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γ-Nonalactone in Beer: Biosynthesis by Yeast

γ-Nonalactone is known as an aroma active and chiral compound in beer and other fermented products. Labeling experiments with tetra deuterated (9,10,12,13-$^{2}H_4$)-linoleic acid, single deuterated 13- and 9- hydroxyoctadecadienoic acids (HODE) and its corresponding oxygen-18 labeled oxidation product [$^{18}O_1$]- 13- and 9- HODE elucidated two different biosynthetic routes from linoleic oxidation products (HODE) to γ-nonalactone in brewers yeast *Saccharomyces cerevisiae* and a model yeast *Sporobolomyces odorus*.

I) 13-Peroxidation of linoleic acid into 13-HODE in barley or during malting and β-oxidation followed by one α-oxidation type step finally resulting in γ-nonalactone with (*S*)-stereospecificity ((*S*) ~ 60 % e.e.).

II) 9-Peroxidation of linoleic acid into 9-HODE in barley or during malting and Baeyer-Villiger type oxidation resulting in 2*E*,4*E*-nonadien-1-ol and azelaic acid. 2*E*,4*E*-nonadien-1-ol was further transformed to γ-nonalactone with (*R*)-stereospecificity((*R*) ~ 46 % e.e.).

Descriptors: gamma Nonalactone, enantiomers, chiral analysis, yeast metabolism, isotope labeling, lipoxygenase

1 Introduction

Lactones: γ-Lactones are widely distributed in nature and they are important flavor compounds, occurring in beer, fruits, fermented food, etc. γ-Lactones, with the exception of γ-butyrolactone are chiral compounds and they are optical active like enantiomers of lactic acid (please refer to http://en.wikipedia.org/wiki/Chirality_%28chemistry%29). The biosynthesis of γ-decalactone and γ-dodecalactone was investigated in fruits and microorganisms [1–3]. The initial step is generally the introduction of oxygen to the carbon chain of a fatty acid. (*R*)-12-Hydroxylation of oleic acid and subsequent degradation of the resultant ricinoleic acid by β-oxidation yields (*R*)-γ-decalactone in *Sporobolomyces odorus* [1]. In addition an epoxygenation pathway of oleic or palmitoleic acid was characterized in *S. odorus*. The Metabolism of epoxy fatty acid by *S. cerevisiae*, e.g. is leading to γ-dodecalactone and γ-decalactone, respectively. The degradation pathways of epoxy fatty acids and of dihydroxy fatty acids by *S. cerevisiae* are very complex and have been described in the literature [2–5].

Lipoxygenase (LOX): Barley and malt lipoxygenases (LOX) transform free and esterified linoleic acid into 9-and 13-hydroperoxyoctadecadienoic acids (HPODE) which can be reduced to 9- or 13-hydroxyoctadecadienoic acids (HODE). The content of HODE in barley and malt and the influence of process parameters during malting and mashing have been studied [6–9]. Purified green malt LOX has been characterized in detail with respect to several lipid substrates [10–14]. In contrast to β-oxidation, formation of 2E-nonenal and further reactions 9-hydroperoxy fatty acid (i.e. 9S-hydroperoxy-10E,12Z-octadecadienoic acid) can also be transformed into trihydroxy fatty acid in barley and malt especially during mashing [15] which show antifungal activity.

Metabolism: Degradation of oxygenated fatty with an even number of carbon atoms (e.g. C_{16}, C_{18} acids) by β-oxidation (degrades C_2 units) must yield products with an even number of carbon atoms. Carbon chain shortening by β-oxidation cannot yield γ-nonalactone (C_9), a species with an odd number of carbon atoms. Therefore, up to now unknown microbial pathways leading to γ-nonalactone must be involved in its biosynthesis. Further more, the chiral compound γ-nonalactone is formed with low stereospecificity compared to γ- or δ-decalactone (Table 1).

Consequently, we investigated the biosynthesis of γ-nonalactone in *Saccharomyces cerevisiae* and *Sporobolomyces odorus* to elucidate new enzymatic steps in the formation of lactones with odd numbered carbon chain from linoleic acid.

2 Materials and methods

Synthesis of [9,10,12,13-$^{2}H_4$]-9,12-octadecadienoic acid and its lipoxygenase products (HPODE and HODE) were published before [16]. [9- or 13-$^{18}O_2$]-9- or 13-HPOD and [9- or 13-$^{18}O_1$]-9- or 13-HODE were synthesized by enzymatic lipoxygenation of linoleic acid with tomato- or soy bean lipoxygenase under [$^{18}O_2$]-oxygen gas (HPODE) and subsequent reduction with $NaBH_4$, respectively. Incubation experiments were performed in 1000 mL shake flasks with 200 ml yeast cultures, 150 or 250 mg/L of substrate were incubated to investigate the metabolism.

The yeast *Saccharomyces cerevisiae* was purchased from the IfGB (Berlin, Germany, IfG 06136, RH-strain (brewery strain)); the yeast *Sporobolomyces odorus* (CBS 2636) was from the Centraalbureau voor Schimmel-Cultures (CBS), The Netherlands, culture conditions for *S. cerevisiae* and *S. odorus* have been described previously [1, 4].

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Tables and figures see Appendix

3 Results and discussion

In a series of labeling experiments two completely different pathways of the γ-nonalactone biosynthesis of in yeast have been observed (Fig. 1).

In pathway I a (*S*)-13-lipoxygenase/reductase pathway with subsequent 4 cycles of β-oxidation of 13-HODE into (*S*)-5-hydroxydecanoic acid (*S*)-δ-decalactone was detected. Isotope labeling experiments using [13-$^{18}O_{1(2)}$]-(*S*)-13- H(P)ODE as well as [9,10,12,13-$^{2}H_{2}$]-(*S*)-13-H(P)ODE and [13-$^{2}H_{1}$]-(±)-13- HODE showed a transformation of the (*S*)-13-oxygenated linoleic acid precursors into labeled (*S*)-δ-decalactone with an enantiomeric excess of (*S*) ~ 60–100 % (Fig. 2).

(*S*)-δ-Decalactone respectively its hydrolysis product (*S*)-5-hydroxydecanoic acid is further metabolized in *S. cerevisiae* by a α-oxidation type enzyme system into (*S*)-4-hydroxynonanoic acid, which has not been detected previously in yeasts.

α-Oxidation pathways are known in plants and were recently found in mammalian liver tissue to metabolize 3-methyl branched fatty acids [17, 18]. Our results clearly demonstrate the metabolism of 5-hydroxydecanoic acid into 4-hydroxynonanoic acid by isotopic labeling in yeast. Therefore, the yeast *S. cerevisiae* seems to use enzymes with α-oxidation type activity to transform (*S*)-5-hydroxydecanoic acid into (*S*)-4-hydoxynonanoic acid and subsequent lactonization to (*S*)-γ-nonalactone. There is another enzyme activity known that could be responsible for the C-1 carbon chain shortening: Cytochrom P450 enzymes have been shown to hydroxylate and cleave carbon chains [19, 20]. The origin of γ-nonalactone by this pathway C-1 degrading pathway could be demonstrated unequivocally by labeling experiments with ^{2}H- and ^{18}O-labeled precursors.

γ-Nonalactone and 4-hydroxynonanoic acid are further metabolized in *S. cerevisiae e.g.* by α-oxidation. The resulting 3-hydroxyoctanoic acid exhibits (*S*)-stereochemistry with high enantiomeric purity (R:S ~ 2:98). In addition, (*R*)- 3-hydroxyoctanoic acid can by degraded by yeast β-oxidation [21]. Two α-oxidation type steps transform (*S*)-3-hydroxyoctanoic acid into hexanoic acid or (*S*)-3-hydroxyoctanoic acid is degraded otherwise.

The initial (*S*)-stereochemistry of 13-hydro(pero)xy fatty acids by lipoxygenation is in agreement with the (*S*)-stereochemistry of the analyzed δ-decalactone (92 % e.e. (*S*)). The configuration of analyzed γ-nonalactone coming from this pathway shows (*S*)-configuration with (*S*) ~ 50–70 % e.e..

Pathway II into γ-nonalactone in yeast was characterized as 9-lipoxygenase/reductase pathway of linoleic acid. 9-LOX products 9-HPODE and 9-HODE have been analyzed in barley and malt. During mashing, the 9-HODE is released, transferred into wort and degraded by yeast. By this metabolism labeled linoleic acid derivatives [9,10,12,13-$^{2}H_{4}$]-(*S*)-9-hydro(pero)xy-10*E*,12*Z*-octadecadienoic acid as well as [9-$^{2}H_{1}$]-(±)-9-hydroxy-10*E*,12*Z*-octadecadienoic acid are converted to 9-oxo-10*E*,12*Z*-octadecadienoic acid. The double bonds are isomerized to 9-oxo-11*E*,13*E*-octadecadienoic acid and the molecule is subsequently oxygenated by a Baeyer-Villiger type reaction. Consecutively, the Baeyer-Villiger ester is hydrolyzed to azelaic acid and 2*E*,4*E*-nonadien-1-ol. 2*E*,4*E*-Nonadien-1-ol is further oxidized to 2*E*,4*E*-nonadienoic acid, transformed to 3*Z*-nonenoic acid by acyl-CoA-reducto-isomerase and metabolized within the "epoxyde pathway" of yeast to yield γ-nonalactone with (*R*)-stereospecificity. This "epoxyde pathway" was intensively studied in the yeast *S. odorus* [22]. Incubation experiments using deuterated [9-$^{2}H_{1}$] (±)-9-hydro(pero)xy-10*E*,12*Z*-octadecadienoic acids yielded γ-nonalactone with an enantiomeric excess of ca. 50 % e.e. (*R*) (Fig. 3). The ability of Baeyer-Villiger type oxidation in *S. odorus* has been demonstrated previously in the context of lactone metabolism [23]. A comparable degradation of 9-HODE in *S. cerevisiae* has been observed (data not shown).

4 Conclusion

The presented results clearly demonstrate two completely different pathways for the degradation of linoleic acid derived 13- and 9-hydro(pero)xyoctadecadienoic acids in yeasts.

I. A (*S*)-13-lipoxygenase pathway to (*S*)-13-hydroperoxy-9*Z*,11*E*-octadecadienoic acid in barley or during malting/mashing and β-oxidation of (*S*)-13-hydroxy-9*Z*,11*E*-octadecadienoic acid to (*S*)-5-hydroxydecanoic acid in yeast with subsequent α-oxidation type activity leading to 4-hydroxynonanoic acid which is lactonized into (*S*)-γ-nonalactone.

II. A 9-lipoxygenase pathway in barley/malt and Baeyer-Villiger type oxidation activity in yeast leading to C-9/C-9 carbon chain cleavage and further transformation of the cleavage product 2,4-nonadienol into (*R*)-γ-nonalactone within the yeast epoxide pathway.

Therefore, (*R*)- and (*S*)- γ-nonalactone enantiomers can alternate depending on the raw material, yeast enzymes, and depending on storage.

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Appendix

Table 1 Configuration and enantiomeric distribution of natural occurring γ-nonalactone (e.e. = enantiomeric excess)

Source	Configuration	Enantiomeric purity % e.e.
S. odorus ATCC 24259	(R)	44
S. odorus ATCC 26697	(R)	66
Milk	(R)	80
Cheddar cheese	**(S)**	**13**

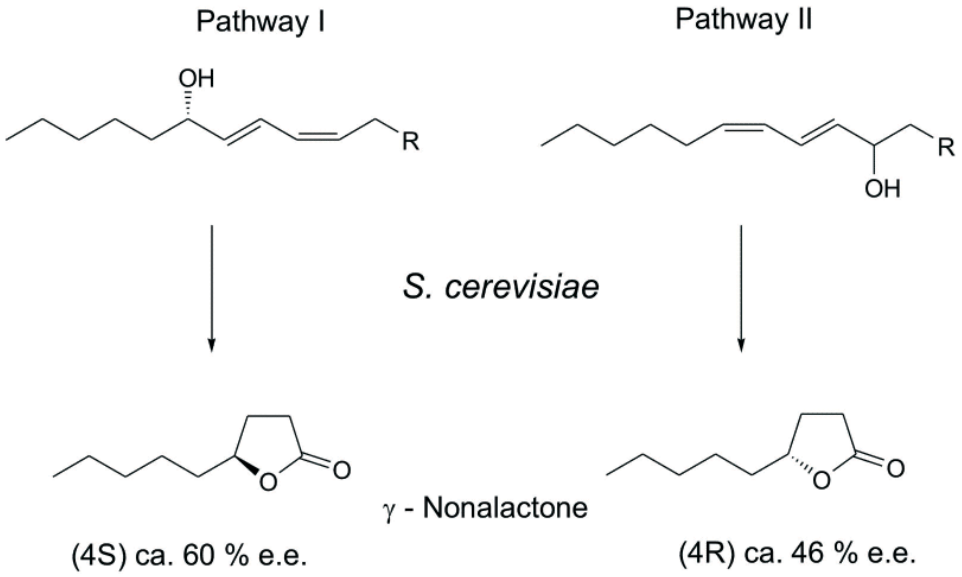


Fig. 1 Two pathways of γ-nonalactone formation by yeast (R = -$(CH_2)_6$-COOH)

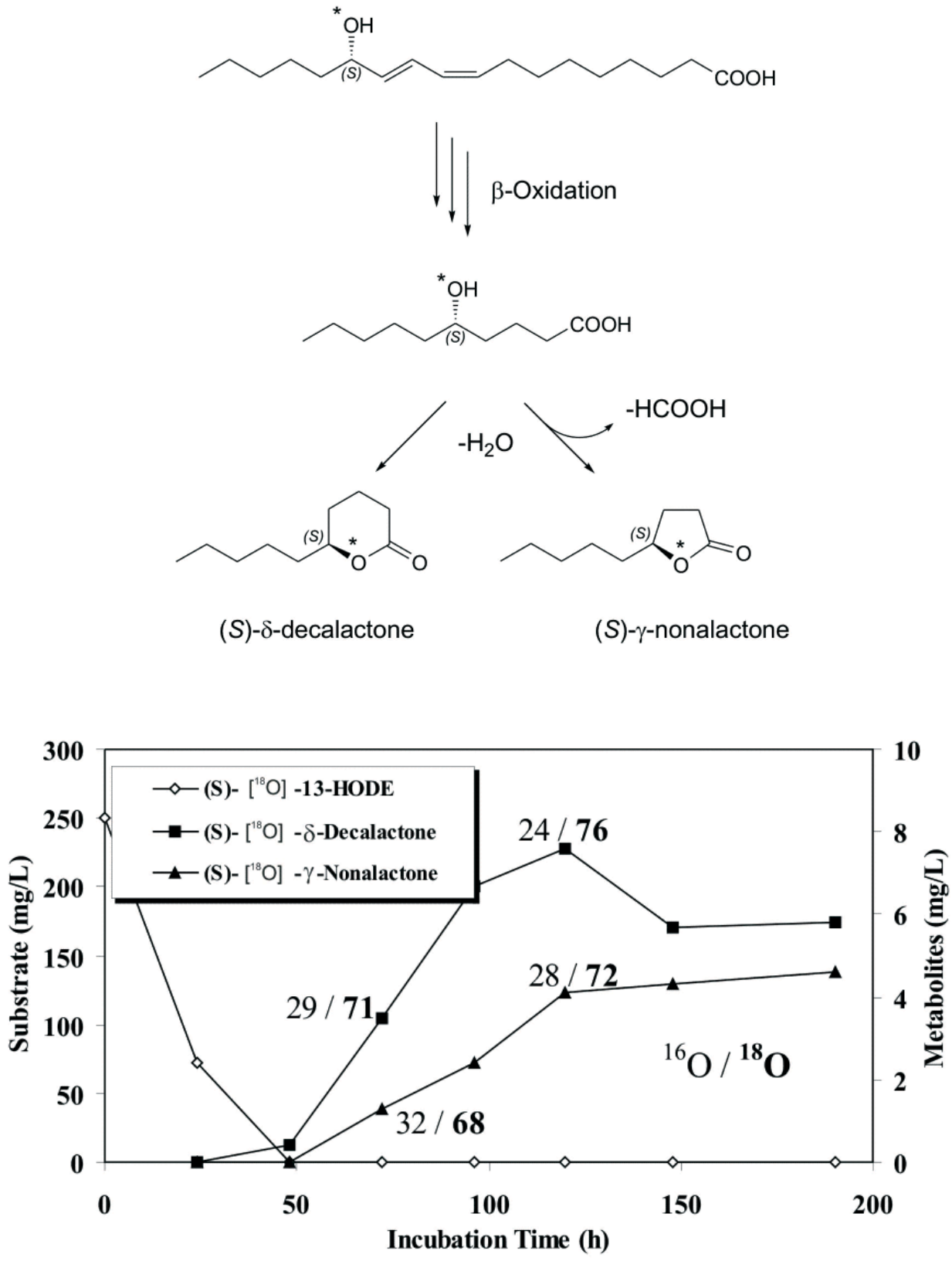
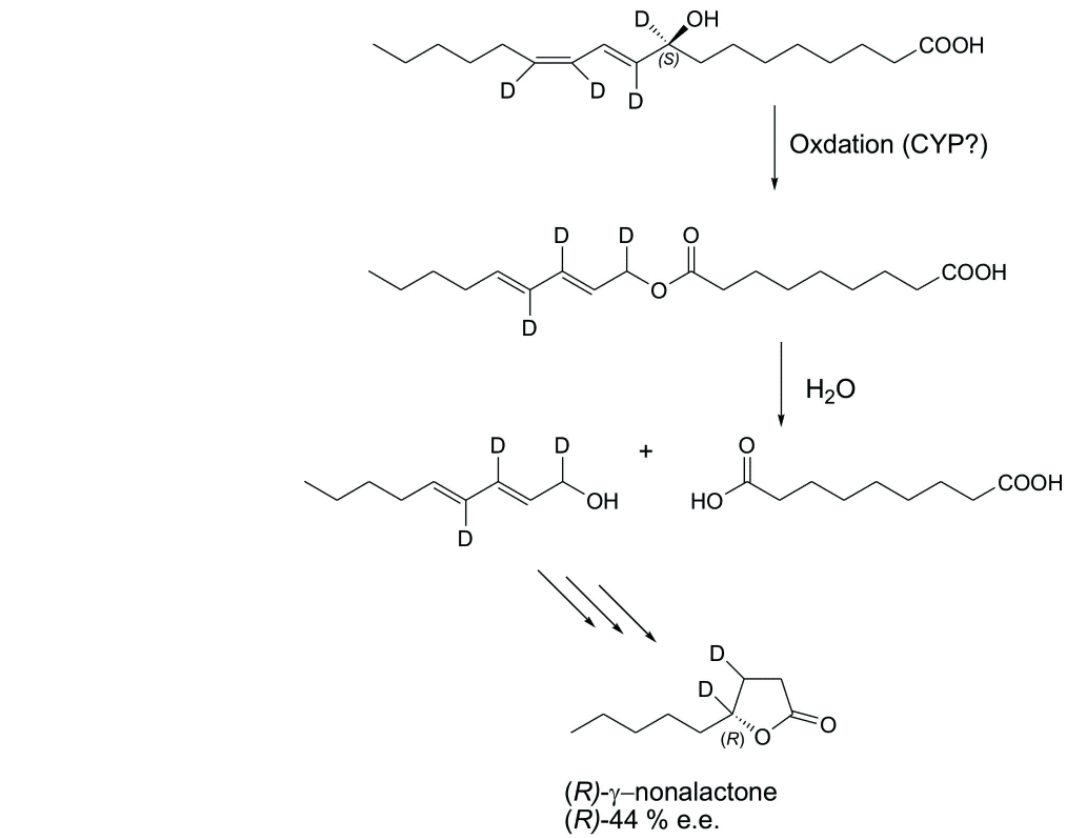


Fig. 2 **Metabolism of 150 mg/L [13-$^{18}O_1$]-(*S*)-13-hydroxy-9Z,11*E*-octadecadienoic acid (13-HODE) in *S. cerevisiae* numbers are given as O-18 labelling (O-18 = *) (e.g. 28/72 means 28 % O-16 and 72 % O-18)**



(*R*)-γ–nonalactone
(*R*)-44 % e.e.

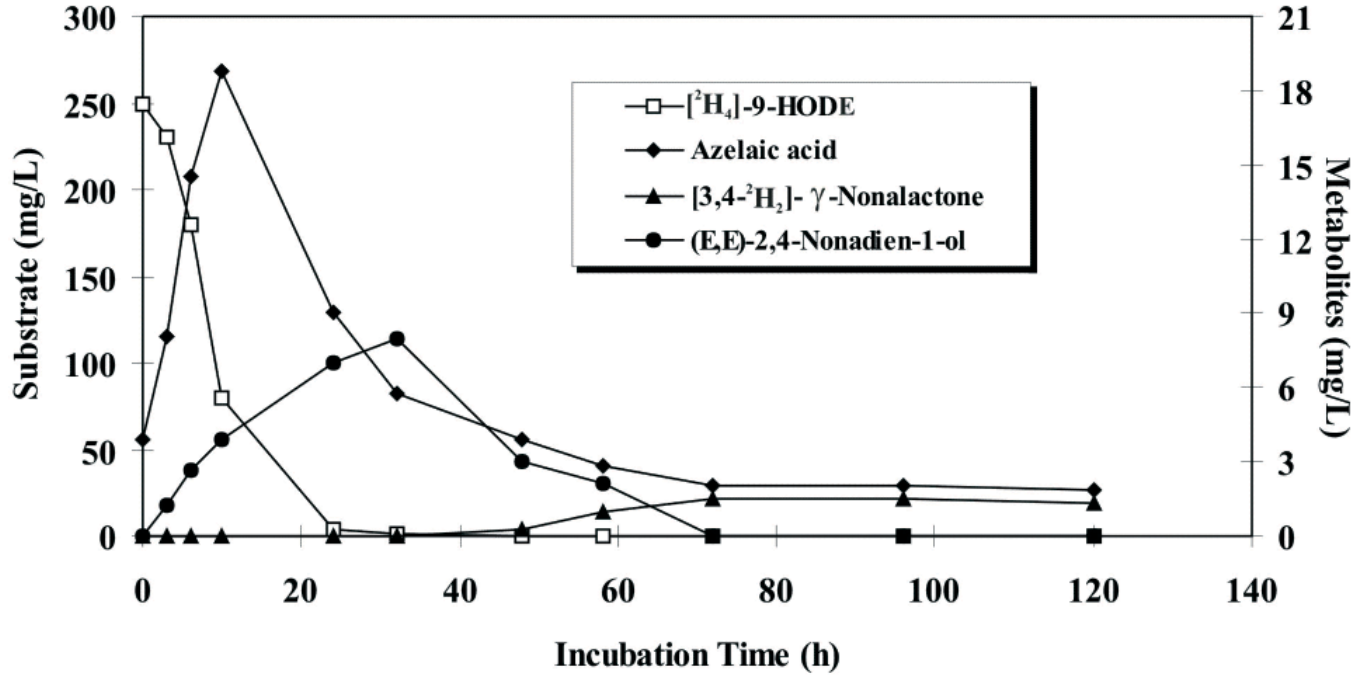


Fig. 3 **Metabolism of 250 mg/L [9,10,12,13-$^2H_4$]-(S)-9-hydroxy-10*E*,12*Z*-octadecadienoic acid (q-HODE) in *S. odorus***