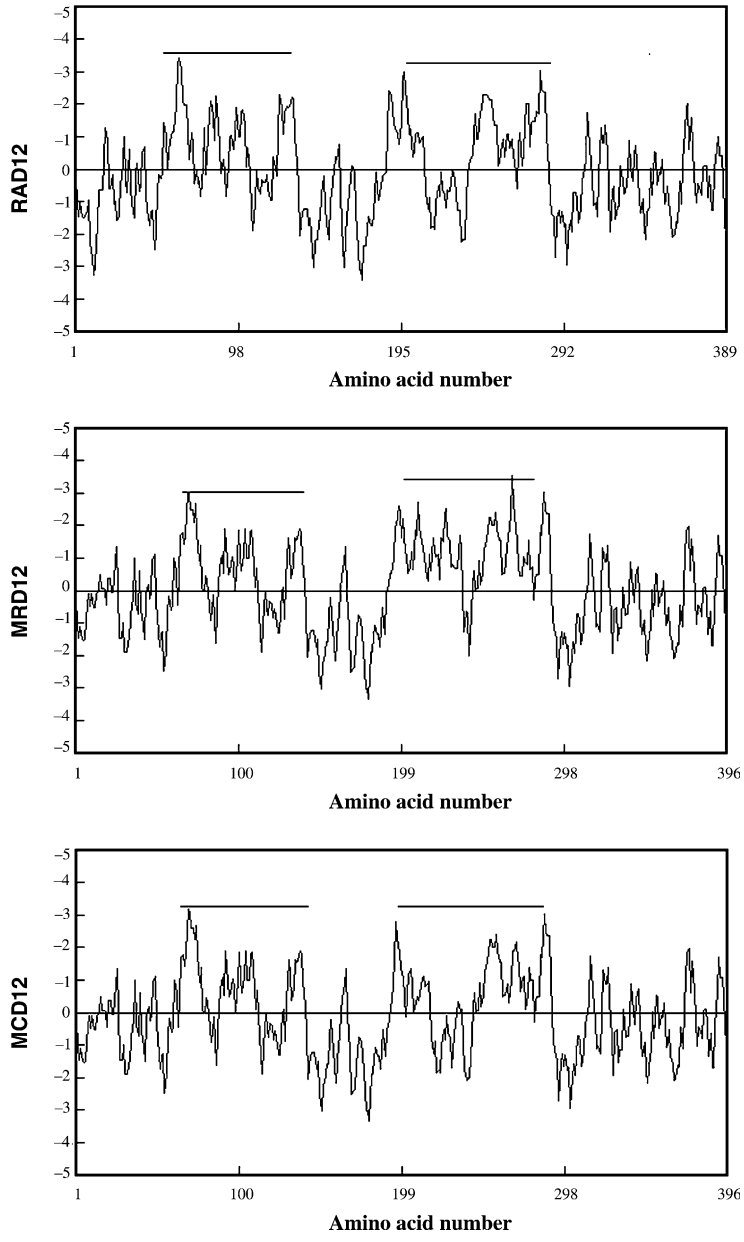

Fig. 2. Hydropathy plot of Δ^{12}-fatty acid desaturase of *R. arrhizus* (RAD12), *M. circinelloides* (MCD12) and *M. rouxii* (MRD12). Hydropathy profiles were analyzed using a Kyte-Doolittle scale. Numbers of the *X*-axis are amino acid residues. Two hydrophobic domains are indicated by bars.

3.2. Sequence comparison of Δ^{12}-fatty acid desaturase genes from fungi

The deduced amino acid sequence of the cloned Δ^{12}-fatty acid desaturase designated RAD12 was compared with those of some Δ^{12}-fatty acid desaturase genes from other organisms [24–27]. The result showed that RAD12 exhibited high identity to those fungal Δ^{12}-fatty acid desaturase genes (Table 1). It comprises three conserved histidine-rich motifs, the homology occurs mainly in the three conserved histidine-rich motifs and its surrounding sequence (Fig. 1). Hydropathy analysis showed that RAD12 has two hydrophobic regions (Fig. 2) known to all membrane-bound desaturases [28]. These results suggested that this gene encode for a putative Δ^{12}-fatty acid desaturase involved in the synthesis of LA in the *R. arrhizus*.

3.3. Functional analysis of the R. arrhizus Δ^{12}-fatty acid desaturase

In order to identify the function of the putative Δ^{12}-desaturase cDNA, we examined its expression in *S. cerevisiae*. The cloned cDNA was first ligated into the yeast expression vector pYES2.0. The resultant plasmid, designated pYRAD12, was then transformed into *S. cerevisiae* strain INVScl and expressed under the control of the inducible GAL1 promoter. A selected transformant, RAD12-1, were used to analyze the fatty acid composition by GC analysis. The result revealed that a novel fatty acid peak in the chromatogram of FAME from RAD12-1 had an identical retention time to the FAME standard LA, which was absent from the yeast containing the empty vector pYES2.0 (Fig. 3). The percentage of this new

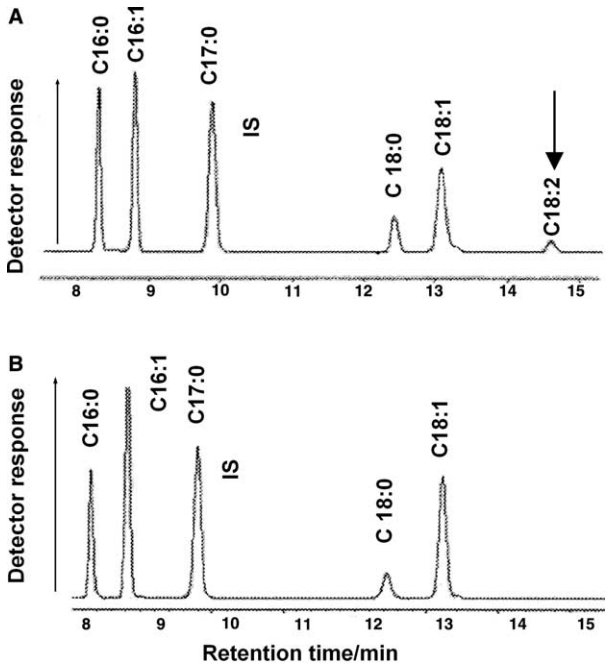

Fig. 3. Identification of LA in transgenic *S. cerevisiae* by GC analysis with Heptadecanoic acid (C17:0) as the internal standard (IS). (A) *S. cerevisiae* transformed with control vector pYES2.0. (B) *S. cerevisiae* transformed with recombinant plasmid pYRAD12. The arrowhead indicates the novel peak of LA.

fatty acid was 5.3% of total fatty acids (Table 2). In addition, the increase in LA caused a decrease in oleic acid. GC-MS analysis of this fatty acid methyl derivative demonstrated that the mass peak was $m/z = 294$, indicated the molecular mass of the methyl derivative LA and also, the fragmentation pattern was identical to that of the standard (Fig. 4). These results suggested that the cDNA encoded a Δ^{12}-fatty acid desaturase that converted oleic acid to LA specifically. This result again

Table 2
Fatty acid composition (wt%) of total lipid from yeast transformant containing pYES2.0 and pYRAD12

Transformant	Relative fatty acid composition				
	C16:O	C16:1	C18:0	C18:1	C18:2
pYES2	23.4	35.8	8.2	32.6	–
pYRAD12	25.4	30.6	10.8	27.9	5.3

reinforced the previous conclusion that Δ^{12}-fatty acid desaturase from filamentous fungi were only able to convert from C18:1 to C18:2. whereas, the recently reported Δ^{12}-fatty acid desaturase from the *Saccharomyce kluyveri* can convert C16:1 and C18:1 to C16:2 and C18:2 [27]. Athough the reason remain far to be elucidated, the different hydrophobic profile (data not shown) which indicated different transmembrane topology between *S. kluyveri* Δ^{12}-fatty acid desaturase and filamentous fungi Δ^{12}-fatty acid desaturases may be useful to make this puzzlement clear.

In conclusion, although several Δ^{12}-fatty acid desaturase genes has been cloned from various resources, due to the extreme difficulty to purify the membrane integral enzyme, the details of the structure-function relation of the enzyme remain to be elusive. To steer clear of the obstacle, comparison of the sequence with identical function but with different resources may be a useful tool to tackle the problem. In this short report, we describe the cloning and characterization of a new Δ^{12}-fatty acid desaturase gene from *R. arrhizus* which is phylogenetically related to *Mucor* sp. and also synthesize PUFAS only up to 18-carbon with γ isomer of linolenic acid. Recently, a gene encoding for Δ^{6}-fatty acid desaturase has been cloned from *R. arrhizus* [29] which also showed a high amino acid sequence identity to Δ^{6}-fatty acid desaturase of *M. circinelloide*, especially the sequence surrounding the histidine-rich motif areas. Comparing of these sequences combined with the use of site-directed mutagenesis method would be useful to identify the active sites that may be responsible for the chain-length

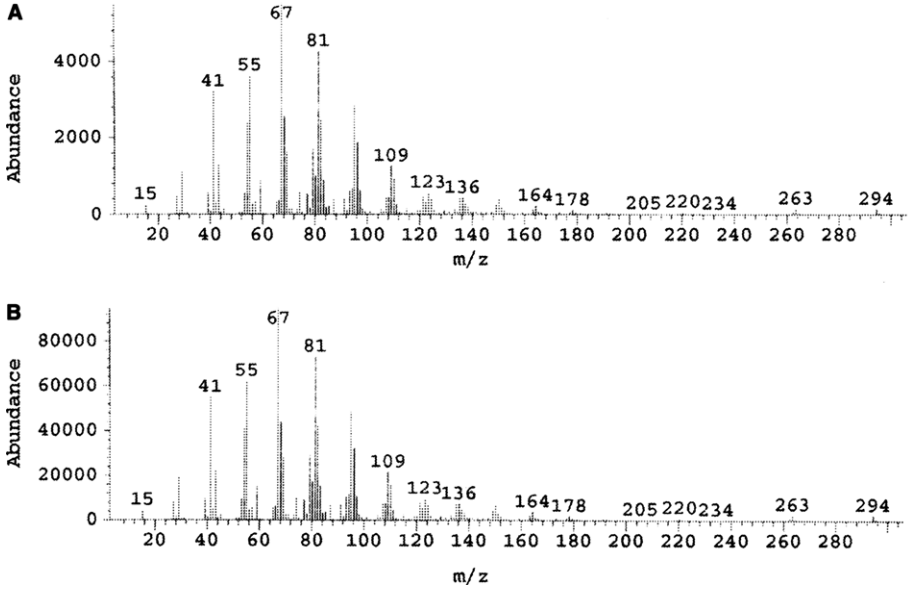

Fig. 4. GC-MS analysis of the novel peak identified in *S. cerevisiae* transformed with pYRAD12. (A) *S. cerevisiae* transformed with pYRAD12. (B) LA standard.

specificity and bound specificity, furthermore for illustrating the metabolic pathways.

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