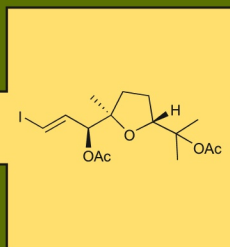
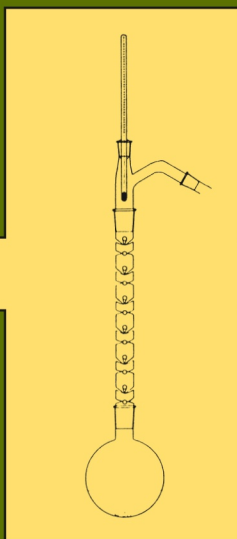


# Studies in Natural Products Chemistry

Atta-ur-Rahman, FRS

Editor

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Volume 74

Bioactive Natural Products



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Bioactive Natural Products

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Edited by Atta-ur-Rahman

- |         |                                                                           |         |                            |
|---------|---------------------------------------------------------------------------|---------|----------------------------|
| Vol. 1  | Stereoselective Synthesis (Part A)                                        | Vol. 37 | Bioactive Natural Products |
| Vol. 2  | Structure Elucidation (Part A)                                            | Vol. 38 | Bioactive Natural Products |
| Vol. 3  | Stereoselective Synthesis (Part B)                                        | Vol. 39 | Bioactive Natural Products |
| Vol. 4  | Stereoselective Synthesis (Part C)                                        | Vol. 40 | Bioactive Natural Products |
| Vol. 5  | Structure Elucidation (Part B)                                            | Vol. 41 | Bioactive Natural Products |
| Vol. 6  | Stereoselective Synthesis (Part D)                                        | Vol. 42 | Bioactive Natural Products |
| Vol. 7  | Structure and Chemistry (Part A)                                          | Vol. 43 | Bioactive Natural Products |
| Vol. 8  | Stereoselective Synthesis (Part E)                                        | Vol. 44 | Bioactive Natural Products |
| Vol. 9  | Structure and Chemistry (Part B)                                          | Vol. 45 | Bioactive Natural Products |
| Vol. 10 | Stereoselective Synthesis (Part F)                                        | Vol. 46 | Bioactive Natural Products |
| Vol. 11 | Stereoselective Synthesis (Part G)                                        | Vol. 47 | Bioactive Natural Products |
| Vol. 12 | Stereoselective Synthesis (Part H)                                        | Vol. 48 | Bioactive Natural Products |
| Vol. 13 | Bioactive Natural Products (Part A)                                       | Vol. 49 | Bioactive Natural Products |
| Vol. 14 | Stereoselective Synthesis (Part I)                                        | Vol. 50 | Bioactive Natural Products |
| Vol. 15 | Structure and Chemistry (Part C)                                          | Vol. 51 | Bioactive Natural Products |
| Vol. 16 | Stereoselective Synthesis (Part J)                                        | Vol. 52 | Bioactive Natural Products |
| Vol. 17 | Structure and Chemistry (Part D)                                          | Vol. 53 | Bioactive Natural Products |
| Vol. 18 | Stereoselective Synthesis (Part K)                                        | Vol. 54 | Bioactive Natural Products |
| Vol. 19 | Structure and Chemistry (Part E)                                          | Vol. 55 | Bioactive Natural Products |
| Vol. 20 | Structure and Chemistry (Part F)                                          | Vol. 56 | Bioactive Natural Products |
| Vol. 21 | Bioactive Natural Products (Part B)                                       | Vol. 57 | Bioactive Natural Products |
| Vol. 22 | Bioactive Natural Products (Part C)                                       | Vol. 58 | Bioactive Natural Products |
| Vol. 23 | Bioactive Natural Products (Part D)                                       | Vol. 59 | Bioactive Natural Products |
| Vol. 24 | Bioactive Natural Products (Part E)                                       | Vol. 60 | Bioactive Natural Products |
| Vol. 25 | Bioactive Natural Products (Part F)                                       | Vol. 61 | Bioactive Natural Products |
| Vol. 26 | Bioactive Natural Products (Part G)                                       | Vol. 62 | Bioactive Natural Products |
| Vol. 27 | Bioactive Natural Products (Part H)                                       | Vol. 63 | Bioactive Natural Products |
| Vol. 28 | Bioactive Natural Products (Part I)                                       | Vol. 64 | Bioactive Natural Products |
| Vol. 29 | Bioactive Natural Products (Part J)                                       | Vol. 65 | Bioactive Natural Products |
| Vol. 30 | Bioactive Natural Products (Part K)                                       | Vol. 66 | Bioactive Natural Products |
| Vol. 31 | Studies in Natural Products<br>Chemistry: Cumulative Indices<br>Vol. 1-30 | Vol. 67 | Bioactive Natural Products |
| Vol. 32 | Bioactive Natural Products (Part L)                                       | Vol. 68 | Bioactive Natural Products |
| Vol. 33 | Bioactive Natural Products (Part M)                                       | Vol. 69 | Bioactive Natural Products |
| Vol. 34 | Bioactive Natural Products (Part N)                                       | Vol. 70 | Bioactive Natural Products |
| Vol. 35 | Bioactive Natural Products (Part O)                                       | Vol. 71 | Bioactive Natural Products |
| Vol. 36 | Bioactive Natural Products                                                | Vol. 72 | Bioactive Natural Products |
|         |                                                                           | Vol. 73 | Bioactive Natural Products |

# Studies in Natural Products Chemistry

Bioactive Natural Products

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# Contents

|                                                                                                              |           |
|--------------------------------------------------------------------------------------------------------------|-----------|
| Contributors                                                                                                 | xi        |
| Preface                                                                                                      | xv        |
| <b>1. Recent advances of the stereoselective bimodal glycosylations for the synthesis of various glucans</b> | <b>1</b>  |
| <i>Feiqing Ding, Akihiro Ishiwata, and Yukishige Ito</i>                                                     |           |
| <b>Introduction</b>                                                                                          | <b>2</b>  |
| Glucans                                                                                                      | 2         |
| Stereoselective glycosylation for the synthesis of D-glucans                                                 | 7         |
| Nonclassical NGP strategies for stereoselective glycosylations                                               | 9         |
| <b>Bimodal approach toward D-glucan synthesis using 2-O-TAB group</b>                                        | <b>10</b> |
| 2-O-TAB group for Stereoselective glycosylation the synthesis of D-glucans                                   | 10        |
| Effect of 2-O-TAB group on for $\alpha$ - and $\beta$ -selective glucosylations                              | 12        |
| Application of 2-O-TAB group toward stereoselective galactosylation                                          | 13        |
| Deprotection of 2-O-TAB group for further elongation                                                         | 13        |
| <b>Application of 2-O-TAB group toward stereoselective mannosylation</b>                                     | <b>16</b> |
| Stereoselective mannosylation                                                                                | 16        |
| 2-O-TAB donor for stereoselective mannosylation                                                              | 17        |
| Effect of 2-O-TAB group on $\alpha$ - and $\beta$ -selective mannosylations                                  | 20        |
| Assembly of D-Man-(1 $\rightarrow$ 2)-D-Man-(1 $\rightarrow$ 6)-D-Glc structure                              | 21        |
| <b>Synthesis of various D-glucan using 2-O-TAB approach</b>                                                  | <b>23</b> |
| Assembly of linear (1 $\rightarrow$ 6)-D-glucan linkages                                                     | 23        |
| Assembly of (1 $\rightarrow$ 3)-D-glucan structures                                                          | 25        |
| Assembly of (1 $\rightarrow$ 4)-D-glucan, cellooligosaccharide structures                                    | 27        |
| Assembly of (1 $\rightarrow$ 3)- $\alpha$ -glucose-branched (1 $\rightarrow$ 6)-D-glucan                     | 28        |
| Summary                                                                                                      | 29        |
| <b>Acknowledgment</b>                                                                                        | <b>29</b> |
| <b>References</b>                                                                                            | <b>30</b> |

|                                                                                                                                                                                                                                        |           |
|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------|
| <b>2. Plant-derived diterpenes for breast cancer treatment: New perspectives and recent advances</b>                                                                                                                                   | <b>41</b> |
| <i>Fernanda Tomiotto-Pellissier, Manoela Daiele Gonçalves, Taylon Felipe Silva, Virgínia Márcia Concato, Bruna Taciane da Silva Bortoleti, Nilton Syogo Arakawa, Ivete Conchon-Costa, Wander Rogério Pavanelli, and Carolina Panis</i> |           |
| Introduction                                                                                                                                                                                                                           | 42        |
| Identification of new diterpenes with anti-breast cancer activity                                                                                                                                                                      | 45        |
| Diterpenes with antiproliferative activity                                                                                                                                                                                             | 53        |
| Inhibition of tumor angiogenesis and metastasis by diterpenes                                                                                                                                                                          | 54        |
| Induction of cell death mechanisms by diterpenes                                                                                                                                                                                       | 57        |
| Diterpenes as a therapeutic option in drug-resistant cell lineages                                                                                                                                                                     | 62        |
| Nanotechnology as an enhancer of diterpene activity                                                                                                                                                                                    | 64        |
| Sites of interaction and structure–activity relationship                                                                                                                                                                               | 66        |
| Conclusion                                                                                                                                                                                                                             | 72        |
| References                                                                                                                                                                                                                             | 72        |
| <b>3. Flavonoids and anticancer activity: Structure–activity relationship</b>                                                                                                                                                          | <b>81</b> |
| <i>Sümeyra Çetinkaya, Kevser Taban Akça, and Ipek Süntar</i>                                                                                                                                                                           |           |
| Introduction                                                                                                                                                                                                                           | 82        |
| General aspects of flavonoids                                                                                                                                                                                                          | 84        |
| Classification of flavonoids                                                                                                                                                                                                           | 84        |
| Flavones                                                                                                                                                                                                                               | 85        |
| Flavonols                                                                                                                                                                                                                              | 85        |
| Isoflavones                                                                                                                                                                                                                            | 86        |
| Flavanols                                                                                                                                                                                                                              | 86        |
| Flavanones                                                                                                                                                                                                                             | 88        |
| Anthocyanidins                                                                                                                                                                                                                         | 88        |
| Chalcones                                                                                                                                                                                                                              | 90        |
| Aurones                                                                                                                                                                                                                                | 90        |
| Biflavonoids                                                                                                                                                                                                                           | 90        |
| Overview of SAR studies of flavonoids                                                                                                                                                                                                  | 90        |
| Structure–activity relationship of flavones                                                                                                                                                                                            | 92        |
| Structure–activity relationship of flavonols                                                                                                                                                                                           | 94        |
| Structure–activity relationship of isoflavones                                                                                                                                                                                         | 96        |
| Structure–activity relationship of flavanols                                                                                                                                                                                           | 98        |
| Structure–activity relationship of flavanones                                                                                                                                                                                          | 99        |
| Structure–activity relationship of anthocyanidins                                                                                                                                                                                      | 101       |
| Structure–activity relationship of chalcones                                                                                                                                                                                           | 101       |
| Structure–activity relationship of aurones                                                                                                                                                                                             | 102       |
| Structure–activity relationship of biflavonoids                                                                                                                                                                                        | 103       |
| The role of epigenetic modifications in SAR studies                                                                                                                                                                                    | 103       |
| Multidrug resistance (MDR) and SAR                                                                                                                                                                                                     | 104       |

|                                                                                                       |            |
|-------------------------------------------------------------------------------------------------------|------------|
| Recent three-dimensional quantitative structure–activity relationship (3D-QSAR) studies on flavonoids | 105        |
| Conclusion                                                                                            | 106        |
| Abbreviations                                                                                         | 107        |
| References                                                                                            | 108        |
| <br>                                                                                                  |            |
| <b>4. Bioactive dihydrophenanthrenes from plants</b>                                                  | <b>117</b> |
| <i>Jiaxin Qi, Di Zhou, Gang Chen, and Ning Li</i>                                                     |            |
| Introduction                                                                                          | 117        |
| Biosynthesis of dihydrophenanthrenes                                                                  | 119        |
| Separation and identification of dihydrophenanthrenes                                                 | 120        |
| Structural types                                                                                      | 120        |
| Subtype A: DP monomers                                                                                | 120        |
| Subtype A-1: Simple DP monomers                                                                       | 121        |
| Subtype A-2: Dihydrophenanthrenequinones                                                              | 121        |
| Subtype A-3: DP monomers with C6-C3 fragment                                                          | 121        |
| Subtype A-4: Others                                                                                   | 121        |
| Subtype B: DP polymers                                                                                | 121        |
| Subtype B-1: DPs and DPs                                                                              | 121        |
| Subtype B-2: DPs and phenanthrenes                                                                    | 125        |
| Subtype B-3: DPs and bibenzyls                                                                        | 150        |
| Biological activities                                                                                 | 151        |
| Cytotoxic activity                                                                                    | 152        |
| Antiinflammatory activity                                                                             | 152        |
| Antimicrobial activity                                                                                | 153        |
| Antioxidant activity                                                                                  | 154        |
| Other activities                                                                                      | 155        |
| Conclusions                                                                                           | 155        |
| Abbreviations                                                                                         | 155        |
| References                                                                                            | 156        |
| <br>                                                                                                  |            |
| <b>5. Metabolomics applied to the discovery of new bioactive pharmaceuticals in complex matrices</b>  | <b>165</b> |
| <i>Caroline Schmitz, Aline Nunes, Deise Munaro, Thaise Gerber, and Marcelo Maraschin</i>              |            |
| Introduction                                                                                          | 165        |
| Analytical challenges on complex mixtures                                                             | 167        |
| Analytical methods                                                                                    | 169        |
| GC-MS and LC-MS                                                                                       | 169        |
| NMR                                                                                                   | 172        |
| Bioinformatic tools                                                                                   | 174        |
| Data (pre)processing                                                                                  | 174        |
| Software and databases                                                                                | 177        |

|                                                                                                                    |         |
|--------------------------------------------------------------------------------------------------------------------|---------|
| Biological models                                                                                                  | 178     |
| Algae                                                                                                              | 180     |
| Maize ( <i>Z. mays</i> L.)                                                                                         | 183     |
| Propolis                                                                                                           | 184     |
| Conclusion                                                                                                         | 186     |
| Acknowledgment                                                                                                     | 187     |
| References                                                                                                         | 188     |
| <br>6. Biosynthesis of polyunsaturated fatty acids by metabolic engineering of yeast <i>Yarrowia lipolytica</i>    | <br>197 |
| <i>Andrea Palyzová, Jaroslav Spížek, Milada Vítová, and Tomáš Řezanka</i>                                          |         |
| Introduction                                                                                                       | 197     |
| Genetic engineering strategy inducing PUFA production                                                              | 202     |
| Biosynthesis of microbial PUFAs                                                                                    | 204     |
| Biosynthesis of lipids in modified <i>Yarrowia lipolytica</i>                                                      | 209     |
| Eicosapentaenoic acid and docosahexaenoic acid                                                                     | 209     |
| Triacylglycerols                                                                                                   | 212     |
| Enhanced PUFA production using different carbon sources                                                            | 212     |
| Pilot—Scale process for microbial PUFA production                                                                  | 213     |
| Engineering <i>Y. lipolytica</i> for production of lipid compounds other than PUFAs                                | 214     |
| Engineering <i>Y. lipolytica</i> for biodiesel production                                                          | 215     |
| Conclusion                                                                                                         | 217     |
| Acknowledgment                                                                                                     | 217     |
| Abbreviations                                                                                                      | 217     |
| References                                                                                                         | 218     |
| <br>7. <i>Aspergillus versicolor</i> as a source of diversified metabolic products with pharmacological activities | <br>225 |
| <i>Shuai-Shuai Zhang, Zhi-Hui Meng, Guo-Zheng Zhao, Hui-Tao Wu, and Fei Cao</i>                                    |         |
| Introduction                                                                                                       | 225     |
| Isolation of secondary metabolites                                                                                 | 226     |
| Anthraquinones                                                                                                     | 226     |
| Xanthones                                                                                                          | 241     |
| Terpenes                                                                                                           | 244     |
| Peptides and diketopiperazines                                                                                     | 246     |
| Alkaloids                                                                                                          | 253     |
| Others                                                                                                             | 259     |
| Conclusion                                                                                                         | 267     |
| Classification of secondary metabolites of <i>A. versicolor</i>                                                    | 267     |
| Source classification                                                                                              | 268     |
| Biological activity classification of secondary metabolites                                                        | 269     |
| References                                                                                                         | 270     |

|                                                                                                                                                                |            |
|----------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| <b>8. Antihyperuricemic peptides: A review focused on xanthine oxidase inhibitory activities</b>                                                               | <b>279</b> |
| <i>Siyong You, Guiqing Wang, Fang Zhou, Haixia Wu, Yanqing Han, Wenshuang Xue, Yuchen Ma, Chunxue Zhang, Lehao Zhou, Fen Yan, Caili Fu, and Alideertu Dong</i> |            |
| Introduction                                                                                                                                                   | 279        |
| Preparation and properties of antihyperuricemic peptides                                                                                                       | 281        |
| Preparation and structural characterization                                                                                                                    | 281        |
| Properties of antihyperuricemic peptides in vivo and in vitro                                                                                                  | 284        |
| The interactions between XOD and antihyperuricemic peptides                                                                                                    | 287        |
| XOD                                                                                                                                                            | 287        |
| Molecular docking of antihyperuricemic peptides                                                                                                                | 287        |
| Conclusion and perspectives                                                                                                                                    | 290        |
| Acknowledgment                                                                                                                                                 | 290        |
| Declaration of competing interest                                                                                                                              | 291        |
| References                                                                                                                                                     | 291        |
| <b>9. Novel cold process applications for the preservation of bioactive components of several natural functional foods</b>                                     | <b>295</b> |
| <i>Aydin Kilic</i>                                                                                                                                             |            |
| Introduction                                                                                                                                                   | 295        |
| General considerations about novel LTHV-assisted fluidized bed drying                                                                                          | 299        |
| Novel low-temperature unit operation applications in pollen                                                                                                    | 302        |
| Novel low-temperature unit operations in fish                                                                                                                  | 306        |
| Novel low-temperature unit operations in white tea                                                                                                             | 312        |
| Novel low-temperature unit operations in spice ( <i>C. sativum</i> )                                                                                           | 319        |
| Conclusion                                                                                                                                                     | 322        |
| Acknowledgments                                                                                                                                                | 322        |
| Conflict of Interest                                                                                                                                           | 323        |
| Funding                                                                                                                                                        | 323        |
| Abbreviations                                                                                                                                                  | 323        |
| References                                                                                                                                                     | 324        |
| <b>10. Advances in biological activities of essential oils</b>                                                                                                 | <b>331</b> |
| <i>Aysegul Mutlu-Ingok, Dilara Devecioglu, Dilara Nur Dikmetas, and Funda Karbancioglu-Guler</i>                                                               |            |
| Introduction                                                                                                                                                   | 331        |
| Encapsulation of essential oils                                                                                                                                | 332        |
| Interactions of essential oils                                                                                                                                 | 336        |
| ACE-inhibitory activities of essential oils                                                                                                                    | 342        |
| $\alpha$ -Amylase and $\alpha$ -glucosidase inhibitory activities of essential oils                                                                            | 346        |
| Xanthine oxidase inhibitory activities of essential oils                                                                                                       | 351        |
| Conclusions                                                                                                                                                    | 358        |
| References                                                                                                                                                     | 358        |

|                                                                                                  |            |
|--------------------------------------------------------------------------------------------------|------------|
| <b>11. Natural polymers for wound dressing applications</b>                                      | <b>367</b> |
| <i>Gökçen Yaşayan, Emine Alarçin, Ayça Bal-Öztürk, and Meltem Avcı-Adalı</i>                     |            |
| <b>Introduction</b>                                                                              | <b>368</b> |
| Wounding and wound types                                                                         | 369        |
| Wound healing phases                                                                             | 370        |
| Parameters to consider during wound care                                                         | 372        |
| Dressings for wound care                                                                         | 372        |
| The impact of distinctive functions of natural materials on wound dressing design                | 378        |
| <b>Commonly used polymers and elements in fabrication of wound dressings</b>                     | <b>381</b> |
| Natural polymers                                                                                 | 381        |
| Synthetic polymers used in combination with natural polymers                                     | 394        |
| Other compositional elements                                                                     | 396        |
| <b>Novel strategies in fabrication of wound dressings</b>                                        | <b>398</b> |
| Lithography                                                                                      | 398        |
| Electrospinning                                                                                  | 401        |
| Bioprinting                                                                                      | 403        |
| Tissue engineered skin substitutes                                                               | 410        |
| Decellularized skin ECM (dECM)                                                                   | 412        |
| <b>Commercial wound dressings by natural polymers</b>                                            | <b>413</b> |
| <b>Conclusions and future outlook</b>                                                            | <b>413</b> |
| <b>References</b>                                                                                | <b>417</b> |
| <br>                                                                                             |            |
| <b>12. Insight into coronaviruses and natural products-based approach for COVID-19 treatment</b> | <b>443</b> |
| <i>P.S. Suresh, S.S. Gupta, Anmol, and U. Sharma</i>                                             |            |
| <b>Introduction</b>                                                                              | <b>443</b> |
| <b>Coronavirus</b>                                                                               | <b>445</b> |
| Morphology and genome                                                                            | 445        |
| Mode of action and pathogenesis                                                                  | 447        |
| Challenges and opportunity in COVID-19 treatment                                                 | 449        |
| <b>Potential therapeutic targets for COVID-19 treatment</b>                                      | <b>450</b> |
| <b>Anticoronaviral natural products</b>                                                          | <b>450</b> |
| <b>Molecular docking and pharmacokinetic study: For support</b>                                  | <b>453</b> |
| <b>Conclusion</b>                                                                                | <b>461</b> |
| <b>Acknowledgments</b>                                                                           | <b>461</b> |
| <b>Conflict of Interest</b>                                                                      | <b>461</b> |
| <b>Abbreviations</b>                                                                             | <b>461</b> |
| <b>References</b>                                                                                | <b>462</b> |

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# Preface

Volume 74 of the book *Studies in Natural Products Chemistry* contains a number of remarkable reviews covering various important aspects of bioactive natural products. [Chapter 1](#) by Ishiwata et al. presents a broad review on various glucans and their synthesis by employing the recent advances in stereoselective bimodal glycosylation. Many diterpenes exhibit a wide range of biological activities including antitumor effects. In [Chapter 2](#), Panis et al. present the new perspectives and current advances in plant-derived diterpenes for the treatment of breast cancer. In [Chapter 3](#), Süntar et al. provide a detailed review on anticancer activity of selected flavonoids and their chemical structures.

Dihydrophenanthrenes have been frequently isolated from plants. Li et al. have summarized the recent literature on natural dihydrophenanthrenes and their activities in [Chapter 4](#). In [Chapter 5](#), Maraschin et al. review the applications of metabolomics in the discovery of novel bioactive compounds having pharmaceutical potential. Metabolic engineering involves the intentional modification of cellular metabolism for the production of desired compounds. A review on the current metabolic engineering strategies used in the manufacturing and biosynthesis of some nutraceuticals is presented by Řezanka et al. in [Chapter 6](#). In [Chapter 7](#), Cao et al. present a comprehensive review on the isolation, characterization, and biological activities of *Aspergillus versicolor*, which was isolated from a marine sponge and served as a source of several interesting bioactive metabolites.

Hyperuricemia and gout are clinical conditions in which there is excess production of uric acid in the body, which leads to inflammation and intense pain in the joints. Fu et al. provide a detailed review on the understanding of the mechanism of antihyperuricemic peptides in [Chapter 8](#). In [Chapter 9](#), Kilic has discussed some novel cold unit operations for the preservation of bioactive components of natural functional foods.

Essential oils are secondary metabolites extracted from potentially beneficial plants having antimicrobial and some other biological activities. Mutlu-Ingok et al. present a review on the recent advances in biological activities of essential oils in [Chapter 10](#). Some natural polymers are significantly attractive wound dressing materials owing to their bioactivity and biocompatibility. [Chapter 11](#) by Yaşayan et al. emphasizes the use of natural polymers and their functions in the wound healing process. Finally, [Chapter 12](#) by Sharma et al. focuses on the hot topic of the recent pandemic year with

insights into coronaviruses and natural product-based therapeutic targets and strategies.

This volume contains many excellent reviews by eminent experts on bioactive natural products. The volume proves to be of great interest to researchers working in the field of medicinal and natural products chemistry.

I express my sincere thanks to Mr. Mahmood Alam for his support in the preparation of this volume. I also thank Dr. Taqdees Malik for her secretarial assistance.

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## Chapter 6

# Biosynthesis of polyunsaturated fatty acids by metabolic engineering of yeast *Yarrowia lipolytica*

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### Chapter Outline

|                                                               |     |                                                                                     |     |
|---------------------------------------------------------------|-----|-------------------------------------------------------------------------------------|-----|
| Introduction                                                  | 197 | Pilot—Scale process for microbial PUFA production                                   | 213 |
| Genetic engineering strategy inducing PUFA production         | 202 | Engineering <i>Y. lipolytica</i> for production of lipid compounds other than PUFAs | 214 |
| Biosynthesis of microbial PUFAs                               | 204 | Engineering <i>Y. lipolytica</i> for biodiesel production                           | 215 |
| Biosynthesis of lipids in modified <i>Yarrowia lipolytica</i> | 209 | Conclusion                                                                          | 217 |
| Eicosapentaenoic acid and docosahexaenoic acid                | 209 | Acknowledgment                                                                      | 217 |
| Triacylglycerols                                              | 212 | Abbreviations                                                                       | 217 |
| Enhanced PUFA production using different carbon sources       | 212 | References                                                                          | 218 |

### Introduction

The rapid development of society, especially Western civilization after World War II, has led to a change in diets. Mainly due to the high consumption of vegetable oils, a change in the type of fatty acids consumed has occurred, especially in the amounts of  $\omega$ -6 and  $\omega$ -3 polyunsaturated fatty acids (PUFAs)

**TABLE 6.1** Names of PUFAs

| $\omega$ -x Nomenclature | Abbreviation   | Systematic Abbreviation |
|--------------------------|----------------|-------------------------|
| 16:0                     | Palmitic       | 16:0                    |
| 18:0                     | Stearic        | 18:0                    |
| 18:1 $\omega$ 9          | Oleic          | 9-18:1                  |
| 18:2 $\omega$ 6          | Linoleic or LA | 9,12-18:2               |
| 18:3 $\omega$ 3          | ALA            | 9,12,15-18:3            |
| 18:3 $\omega$ 6          | GLA            | 6,9,12-18:3             |
| 18:4 $\omega$ 3          | STA            | 6,9,12,15-18:4          |
| 20:2 $\omega$ 6          | EDA            | 11,14-20:2              |
| 20:3 $\omega$ 6          | DGLA           | 8,11,14-20:3            |
| 20:3 $\omega$ 3          | ETrA           | 11,14,17-20:3           |
| 20:4 $\omega$ 6          | ARA            | 5,8,11,14-20:4          |
| 20:4 $\omega$ 3          | ETA            | 8,11,14,17-20:4         |
| 20:5 $\omega$ 3          | EPA            | 5,8,11,14,17-20:5       |
| 22:4 $\omega$ 6          | DTA            | 7,10,13,16-22:4         |
| 22:5 $\omega$ 3          | DPA            | 7,10,13,16,19-22:5      |
| 22:6 $\omega$ 3          | DHA            | 4,7,10,13,16,19-22:6    |

All double bond(s) have *cis* configuration(s), e.g., all-*cis* 6,9,12,15-18:4 or better (6Z,9Z,12Z,15Z-18:4)<sup>a</sup>.

<sup>a</sup>According to the current nomenclature, the geometric isomers should be used instead of the *cis*-*trans* nomenclature, the so-called E-Z nomenclature (see below), but by convention is still an outdated designation, i.e., *cis*. Configuration E-Z is the IUPAC preferred method for describing the absolute stereochemistry of double bonds in organic chemistry according to the Cahn-Ingold-Prelog priority rules [15], i.e., the configuration E (from *entgegen*, the German word for "opposite") and the configuration Z (from *zusammen*, the German word for "together").

(Table 6.1 and Fig. 6.1) [1]. According to WHO reports, excessive use of vegetable oils has shifted the ratio of  $\omega$ -6/ $\omega$ -3 from the ideal value of 1:1–1:0.25 to an almost unbelievable 1:0.1–1:0.03, i.e., values typical of European and US residents. This change in ratio brings with it a number of complications including an increased incidence of cardiovascular disease, increased risk of cancer and autoimmune diseases, mental disorders, worsening memory in the form of Alzheimer's disease, developmental retardation, visual disturbances, muscle weakness and tremor, dyslipidemia, insulin resistance, high blood pressure,

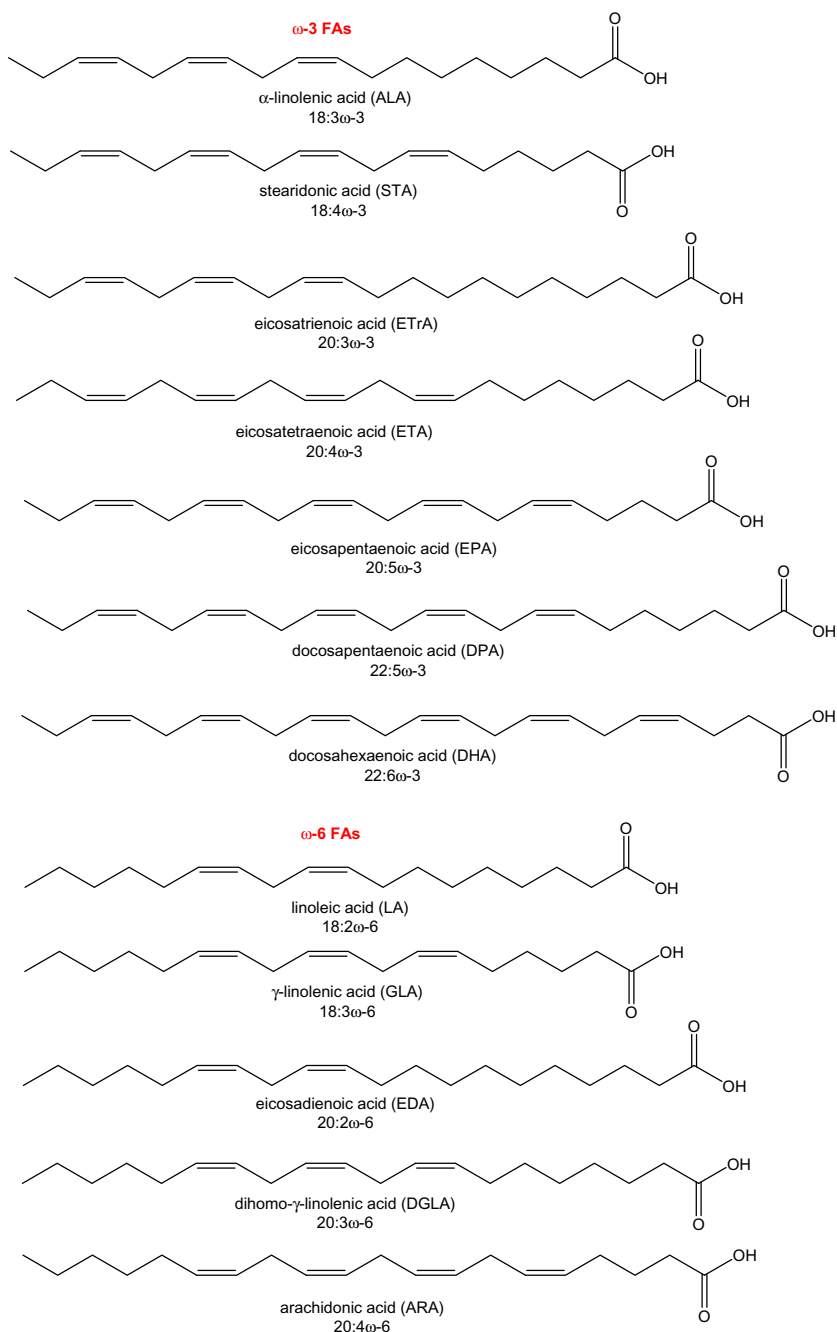


FIG. 6.1 Structures of the most common polyunsaturated fatty acids.

thrombosis, diabetes, asthma, and obesity [2–10]. Humans have the natural ability to biosynthesize eicosapentaenoic acid (EPA) and/or docosahexaenoic acid (DHA) from linolenic acid, but production is insufficient to meet daily needs [11]. In humans, DHA is therefore either obtained from food or is biosynthesized in small amounts from eicosapentaenoic acid (EPA, 20:5,  $\omega$ -3) by “Sprecher’s shunt” [12] via the metabolic intermediate docosapentaenoic acid (DPA, 22:5  $\omega$ -3). The ability of human enzymes to desaturate and elongate essential linoleic acid (LA) and  $\alpha$ -linolenic acid (ALA) to EPA and especially to DHA is low and insufficient to provide adequate levels of LC-PUFA to meet needs for mental and cardiovascular health. In addition, biosynthesis decreases with increasing age and in some diseases [13,14]. Therefore, DHA is now considered an essential PUFA.

The current production of PUFA from natural sources is not sufficient for commercial use. The main source of PUFAs is currently sea fish, but fish stocks are declining and may not represent a truly sustainable resource (Table 6.2) [16].

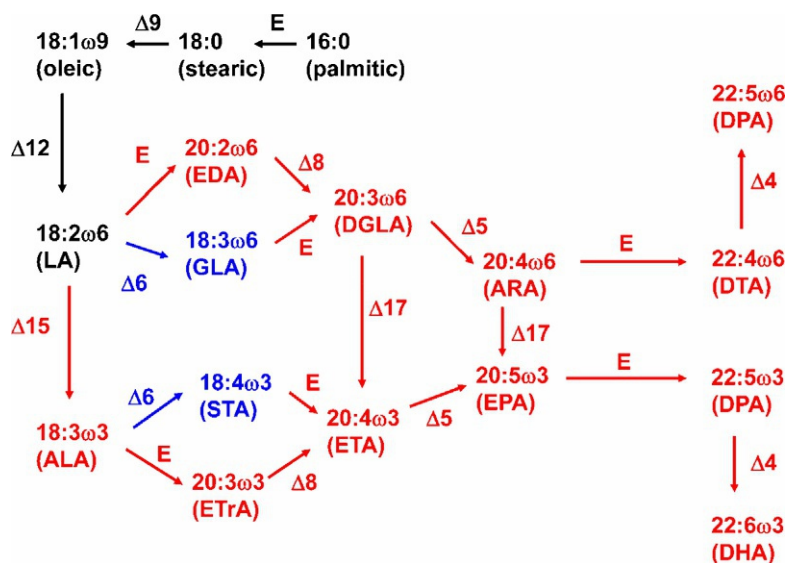
Furthermore, heavy metal pollution, toxic organic molecules, and recently, microplastics represent serious contamination problems for sea fish. Feeding fish on fish farms or freshwater ponds with wheat or corn leads to only minor levels of EPA and DHA in these fish; in addition, the  $\omega$ -6/ $\omega$ -3 ratio has shifted toward the  $\omega$ -3 PUFA [1]. At present, as an alternative to fish oils, some  $\omega$ -3 PUFA-rich oils, mostly from microorganisms [17] or agriculture crops, are added to foods sold on the market. Vegetable oils obtained from agricultural crops such as sunflower, soy, canola, palm and olive oils are relatively inexpensive and are not contaminated with pollutants. However, PUFAs found in commercially available vegetable oils are typically limited to LA (18:2 $\omega$ -6) and ALA (18:3 $\omega$ -3) [18].

In conventional PUFA biosynthesis, saturated long-chain fatty acids produced by fatty acid synthase (FAS) are modified by a series of elongation and desaturation reactions. A number of separate desaturase and elongase enzymes (approximately 30 different enzymes and 70 reactions; [19]) are required to produce unsaturated and longer chain PUFAs, typically EPA and/or DHA. See also a simplified scheme of biosynthesis (Fig. 6.2).

Many plants, including algae and some fungi, have the ability to synthesize LA and ALA. They express genes encoding  $\Delta$ 12-desaturase (*fad2*) and  $\omega$ 3-desaturase (*fad3*). The direct identification of the respective enzymes, i.e.,  $\Delta$ 12-desaturase and  $\omega$ 3-desaturase, has been performed only a few times, for example, for  $\Delta$ 12-desaturase [20] and  $\omega$ 3-desaturase [21] from *Saccharomyces kluyveri*. Coexpression of two genes from *Kluyveromyces lactis*, structurally similar to  $\Delta$ 12-desaturase and  $\omega$ 3-desaturase of *Saccharomyces cerevisiae*, led to the endogenous production of both PUFAs, i.e., LA and ALA [22]. Furthermore, from the fungus *M. alpina*,  $\Delta$ 6-desaturase and  $\Delta$ 12-desaturase cDNA

**TABLE 6.2** Sources of Polyunsaturated Fatty Acids [16]

| PUFA | Conventional Sources                                                                                 | Microbial Sources                                                                                                                                                                                                                                                                                                                                                                                                                                       |
|------|------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| GLA  | Plants (Evening primrose, Borage, Blackcurrant)                                                      | Fungi ( <i>Mucor circinelloides</i> , <i>M. mucedo</i> , <i>Mortierella isabellina</i> , <i>M. ramanniana</i> , <i>Cunninghamella echinulata</i> , <i>C. elegans</i> , <i>C. japonica</i> , <i>Rhizopus arrhizus</i> , <i>Thamnidium elegans</i> )<br><br>Algae ( <i>Spirulina platensis</i> , <i>Chlorella vulgaris</i> )                                                                                                                              |
| DGLA | Human milk, animal tissues, fish ( <i>Scomber scombrus</i> ), mosses ( <i>Pogonatum urnigerum</i> )  | Fungi ( <i>Mortierella</i> spp., <i>Mortierella alpina</i> , <i>Conidiobolus nanodes</i> , <i>Saprolegnia ferax</i> )                                                                                                                                                                                                                                                                                                                                   |
| ARA  | Animal tissues, fish ( <i>Brevoortia</i> , <i>Clupea</i> ), mosses ( <i>Ctenidium molluscum</i> )    | Fungi ( <i>Mortierella</i> spp., <i>M. alpina</i> , <i>Cladonia nanodes</i> , <i>Entomophthora exitalis</i> , <i>Blastocladiella emersonii</i> )<br><br>Algae ( <i>Porphyridium cruentum</i> , <i>Sargassum salicifolium</i> , <i>Euglena gracilis</i> )                                                                                                                                                                                                |
| EPA  | Fish (herring, menhaden), shellfish (blue crab, oyster, lobster, mussel)                             | Fungi ( <i>M. alpina</i> , <i>Mortierella elongata</i> , <i>Pythium irregulare</i> , <i>Pythium ultimum</i> ), algae ( <i>Chlorella minutissima</i> , <i>C. minutissima</i> , <i>Monodus subterraneus</i> , <i>Polysiphonia latifolium</i> , <i>P. cruentum</i> , <i>Phaeodactylum tricornutum</i> , <i>Nannochloropsis oculata</i> , <i>Amphidinium carteri</i> , <i>Thalassiosira pseudonana</i> )<br><br>Bacteria ( <i>Shewanella putrefaciens</i> ) |
| ETA  | Animal tissues                                                                                       | Fungi ( <i>M. alpina</i> )                                                                                                                                                                                                                                                                                                                                                                                                                              |
| DPA  | Fish                                                                                                 | Fungi ( <i>Schyzochytrium</i> sp.)                                                                                                                                                                                                                                                                                                                                                                                                                      |
| DHA  | Fish (tuna, herring, cod, sardine, salmon, menhaden), shellfish (blue crab, mussel, lobster, oyster) | Fungi ( <i>Thraustochytrium aureum</i> , <i>T. roseum</i> , <i>Schyzochytrium</i> SR21, <i>Schyzochytrium aggregatum</i> ), algae ( <i>Cryptocodinium cohnii</i> , <i>Gyrodinium nelsoni</i> , <i>A. carteri</i> , <i>Gonyaulax lyedra</i> ), bacteria ( <i>Vibrio</i> spp., <i>Rhodopseudomonas</i> spp.)                                                                                                                                              |



**FIG. 6.2** Metabolic engineering in *Yarrowia lipolytica* for PUFA production. The native fatty acid pathway is indicated in black and the genetically modified pathway for PUFA production is indicated in red. Undesirable metabolic pathways leading to  $\omega$ -6 PUFAs, i.e., mainly GLA, are indicated in blue.

were isolated and their coexpression in *S. cerevisiae* showed that the yield of  $\gamma$ -linolenic acid reached up to 8% of total fatty acids. The yeast was cultured at 15°C for 2 days in a synthetic medium containing 2% galactose [23].

## Genetic engineering strategy inducing PUFA production

The metabolic engineering of nutraceutical products provides an attractive alternative to chemical synthesis or extraction from natural material, allowing the production of pure compounds under environmentally acceptable conditions. Model microorganisms such as *Escherichia coli*, *Saccharomyces cerevisiae*, or *Corynebacterium glutamicum* are used for this purpose [24–26]. These traditional hosts were usually selected based on their well-described metabolism, safe use status in the pharmaceutical and food industries and high genetic traceability [27]. Despite the prospects provided by metabolic engineering, regulation is required for the use of genetically modified organisms (GMOs) in commercial production processes. In this respect, GMO regulation includes approval processes, risk assessment, and other requirements that vary considerably from country to country [28–30].

From the very beginning, GMO foods [31–34], or food supplements, including PUFAs produced by genetically modified *Yarrowia lipolytica*, have

been widely discussed both as a result of their industrial applications and the possible consequences of their use. Ethical concerns include adverse effects on human health, the decline of overall biodiversity and, most importantly, the risk of release into the wild, as can be observed in some conspiratorial rumors about the escape of Covid-19 virus from the laboratory. These concerns can affect a person's psychological state when consuming GM products. The issue of labeling of GMO foods, for example, in the United States, in contrast to 64 other world nations, including the EU, is different and is also accepted differently by the public. In contrast, GMOs benefit humanity when used for purposes such as increasing the availability and quality of food, medical care and contributing to a cleaner environment. Used wisely, they could result in improved economic activity and alleviate food deficiencies. However, the full potential of GMOs cannot be realized without close attention to the risks associated with each new GMO case.

In recombinant microorganisms, the microbial profile of fatty acids can be altered both by creating new biosynthetic pathways in the host and/or by suppressing undesirable biosynthetic pathways. This can result in increased production of desirable, and reduced production of undesirable PUFA(s). In the case of PUFA production, this primarily means to influence the ratio of  $\omega$ -3 and  $\omega$ -6 fatty acids synthesized. This possibility leads to specific biosynthesis of, e.g., EPA without significant production of, e.g.,  $\gamma$ -linolenic acid (GLA; 18:3 $\omega$ -6). Microbial production of EPA using recombinant strains of microorganisms has advantages over production of EPA from natural microbial sources, e.g., diatoms *Cyclotella* sp. or *Nitzschia* sp., the bacterium *Shewanella*, filamentous fungi of the genera *Pythium* sp., *Mortierella elongata* or isolation from fish oil and/or marine plankton.

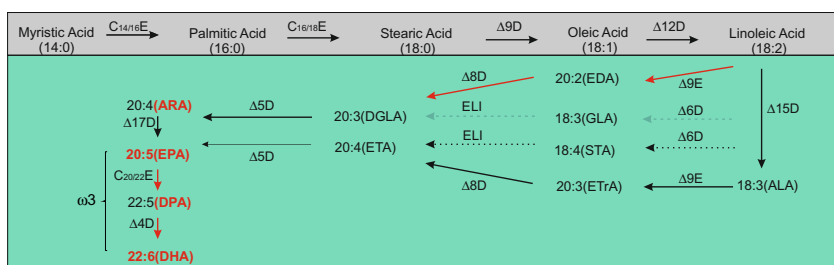
For the development and optimization of *Y. lipolytica* in order to improve production mainly of fatty acids, the whole bioprocess, including the preparation of the starting strain, must be performed on a larger scale, i.e., at least in a pilot plant in fermenters of tens of liters. Production must also be optimized. A study has already been published [35] that convincingly demonstrates that metabolic engineering, systems biology integration, synthetic biology, and evolutionary engineering can enable microbial strains to produce chemicals efficiently. One way to optimize cellular metabolism is to reduce the production of undesirable metabolites (i.e., biosynthesis of certain fatty acids, e.g., GLA) [36,37]. Other low-cost carbon sources can be used to reduce fermentation costs, especially single-carbon substrates, which can be metabolized to fatty acids by metabolic engineering of *Y. lipolytica*. We believe that genetically modified strains of *Y. lipolytica* can be used to produce fatty acid-based chemicals, holding great promise for researchers working in this field.

As a basis for the successful production of lipids in yeast, knowledge of the complete genome is necessary. In addition, genome-scale models

(GSMs) of *Y. lipolytica* [38,39] should cover multiple reactions, and its reconstruction should involve annotations of unknown protein sequences predicted from the entire genome. Furthermore, much more attention must be paid to the physiological conditions under which the yeast is grown. One possibility is to influence the carbon-to-nitrogen ratio, the amount of phosphorus, and other elements such as magnesium. High transformation efficiency is also important to avoid time losses due to the need for repetitive experiments. It is necessary to integrate genes into specific loci and to insert the entire pathway, which would allow integration of more than one DNA fragment and thus significantly speed up strain engineering. In the field of metabolite analysis, in this case PUFAs, it is first necessary to simplify the preparation of methyl esters or even to analyze free fatty acids and to reduce the time required for analysis, see, e.g., Rezanka et al. [40]. It is also necessary to suppress the activity of enzymes causing, for example, the hydrolysis of triacylglycerols as end products of lipid biosynthesis and the degradation of fatty acids ( $\beta$ -oxidation). There are many possible solutions for implementation of more efficient engineering of *Y. lipolytica*. Tools and methods developed in the past could serve as starting points for the development of more productive strains. Furthermore, it would be possible to include other yeasts such as *S. cerevisiae* in screenings, which could lead to an expansion of possible strains to use and may be of great practical importance.

## Biosynthesis of microbial PUFAs

For a long time, species of yeasts *S. cerevisiae* or *Schizosaccharomyces pombe*, used in the production of beer or bread, have served as model organisms in the development of metabolic engineering strategies for the synthesis of certain metabolites [41]. Wild strains of *S. cerevisiae* produce saturated and monounsaturated fatty acids, mainly with 16- and 18-carbons, because they contain only one fatty acid desaturase, i.e.,  $\Delta 9$ -desaturase (OLE1), which is capable of producing monounsaturated palmitoleic (16:1 $\omega$ -7) and oleic (18:1 $\omega$ -9) acids [42]. Both *S. cerevisiae* and *Sch. pombe* are unable to synthesize linoleic acid (18:2 $\omega$ -6) [43,44]. However, in some yeasts, oleic acid can be desaturated by introducing a second double bond at the  $\Delta 12$  position with  $\Delta 12$ -fatty acid desaturase, followed by desaturation of  $\omega 3$ -fatty acids (Fig. 6.3) to form ALA. Examples include *S. kluyveri* or *K. lactis* that can produce both PUFAs (18:2 $\omega$ -6 and 18:3 $\omega$ -3) [22,44]. In addition to *S. kluyveri* and *K. lactis*, *Candida albicans* and *C. tropicalis* are able to produce ALA to a greater or lesser extent, but other yeasts such as *Candida boidinii* and *Zygosaccharomyces rouxii* are only able to produce LA [43]. Oleaginous yeasts include *Y. lipolytica* (max lipid content 36%) and *Trichosporon pullulans* (65%) producing about 1% ALA, but *Lipomyces starkeyi* (63%), *Cryptococcus albidus* (65%), and *Rhodotorula glutinis* (72%) can only produce LA [45].



**FIG. 6.3** Metabolic engineering in *Yarrowia lipolytica* for omega-3 EPA and DHA production (indicated in red). The native fatty acid pathway is indicated in the gray box and the engineered pathway for  $\omega$ -3 EPA and DHA production is indicated in green.

PUFA synthesis occurs in mammals, including humans, predominantly by the  $\Delta 6$ -desaturation route, with dietary LA and/or linolenic acids being desaturated by  $\Delta 6$  desaturase to  $\gamma$ -linolenic (18:3 $\omega$ -6) and stearidononic (18:4 $\omega$ -3) acids, see Fig. 6.3. Hydrocarbon chain elongation and further desaturation leads to arachidonic (20:4 $\omega$ -6), possibly dihomo- $\gamma$ -linolenic acid (20:3 $\omega$ -6) in the  $\omega$ -6 pathway, and eicosapentaenoic (20:5 $\omega$ -3) and docosahexaenoic (22:6 $\omega$ -3) acids via eicosatetraenoic acid (20:4 $\omega$ -3) in the  $\omega$ -3 pathway (Fig. 6.3). Therefore, suitable desaturase and elongase genes must be introduced into yeast for the production of PUFAs; see several examples in Table 6.3.

In order for *S. cerevisiae* to produce PUFAs with 20 or 22 carbon atoms, it is necessary to insert genes encoding  $\Delta 4$ -,  $\Delta 5$ -, and  $\Delta 6$ -fatty acid desaturases. As mentioned above, (see Table 6.3), various organisms have been used, including higher plants, algae, mosses, fungi, nematodes, and also mammals. In addition, elongase genes of *S. cerevisiae* are necessary. These are the three elongations of fatty acids genes that unfortunately are not functional for the biosynthesis of the PUFAs described above. The first of these, i.e., Elo1p mainly elongates myristic and palmitic acids, and Elo2p and Elo3p are involved in the formation of very long-chain saturated fatty acids, which are a part of sphingolipids. Therefore, elongase genes from other organisms were used. In essence, these organisms produce C<sub>20</sub> and C<sub>22</sub> PUFAs. Because  $\Delta 5$  and  $\Delta 6$  desaturases are active in both series ( $\omega$ -6 and  $\omega$ -3), the resulting products can be both arachidonic and eicosapentaenoic fatty acids. For examples see paper [46], which used elongase (*Caenorhabditis elegans*),  $\Delta 5$ -desaturase (*M. alpine*), and  $\Delta 6$ -desaturase (borage). *S. cerevisiae* was cultured for 4 days at 25°C in a synthetic medium containing 2% galactose, and two fatty acid starters (LA and/or ALA) were added at a concentration of 0.5 mM. After cultivation, the fatty acid composition was as follows:  $\gamma$ -linolenic 6.8%, dihomo- $\gamma$ -linolenic 1.4%, arachidonic 0.2%, and eicosapentaenoic 0.2%.

**TABLE 6.3** Examples of Some Enzymes Used for the Biosynthesis (Production) of PUFAs

| Biological Material                         | Enzymes                          | GenBank Accession No.              |
|---------------------------------------------|----------------------------------|------------------------------------|
| <i>Pavlova lutheri</i>                      | $\Delta 4$ fatty acid desaturase | AY332747                           |
| <i>Caenorhabditis elegans</i>               | $\Delta 5$ fatty acid desaturase | AAC95143.1                         |
| <i>Dictyostelium discoideum</i>             | $\Delta 5$ fatty acid desaturase | AB022097                           |
| <i>Homo sapiens</i>                         | $\Delta 5$ desaturase            | AF199596                           |
| <i>Mortierella alpina</i>                   | $\Delta 5$ fatty acid desaturase | AF067654                           |
| <i>Mus musculus</i>                         | $\Delta 5$ desaturase D5D        | AB072976                           |
| <i>Phytophthora megasperma</i>              | $\Delta 5$ fatty acid desaturase | AJ510244                           |
| <i>Pythium irregulare</i>                   | $\Delta 5$ fatty acid desaturase | AF419297                           |
| <i>Rattus norvegicus</i> liver              | $\Delta 5$ desaturase            | AF320509                           |
| <i>Thraustochytrium</i> sp.                 | $\Delta 5$ fatty acid desaturase | AF489588                           |
| <i>Argania spinosa</i>                      | $\Delta 6$ desaturase            | AY131238                           |
| <i>Borago officinalis</i>                   | $\Delta 6$ desaturase            | AF007561                           |
| <i>Ceratodon purpureus</i>                  | $\Delta 6$ fatty acid desaturase | AJ250735                           |
| <i>Echium gentianoides</i>                  | $\Delta 6$ desaturase            | AY055117                           |
| <i>Echium pitardii</i> var. <i>pitardii</i> | $\Delta 6$ desaturase            | AAL23581.1                         |
| <i>H. sapiens</i>                           | $\Delta 6$ fatty acid desaturase | AF126799                           |
| <i>Mortierella alpina</i>                   | $\Delta 6$ fatty acid desaturase | AF465283                           |
| <i>Mortierella isabellina</i>               | $\Delta 6$ fatty acid desaturase | AF465281,<br>AF110510,<br>AF465282 |
| <i>Mucor circinelloides</i>                 | $\Delta 6$ fatty acid desaturase | AB052086                           |
| <i>Mucor rouxii</i>                         | $\Delta 6$ desaturase            | AF296076                           |
| <i>M. musculus</i>                          | $\Delta 6$ fatty acid desaturase | AF126798                           |
| <i>Pythium irregulare</i>                   | $\Delta 6$ fatty acid desaturase | AF419296                           |
| <i>Synechocystis</i> sp.                    | $\Delta 6$ desaturase            | L11421                             |
| <i>Acheta domesticus</i>                    | $\Delta 9$ desaturase 3          | AF338466                           |

**TABLE 6.3** Examples of Some Enzymes Used for the Biosynthesis (Production) of PUFAs—Cont'd

| Biological Material                      | Enzymes                                             | GenBank Accession No. |
|------------------------------------------|-----------------------------------------------------|-----------------------|
| <i>Anabaena</i> sp.                      | $\Delta 9$ desaturase                               | E11368                |
| <i>Emericella nidulans</i>               | $\Delta 9$ stearic acid desaturase                  | AY504633              |
| <i>Mortierella alpina</i>                | $\Delta 9$ desaturase                               | AF085500              |
| <i>Ogataea (Pichia) angusta</i>          | $\Delta 9$ fatty acid desaturase                    | D83185                |
| <i>Picea glauca</i>                      | $\Delta 9$ desaturase                               | AF438199              |
| <i>Synechococcus vulcanus</i>            | $\Delta 9$ acyl-lipid fatty acid desaturase         | U90417                |
| <i>Synechococcus vulcanus</i>            | $\Delta 9$ acyl-lipid fatty acid desaturase         | U90417                |
| <i>Arabidopsis thaliana</i>              | $\Delta 12$ desaturase microsomal                   | BAB01960.1            |
| <i>C. elegans</i>                        | $\Delta 12$ fatty acid desaturase FAT-2             | AF240777              |
| <i>Emericella (Aspergillus) nidulans</i> | $\Delta 12$ desaturase oleate                       | AAG36933              |
| <i>Chlorella vulgaris</i>                | $\Delta 12$ desaturase CvFad2                       | AB075526              |
| <i>Mortierella alpina</i>                | $\Delta 12$ fatty acid desaturase                   | AAL13300              |
| <i>Mortierella alpina</i>                | $\Delta 12$ fatty acid desaturase                   | AB020033              |
| <i>Mortierella alpina</i>                | $\Delta 12$ fatty acid desaturase                   | AF110509              |
| <i>Mortierella alpina</i>                | $\Delta 12$ fatty acid desaturase                   | AF417244              |
| <i>Mucor rouxii</i>                      | $\Delta 12$ desaturase                              | AF161219              |
| <i>Spirulina platensis</i>               | $\Delta 12$ desaturase                              | X86736                |
| <i>Perilla frutescens</i>                | $\Delta 15$ desaturase                              | AAL36934              |
| <i>Synechocystis</i> sp.                 | $\Delta 15$ desaturase                              | BAA18302.1            |
| <i>Synechocystis</i> sp.                 | $\Delta 15$ desaturase                              | BAA02924              |
| <i>Chlamydomonas reinhardtii</i>         | $\omega 6$ desaturase for chloroplast               | AB007640              |
| <i>C. elegans</i>                        | Elongation of very long chain fatty acids protein 6 | NM_068396             |
| <i>Mortierella alpina</i>                | Elongase gene                                       | AX464731              |

Continued

**TABLE 6.3** Examples of Some Enzymes Used for the Biosynthesis (Production) of PUFAs—Cont'd

| Biological Material      | Enzymes                                                     | GenBank Accession No.      |
|--------------------------|-------------------------------------------------------------|----------------------------|
| <i>M. musculus</i>       | ELOVL family member 5, elongation of long chain fatty acids | <a href="#">NM_134255</a>  |
| <i>R. norvegicus</i>     | ELOVL fatty acid elongase 5                                 | <a href="#">NM_134382</a>  |
| <i>R. norvegicus</i>     | ELOVL fatty acid elongase 6                                 | <a href="#">NM_134383</a>  |
| <i>Brassica oleracea</i> | Stearoyl-ACP desaturase                                     | <a href="#">AAK14969.1</a> |
| <i>A. thaliana</i>       | 3-Ketoacyl-CoA synthase 18                                  | <a href="#">NM_119617</a>  |

Another long-chain fatty acid, docosahexaenoic acid, was also produced by introducing genes into *S. cerevisiae*. This involved coexpression of  $\Delta 6$ -elongase (alga *Thalassiosira pseudonana*) and  $\Delta 5$ -elongase (alga *Ostreococcus tauri*) with algal  $\Delta 5$ -desaturase (*Phaeodactylum tricornutum*) and  $\Delta 4$ -desaturase (*Euglena gracilis*) or three bifunctional elongases (fish *Oncorhynchus mykiss*), algal  $\Delta 5$ -desaturase (*P. tricornutum*) and  $\Delta 4$ -desaturase (*E. gracilis*) [47]. The yeasts were cultured at 20°C for 4 days in a synthetic medium containing 2% galactose and 0.25–0.5 mM stearidonic acid (18:4 $\omega$ -3) as a precursor. The yield was about 0.5% DHA, based on total fatty acids.

Although not all examples of successful gene transfer responsible for the production of PUFAs in *S. cerevisiae* are given above, it can be seen (see also paper of [48]) that the yields are not completely comparable with sources used for production of PUFAs. Therefore, attention was focused on another yeast, e.g., *Y. lipolytica*. There is, of course, the possibility of increasing the production of lipids in *S. cerevisiae*, either by the classical procedure, i.e., by changing the culture conditions, or by means of genetically modified yeast. As an example, we should mention article [49] where the authors successfully reprogramed yeast metabolism from alcoholic fermentation to lipogenesis. In essence, the key step was to prevent the formation of acetyl-CoA in the cytosol and the transfer of pyruvate formed from glucose via glucose  $\beta$ -phosphate and phosphoenolpyruvate into the mitochondrial matrix. Here, pyruvate is involved in the tri-carboxylic acid (Krebs) cycle and the resulting acetyl-CoA is further metabolized to malonyl-CoA and fatty acids. Although the goal was to create a strain producing free fatty acids (predominantly C10 to C18, palmitoleic and oleic acids, with palmitic, palmitoleic and oleic acids being identified as the majority), it is certainly possible to create a strain producing PUFAs.

## Biosynthesis of lipids in modified *Yarrowia lipolytica*

Next to standard organisms, many other microorganisms have been investigated for the production of nutraceuticals, e.g., *Y. lipolytica* [50,51] has been extensively studied for its innate ability to produce large quantities of lipids [52]. Oleaginous yeasts are defined as yeasts that are naturally capable of biosynthesis and oil storage, with an oil content of at least 25% dry cell weight (DCW). Thanks to metabolic engineering efforts, PUFAs have also been produced (e.g., Table 6.4).

*Y. lipolytica* belongs to the Ascomycota family. The ascigerous teleomorph of *Candida lipolytica*, previously classified as *Endomycopsis lipolytica* and *Saccharomycopsis lipolytica*, has been assigned to the new genus *Yarrowia*. *Yarrowia lipolytica* is a typical species of this genus [53]. The complete genome of the strain *Y. lipolytica* CLIB122 has been published (Gen Bank: GCA000002525). It is approximately 20.5 Mb in size and is organized into 6 chromosomes that encode ~6472 genes. *Y. lipolytica* is a nonpathogenic yeast that can utilize various fats or hydrocarbons as carbon sources. Therefore, it is often isolated from foods such as cheese and sausage, or from natural sources such as oil fields. *Y. lipolytica* is only very distantly associated with other yeasts. It shares several properties with filamentous fungi, including resolution to true mycelial form under certain growth conditions. *Y. lipolytica* does not undergo mating type switching and is used as a model organism in basic research as well as for industrial applications such as the production of nutraceuticals [54].

## Eicosapentaenoic acid and docosahexaenoic acid

Considerable efforts have been made at the laboratory level to express elongases and desaturases for PUFA biosynthesis in various microorganisms such as *S. cerevisiae* [59], *Mortierella alpina* [60], and *Lipomyces starkeyi* [61]. In the field of commercialization, only modified *Y. lipolytica* strain Y4305, which was developed by the DuPont company, has been used in a genetic engineering strategy [56,62–64]. The following genes encoding proteins were used for PUFA biosynthesis: synthetic  $\Delta 9$  elongase derived from *E. gracilis*, C16/18 elongase from *Mortierella alpina*,  $\Delta 5$  desaturase from *Peridinium* sp.,  $\Delta 8$  desaturase from *E. gracilis*,  $\Delta 12$  and  $\Delta 15$  desaturases from *Fusarium moniliforme*,  $\Delta 17$  desaturase from *Phytophthora ramorum*; codons were always optimized for expression in *Y. lipolytica* [64]. Various strains of *Y. lipolytica* from the American Type Culture Collection were first screened and strain ATCC 20362 was selected because its DCW production was greater than 100 g/L, with lipid production up to 30% DCW. This yield meant that lipid productivity was more than 1 g/L/h. In the next step, this strain was transformed with three desaturases ( $\Delta 5$ ,  $\Delta 6$ , and  $\Delta 17$ ) and C18/20 elongases under the control of the translation elongation factor (TEF) promoter. As a result of this transformation, a yeast producing 3% EPA was obtained [65].

**TABLE 6.4** Production of PUFAs in Engineered *Y. lipolytica*

| Product                  | Production                         | Strain     | Medium, Fermentation Type and Parameters                                                                                                                                                 | References           |
|--------------------------|------------------------------------|------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------|
| $\alpha$ -Linolenic acid | 1400mg/L                           | L36DGA1    | YSC medium contained 80 g/L glucose; a pulse of 80 g glucose was added at 72 h/fed-batch 2 L bioreactor, 20°C                                                                            | <a href="#">[55]</a> |
| EPA                      | 56.6% of total lipids with 15% DCW | ATCC 20362 | Nitrogen-rich medium contained 20 g/L glucose for the first stage fermentation, nitrogen-limited medium contained 80 g/L glucose for the second stage fermentation/two-stage flask, 30°C | <a href="#">[56]</a> |
| EPA                      | 50% of total lipids with 25% DCW   | Y4305      | Two-stage 2 L bioreactor, 30°C                                                                                                                                                           | <a href="#">[57]</a> |
| DHA                      | 5.6% of total lipids               | Y4305      | Cells were grown in MM medium containing 20 g/L glucose for 48 h, then transferred to HGM medium containing 80 g/L glucose for additional 72 h fermentation/two-stage fermentation, 30°C | <a href="#">[58]</a> |

Unfortunately, although this procedure proved to be viable, three basic deficiencies were identified and gradually eliminated in the next steps: low flow of metabolites into the engineered pathway, low efficiency of elongases and low levels of gene expression. Improvements have been achieved by eliminating these shortcomings. To increase flow to the  $\Delta 6$  pathway, over-expression of elongase from *M. alpina* was performed, which increased the conversion of palmitic acid to stearic acid and  $\Delta 12$  desaturase from *F. moniliforme*. This strain already contained 19 copies of various heterologous genes and was capable of producing up to 40% EPA relative to DCW. However, expression also had an adverse effect. The expressed  $\Delta 6$  desaturase, and lack of CoA pools in the membrane of the endoplasmic reticulum, produced undesirable PUFA  $\omega$ -6 fatty acids, and especially 21% of GLA.

Therefore, a completely different procedure for EPA biosynthesis was developed, based on replacing the  $\Delta 6$  pathway with the  $\Delta 9$  pathway, see Fig. 6.1. Multiple copies of selected genes were integrated into the genome, and an optimized codon with a strong promoter was obtained, including genes encoding C16/18-elongase,  $\Delta 5$ ,  $\Delta 8$ ,  $\Delta 12$ ,  $\Delta 17$ -desaturases, and choline phosphotransferase. The strain prepared in this way already contained 30 copies of nine different genes and production achieved 56.6% EPA as a proportion of total fatty acids and DCW yield was approximately 15%. GLA was not produced. This strain was further improved to produce a mutant strain that contained a total of 35 copies of 17 different genes. EPA production reached 58% of total FAs, and the DCW yield was approximately 20%. To fully optimize the culture conditions, an industrially usable strain was constructed containing 41 copies of 19 different genes and EPA production was about 50% based on total FAs; DCW yield was approximately 25%. This yield was achieved by optimizing the fermentation processes.

Interest in the production of PUFAs using genetically modified yeasts has been reflected in the number of patents that have been granted. Just a few of the most significant examples are US patents. Two patents relate mainly to the preparation of modified strains of the oleaginous yeast *Y. lipolytica* capable of producing more than 25% of eicosapentaenoic acid (EPA) based on total lipids [66], or more than 5.6% docosahexaenoic acid (DHA), again based on total lipids [67]. The other two patents [68,69] granted to the same company relate to processes for the production of  $\omega$ -3 and/or  $\omega$ -6 fatty acids in oleaginous yeasts of the genus *Yarrowia*. They have been genetically modified with desaturases and elongases, for example, from *Mortierella alpina* and *Saprolegnia diclina* capable of catalyzing the elongation and desaturation of acids (LA-GLA-DGLA-ARA-EPA, ALA-STA-ETA-EPA-DPA, and/or DGLA-ETA). The patent of Knutzon et al. [70] relates to fatty acid desaturases capable of desaturating C18 acids. This is again a biosynthetic pathway in the O-LA-GLA or ALA-STA sequences. One can also mention some other patents; we chose two of them as examples. The first [71] describes genetically engineered strains of the oil yeast *Y. lipolytica*.

Production of strain Y8672 reached up to 61.8% EPA, based on total fatty acids. Another patent [72], again used the genetically modified yeast *Y. lipolytica* strain Y2214, and describes the production of arachidonic acid to a level of 14% of total fatty acids.

## Triacylglycerols

Major metabolic engineering efforts in *Y. lipolytica* have focused on increasing the production of lipids, especially triacylglycerols (TAGs). Because TAGs are nonpolar, they are not part of the cell membrane phospholipid bilayer, and as lipid bodies, are located within the cell [73]. Many approaches have been developed to increase targeted lipogenesis, in particular the regulation of lipase activity (reverse hydrolysis of TAGs to FFAs), and the reduction of enzymes that catalyze  $\beta$ -oxidation or peroxisome formation. Overexpression of natural diacylglycerols was used for their extensive potential in the biosynthesis of triacylglycerols. These overexpression targets were catalyzed by acyltransferase (DGA1) with concomitant deletions of genes (*pex10* and *mfe1*) that promote  $\beta$ -oxidation and peroxisome formation [74] followed by lipid yields of 25.3 g/L (90% dry matter) in such a modified *Y. lipolytica*. Similarly, expression of DGA1 and acetyl-CoA carboxylase led to the formation of a mutant producing 17.6 g/L lipids (61.7% based on dry matter) [75]. In addition, transcriptomic analysis of the high-production mutant strain of *Y. lipolytica* showed  $\Delta 9$  desaturase activity that reduced the tricarboxylic acid cycle flux and increased the glycolytic flux. It was further reported that overexpression of DGA1 in this mutant strain resulted in lipid production of 25 g/L, which represented 86% of dry matter [76]. Floating cells were also selected after random mutagenesis in *Y. lipolytica* and a strain was found that produced almost 40 g/L lipids (87% of dry matter) [76].

## Enhanced PUFA production using different carbon sources

Further attention has been paid to modifications that allowed cells to utilize less traditional and especially cheaper substrates. Most of the cultures mentioned above were performed on glucose, which is an economically unsuitable source of carbon. On the contrary, lignocellulosic biomass (hemicellulose), which is a polysaccharide composed of xylose, appears to be a suitable (cheap) source. Following genetic engineering of enzymes capable of degrading xylose (xylose reductase, xylitol dehydrogenase, and xylulokinase), *Y. lipolytica* was able to utilize this source, with lipids accounting for 21% of the dry matter [77]. Similarly, Li and Alper [78] used heterologous enzymes from *Scheffersomyces stipitis*, the resulting lipid production was 15 g/L. Many other polymers (oligomers) of carbohydrates have also been used as carbon sources. These include, for example, sugarcane bagasse,

molasses, inulin, etc. From the above overview, it is clear that *Y. lipolytica*, after appropriate genetic modification, is able to utilize essentially any oligo- and/or polysaccharide.

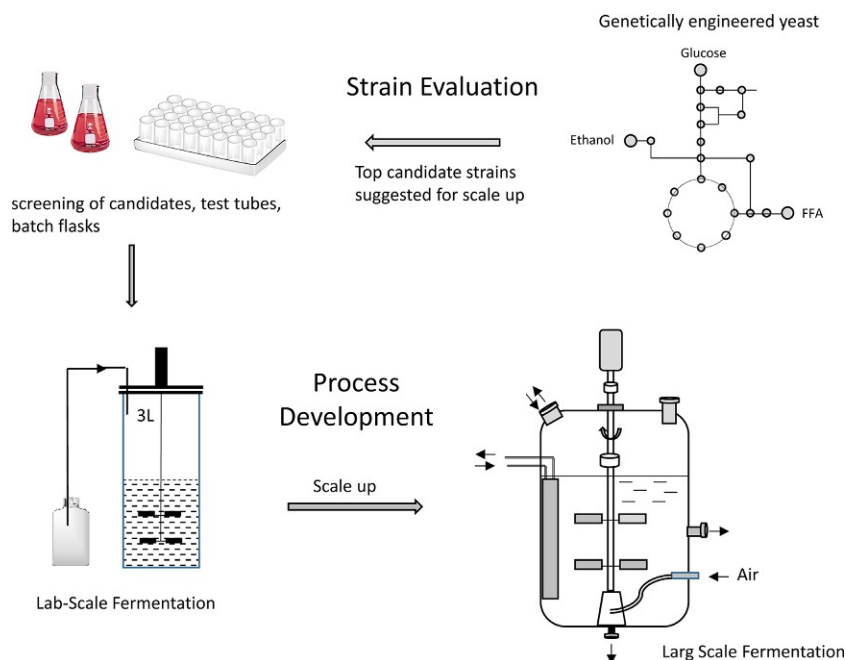
However, by far the most common alternative carbon source is glycerol. Glycerol is basically waste material in the production of biofuels from vegetable oils. *Y. lipolytica* has an innate ability to take advantage of this carbon source and the production of 3.5 g/L lipids (43% by dry cell weight) has been described [79]. Another strain of *Y. lipolytica* was able to produce 4.72 g/L lipid (21% dry matter) in fermenter batch culture using crude glycerol [80]. However, far better results have been achieved with the help of metabolic engineering. Heterologous glycerol dehydratase was expressed in *Y. lipolytica* and the lipid yield was 13 g/L (30% dry cell weight) [81]. Glycerol has also been used as an additive with another carbon source. Experiments were performed in which glycerol was added, for example, to xylose in a fed batch fermenter or was added to animal fats. These examples clearly show that high lipid production in *Y. lipolytica* can be achieved on various, especially waste sources, which considerably reduces the cost of industrial lipid production in genetically modified yeast.

Another way to increase the production of PUFAs is to change the carbon source. For example, after the addition of LA to the culture medium, the conversion of this acid to  $\gamma$ -linolenic acid was up to 69% by *Y. lipolytica* with heterologous overexpression of the  $\Delta 6$ -desaturase from *M. alpine* [82].

## Pilot—Scale process for microbial PUFA production

To test the prepared recombinant strains, the authors used three types of equipment [62]. Firstly, 24-well blocks, test tubes, shake flasks, and micro-fermenters, secondly, several-liter laboratory fermenters and thirdly, a 5000L pilot-scale fermenter. The cultivation of *Y. lipolytica* is a two-stage cultivation, i.e., growth and production (lipids and PUFAs). Therefore, the cells were first cultured in a simple bioreactor to a specific density, and after culture nitrogen had been depleted, glucose was added to produce lipids. The lipid composition of yeast, including the production of EPA and other fatty acids, was determined by gas chromatographic analysis. DCW and total lipid content was also determined in shake flasks and microfermenters. Most production strains were further cultivated in laboratory fermenters.

The inoculum prepared in the flasks was transferred to a 2L laboratory fermenter with 950 mL of culture medium. The course of the culture, including production, is shown in Fig. 6.4, and for more details, see the Table of the US Patent [64]. To optimize the transfer of results from a laboratory fermenter to a pilot plant fermenter (5000L) modeling was performed, where many parameters were optimized, particularly model equations of cell growth, substrate consumption, nitrogen utilization, oxygen uptake, lipid and EPA accumulation.



**FIG. 6.4** Workflow of fermentation of PUFAs. Strains generated by metabolic engineering are first evaluated using test tubes and batch flasks, further tested in laboratory fermenters and finally, large-scale fermentation is performed.

The best strain, which was verified in pilot scale cultures of *Y. lipolytica* for the production of EPA, was finally grown in a 5000L fermenter, and ultimately resulted in patent filing and industrial production of two dietary supplements, i.e., New Harvest™ EPA oil and Verlasso® oil.

### Engineering *Y. lipolytica* for production of lipid compounds other than PUFAs

The above examples show that *Y. lipolytica* can be used for biosynthesis of both “natural and atypical PUFAs,” i.e., fatty acids that are not produced by wild strains, as well as other lipid compounds. For example, ricinoleic acid, a major component of castor oil. Ricinoleic acid (12-hydroxyoctadec-cis-9-enoic acid) has many specialized uses in bioproduction industries, and castor bean is currently the only commercial source of this fatty acid. The paper of Meesapyodsuk et al. [83] describes metabolic engineering of a microbial system (*Pichia pastoris*) to produce ricinoleic acid using a “push” (synthesis) and “pull” (assembly) strategy. CpFAH, a fatty acid hydroxylase from *Claviceps purpurea*, was used for synthesis of ricinoleic acid, and CpDGAT1, a diacylglycerol acyl transferase for triacylglycerol synthesis from the same

species, was used for assembly of the fatty acid. Coexpression of CpFAH and CpDGAT1 produced higher lipid contents and ricinoleic acid levels than expression of CpFAH alone. Coexpression in a mutant haploid strain defective in  $\Delta 12$  desaturase activity resulted in a higher level of ricinoleic acid than in the diploid strain. Intriguingly, the ricinoleic acid produced was mainly distributed in the neutral lipid fractions, particularly the free fatty acid form, but with little in the polar lipids. This work demonstrates the effectiveness of the metabolic engineering strategy for industrial uses and excellent capacity of the microbial system for production of ricinoleic acid as an alternative to plant sources. The genetically modified *Y. lipolytica* strain produced more than 60 mg of ricinoleic acid per 1 g DCW, which was achieved by deletion of  $\beta$ -oxidation, TAG acyltransferases, and  $\Delta 12$  desaturase. This production was achieved by overexpression of heterologous  $\Delta 12$  hydroxylases from the plant *Ricinus communis* and fungus *Claviceps purpurea*, i.e., wild strains that normally produce ricinoleic acid [84].

Another possibility is the production of  $\gamma$ -decalactone by  $\beta$ -oxidation of ricinoleic acid. Specifically, ricinoleyl-CoA can undergo four cycles of  $\beta$ -oxidation to produce 4-hydroxydecanoic acid, which can then be cyclized into  $\gamma$ -decalactone [85]. By optimizing the culture conditions, in particular oxygen transfer rate, cell density, and fed-batch cultivation in which an oil was added, a yield of up to 6.8 g/L was achieved [86].

Production of 4.98 mg/L pentane was achieved by overexpression of the soya lipoxygenase gene [87]. This production was made possible by *Y. lipolytica* having a high tolerance for  $\omega$ -alkanes, aldehydes, and fatty alcohols. This fact was also used for the production (350 mg/L) of C6-aldehyde of [88], where the fatty acid hydroperoxide lyase from green bell pepper was over-expressed in *Y. lipolytica*. This yeast can also be used to produce fatty alcohols after insertion of the acyl-CoA reductase from the uropygial glands of the barn owl (*Tyto alba*). Production was more than 636 mg/L of intracellular and 53 mg/L of extracellular hexadecanol [89]. Acyl-CoA reductase from *Arabidopsis thaliana* inserted into the *Y. lipolytica* knockout strain, allowed the production of more than 500 mg/L decanol [90].

## **Engineering *Y. lipolytica* for biodiesel production**

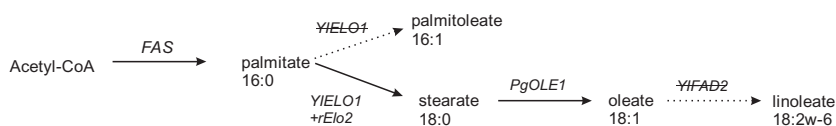
Many different processes have been used for the production of biofuels, and these have undergone further development of various technical and conceptual approaches over time, from first to third generation biodiesel production. For the production of first-generation biodiesel, edible vegetable oils such as soybean, canola, sunflower, palm, etc., were used, now encompassing almost 99% of all biodiesel production. However, the use of edible oils as an energy source has given rise to many objections and controversies regarding their use as fuel and also deforestation in order to obtain free land for the planting of oil palms. The second generation of biodiesel comes from lignocellulosic waste

in agriculture and inedible oils (e.g., *Jatropha curcas*), that also emerged as an attractive alternate raw material for the production of biodiesel on land unsuitable for agriculture. In the third generation, a procedure for more sustainable biodiesel production is being sought, and interest is focused on yeasts and fungi as a source of biodiesel (as well as microalgae).

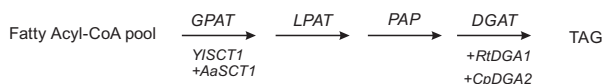
Biodiesel is a mixture of alkyl esters (mostly methyl esters) of fatty acids obtained by transesterification of vegetable oil or animal fat [91]. However, biodiesel must meet existing standards, such as EN 14214 in EU, which limits the proportion of total unsaturated FAs (iodine value), methyl esters of linolenic acid, and PUFAs content with four or more double bonds [92,93]. PUFAs are responsible for low oxidative stability and a tendency to form gums [94]. The goal of high-quality biofuel production is a balanced mixture of 16:1, 18:1 and 14:0 fatty acids in a ratio of 5:4:1 [95]. This ideal mix has been identified as providing a good cetane number along with a low oxidation potential and a cold filter plugging point [96].

In most published articles, the authors focused on the genetic modification of yeast, which then produced PUFAs that wild-type strains do not normally produce [97]. However, the presence of PUFAs is undesirable for producing biofuels. Therefore, Tsakraklides et al. [98] used a completely different approach—to increase the production of oleic acid, see Fig. 6.5. A strain of *Y. lipolytica* that produced TAGs containing a high content of oleic acid was constructed. Deletions and over-expressions in the synthesis of fatty acids and TAGs that increased oleate levels were studied. The accumulation of oleic acid in TAGs was promoted by the exchange of native  $\Delta 9$  fatty acid desaturase and glycerol-3-phosphate acyltransferase for heterologous enzymes, as well as the deletion of  $\Delta 12$  fatty acid desaturase and fatty acid elongase. Deletion of the native  $\Delta 12$  fatty acid desaturase led to the elimination of LA production.

#### Fatty acid synthesis



#### TAG synthesis



**FIG. 6.5** A simplified pathway for fatty acid and TAG synthesis in *Y. lipolytica* was used with gene deletions (*Ylde1*, *Ylsc1*, *Ylfad2*) and heterologous gene expressions (*PgOLE1*, *AaSCT1*, *rELO2*, *RtDGA1*, *CpDGA2*) incorporated into the high-oleate strain. *DGAT*, diacylglycerolacyltransferase; *FAS*, fatty acid synthase; *GPAT*, glycerol-3-phosphate acyltransferase; *LPAT*, lysophosphatidic acid acyltransferase; *PAP*, phosphatidate phosphatase; *TAG*, triacylglycerol.

The wild-type and  $\Delta$ *fad2* strains were grown in nitrogen-free medium for 96 h, and analysis of fatty acid content by GC–MS after cultivation confirmed the absence of LA. Further, heterologous glycerol-3-phosphate acyltransferase expression in *Y. lipolytica* increased lipid production only minimally. By combining these genetic steps, polyunsaturated fatty acids were eliminated and a strain of *Y. lipolytica* was prepared that accumulated TAGs containing more than 90% oleate.

In addition, an oleaginous strain of *Rhodospiridium toruloides* (ATCC20409) for the production of microbial lipids such as biodiesel was investigated. The strain used a consolidated process to produce a high lipid content (76% of the dry weight of the biomass), which was then trans-esterified to produce biodiesel. This had the physicochemical properties in accordance with ASTM standards [99].

Lignocellulosic biomass represents raw material for producing biofuels. A study by Singh et al. [100] describes an integrated microbial fermentation process involving a thermoanaerobe and a marine thraustochytrid, aiming at the coproduction of docosahexaenoic acids and bioethanol using rice straw biomass as a substrate.

## Conclusion

All of these examples, including the production of PUFAs, show that individual GMO strains of *Y. lipolytica* can be designed to produce almost any lipid compound. It is necessary to continue in this field, both with scientific experiments and with industrial uses of GMOs of *Y. lipolytica*, which have GRAS status. This phenomenon is reflected in the recognition of two industrial yeast products, named by DuPont as New Harvest™ EPA oil and oil Verlasso® for dietary supplements in sustainable salmon farming.

## Acknowledgment

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## Abbreviations

|             |                                  |
|-------------|----------------------------------|
| <b>ALA</b>  | $\alpha$ -linolenic acid         |
| <b>ARA</b>  | arachidonic acid                 |
| <b>DGLA</b> | dihomo- $\gamma$ -linolenic acid |
| <b>DHA</b>  | docosahexaenoic acid             |
| <b>DPA</b>  | docosapentaenoic acid            |
| <b>DTA</b>  | docosatetraenoic acid            |
| <b>EDA</b>  | eicosadienoic acid               |

|             |                            |
|-------------|----------------------------|
| <b>EPA</b>  | eicosapentaenoic acid      |
| <b>ETA</b>  | eicosatetraenoic acid      |
| <b>ETrA</b> | eicosatrienoic acid        |
| <b>GLA</b>  | $\gamma$ -linolenic acid   |
| <b>GRAS</b> | generally regarded as safe |
| <b>LA</b>   | linoleic acid              |
| <b>STA</b>  | stearidonic acid           |

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# Index

Note: Page numbers followed by “f” indicate figures, “t” indicate tables, and “s” indicate schemes.

## A

- 7 $\beta$ -Acetoxy-20-hydroxy-19, 20-epoxyroyleanone (AHE), 59
- Aconitum japonicum*, 48
- Akebia quinata*, 52
- Alginates wound dressings, 374–377t, 385–386, 386f
- Alkaloids, 249, 253–259, 253–259f
  - cyclopiazonic acid (CPA)-derived alkaloids, 257–258
  - diketopiperazine, 251–253
- Aloe vera, 397
- $\alpha$ -linolenic acid (ALA), 197–200
- Amentoflavone structure, 90, 91f
- $\alpha$ -Amylase, inhibitory activities of essential oils (EOs), 346–351, 348–350t
- Andrographis aniculate* Nees, 60–61
- Andrographis paniculata*, 57
- Angiotensin-converting enzyme (ACE)
  - inhibitors, 332
  - activities of essential oils (EOs), 342–345, 344–345t
  - effects of essential oils (EOs), 332
- Angiotensin-converting enzyme 2 Receptor (ACE2R), 451–452t
- Anisomeles indica* (L.) Kuntze, 53
- Anthocyanin-rich extracts (AREs), 101
- Anthraquinones, 226–241, 240–241f
- Antibacterial nanoparticles, formulation of, 397
- Anticoronaviral natural products, COVID-19 treatments, 450–453, 454–456t
- Antifungal bioassay-directed fractionation, 241–242, 253–254
- Antihypertensive activity, 342–343, 345
  - of plant-based products, 345
- Antihypertensive drugs, 343
  - synthetic, 342
- Antihyperuricemic peptides
  - interactions between XOD and, 287–290
  - molecular docking of, 287–290, 287f, 289f
  - preparation
    - and amino acid compositions of, 282–283t
    - and structural characterization, 281–284
    - properties of antihyperuricemic peptides in vivo and in vitro, 284–286
- Antiinflammatory activity,
  - dihydrophenanthrenes (DPs), 152–153
- Antimicrobial activity
  - dihydrophenanthrenes (DPs), 153–154
  - to wound dressings, 385
- Antimicrobial medicines, 168–169
- Antioxidant activity (AA), 314
  - dihydrophenanthrenes (DPs), 154–155
- Antioxidant components, oxidation of, 315
- Anti-SARS-CoV-2 agents, 445
- Antiviral activity, 226–241
- Apigenin, 85, 86f
- Arginine-glycine-aspartic acid (RGD) peptides, 385
- Aromatic bisabolene-type sesquiterpenoid, 244–245
- Aspergillethers, 265–266
- Aspergillines, 257–258
- Aspergillus* species, 225–226
  - metabolic products of, 226
- Aspergillus versicolor*
  - classification of secondary metabolites of, 267–268, 268f
  - source classification, 268–269, 269f
  - compounds from, 226, 227–238t
- Aspergoterpenin, 245–246
- Aspersymmetide, 247–249
- Asperversiamides, 250–251
- Aspeverin, 258–259
- Aspvanicin, 264
- Azorella compacta* (Apiaceae), 50–51

## B

- Bacterial cellulose (BC), 384–385
- Baicalin, 85, 86f
- Bee pollen, 296, 302–303
  - bioactive characteristics of, 303, 304f
  - LTHV fluidized bed (LTHV<sub>fb</sub>) drying on
    - bioactive content of, 303–305, 305–306t

- Bioactive foods, 295–296
- Bioactive natural products, against  
  coronaviruses, 457*f*
- Bioactive peptides, 281, 290
- Bioactive phenolic compounds, 317
- Biodiesel production, *Yarrowia lipolytica* for,  
  215–217, 216*f*
- Bioink, 404–406  
  shear-thinning, 403–404
- Biological activities, dihydrophenanthrenes  
  (DPs), 151–155
- Biological dressings/tissue-engineered  
  substitutes, 374–377*t*
- Biological response modifiers (BRMs), 3–7
- Biomass, lignocellulosic, 217
- Biomaterial wound dressings, 374–377*t*
- Bioprinting, 403–410  
  strategies and applications, 404–409  
  wound dressings, 403–410, 405*f*
- Biosynthesis  
  of dihydrophenanthrenes (DPs), 119, 119*f*  
  of lipids in modified *Yarrowia lipolytica*,  
    209–212  
  of polyunsaturated fatty acids (PUFAs), 200,  
    204–208, 205*f*, 206–208*t*
- BiovaFlex<sup>®</sup>, 393
- Blumea lacera*, 57–58
- Bombyx mori* silk, 392–393
- Bonito hydrolysate (BH) group, 284
- Boswellia carterii*, 52
- Brazilian green propolis, 186
- Brazilian propolis, 185–186
- Brazilian yellow propolis, 185
- Breast cancer. *See also* Plant-derived  
  diterpenes, for breast cancer treatment  
    HER2, 44  
    luminal A, 44  
    luminal B, 44  
  metastasis and angiogenesis inhibition in,  
    54–57, 55*f*  
  oncological medicines, 44  
  sites of interaction and structure–activity  
    relationship, 66–72, 67–70*t*  
  triple-negative, 44  
  in vitro studies, experimental design of, 45*f*
- Brevianamides, 249
- BRMs. *See* Biological response modifiers  
  (BRMs)
- Burn, 369, 371*f*
- C**
- Caffeic acid phenethyl ester (CAPE), 186
- Calcium ions, hemostatic ability of, 385–386
- Canine coronavirus (CCoV), 448
- Carbocyclic diterpenes, 56
- Carboxymethylcellulose (CMC), 384, 402–403
- Carnosic acid (CA), 58–59
- Carnosol, 55–56
- Carotenoids, biological activities of, 317*f*
- Casearia graveolens*, 51
- Casearia grewiifolia*, 51
- Catechins, 316
- Catechol-*O*-methyltransferase (COMT),  
  98–99
- Cathepsin L (CTSL), 451–452*t*
- CCoV. *See* Canine coronavirus (CCoV)
- CD147, 451–452*t*
- CDKs. *See* Cyclin-dependent kinases (CDKs)
- CDS. *See* Chondroitin sulfate (CDS)
- Cell-matrix interactions, 378–379
- Cembranoids, 56
- Centrifugal lithography, 400
- Centrosymmetric cyclohexapeptide, 247–249
- Ceriops tagal*, 49
- Chinese propolis, 186
- Chitosan (CS), 333, 382
- Chloroquine phosphate, 449–450
- Chondroitin sulfate (CDS), 388–389
- Chronic diseases, 351
- Chronic wound healing, 395–396
- Chrozophora oblongifolia*, 45
- Chrysin, 85, 86*f*  
  1,2-*cis* glycosylations, for synthesis of  
    α-glucopyranosides, 8–9, 8*f*, 9*s*
- Codium* spp., 180–181
- Coexpression, in mutant haploid, 214–215
- Coffea arabica*, 61
- Cold brewing method, 314
- Collagen, 373–378, 390–391, 407
- Collagen-based dressings, restrictions of, 391
- Coriander foliage (*Coriandrum sativum* L.),  
  296–297
- Coronaviruses, 443–450  
  β-coronavirus, 445–447  
  bioactive natural products against, 457*f*  
  mode of action and pathogenesis, 447–449  
  morphology and genome, 445–447, 446*f*  
  open-reading frames (ORFs) in, 445–447
- Cotteslosin, 246–247
- Cottoquinazoline, 257–258
- COVID-19  
  causes of, 448–449  
  molecular docking and pharmacokinetic  
    study, 453–460, 458–459*t*, 460*f*  
  pathophysiology of, 448–449  
  symptoms of, 448  
  treatments, 444

- anticoronaviral natural products, 450–453, 454–456*t*
  - challenges and opportunity, 449–450
  - potential therapeutic targets, 450, 451–452*t*
  - remdesivir, 450–453
- CpFAH, 214–215
- CREMPs. *See* Cysteine-rich eggshell membrane proteins (CREMPs)
- Crystals, monosodium urate (MSU), 279–280
- CS. *See* Chitosan (CS)
- Curcuma amada*, 53
- Cyclic pentapeptide, 246–247
- Cyclin-dependent kinases (CDKs), 93–94, 152
- Cyclodextrins, 335
- Cyclopentapeptides, 247
- Cyclopiazonic acid (CPA)-derived alkaloids, 257–258
- Cysteine-rich eggshell membrane proteins (CREMPs), 393
- Cytotoxic activity
  - against A549 and MCF7 cell lines, 259–262
  - against cancer cell lines, 242–244
  - cell lines, 257–258
    - against MCF-7 and SMMC-7721 cell lines, 253–254
  - dihydrophenanthrenes (DPs), 152
  - against the HeLa and K562 cell lines, 266–267
  - against HL-60 and K562 cell lines, 242
  - against human solid tumor cell lines, 247–249
  - against MCF-7 and SMMC-7721 cell lines, 253–254
  - against the mouse lymphoma cell line, 264
  - against NCI-H292 and A431 cell lines, 247–249
  - against P388 cell line, 258–259
- D**
  - Daphne genkwa*, 60
  - Data-dependent acquisition method, 170, 171*t*
  - Data-independent acquisition method, 170, 171*t*
  - Delphinium chrysotrichum*, 52
  - Dermagraft™, 411–412
  - D-Glucose, 2–3
  - DHA. *See* Docosahexaenoic acid (DHA)
  - Diabetes, 346
  - Dickkopf-Related Protein 1 (DKK1) gene, 96
  - Dihydrophenanthrenequinones structure, 121, 125*f*
  - Dihydrophenanthrenes (DPs), 117–118, 118*f*
  - activities, 155
  - antiinflammatory activity, 152–153
  - antimicrobial activity, 153–154
  - antioxidant activity, 154–155
  - biological activities, 151–155
  - biosynthesis of, 119, 119*f*
  - cytotoxic activity, 152
  - monomers, 120–121, 122–125*f*
    - dihydrophenanthrenequinones, 121, 125*f*
    - monomers with C6-C3 fragment, 121, 126*f*
    - with other substituents, 121
    - simple, 121
  - names, origins and biological activities of, 127–146*t*
  - polymers, 121
    - DPs and bibenzyls, 150, 151*f*
    - DPs and DPs, 121–125, 148–149*f*
    - DPs and phenanthrenes, 125–149, 150*f*
  - separation and identification of, 120
  - structural types, 120–150
- Diketopiperazines, 246–252, 249–252*f*
  - alkaloids, 251–253
- Dioscorea nipponica, 153
- Diosmetin, 85, 86*f*
- 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay, 154
- Diterpenes. *See* Plant-derived diterpenes, for breast cancer treatment
- D-Man-(1→2)-D-Man-(1→6)-D-Glc structure, assembly of, 21–22, 22*s*
- Docosahexaenoic acid (DHA), 197–200, 209–212, 296
- DPs. *See* Dihydrophenanthrenes (DPs)
- Drug carriers, stimuli-responsive, 373–378
- E**
  - ECM. *See* Extracellular matrix (ECM)
  - Edible oils, 215–216
  - Effusulin, 153
  - Eggshell membrane (EgM), 393
  - EgM. *See* Eggshell membrane (EgM)
  - Egyptian propolis, 185
  - Eicosapentaenoic acid (EPA), 182, 197–200, 209–212, 296
  - Elastomer, 399–400
  - Electronic circular dichroism (ECD) analysis, 226–241
  - Electron impact ionization (EI) techniques, 170
  - Electrospinning, wound dressings, 401–403, 401*f*
  - Electrospun nano-meshes, 402–403
  - Electrospun silk fibroin nanofibers, 392
  - Enzymatic hydrolysis, 281–284

## Enzymes

- fibrinolytic, 397
- heterologous, 212–213
- lytic, 397
- proteolytic, 397
- xanthine oxidase, 332

EOs. *See* Essential oils (EOs)

EPA. *See* Eicosapentaenoic acid (EPA)

Ephemeranthoquinone, 153–154

Ephenmeranthoquinone, 152

Epicel™, 411

Epidermal growth factor (EGF) receptor, 63–64

ERBB2, 63–64

Eriocalyxin B (EriB), 56–57, 62

Essential oils (EOs), 331–332

- ACE-inhibitory activities of, 342–345, 344–345*t*

- ACE-inhibitory effects of, 332

- $\alpha$ -amylase and  $\beta$ -glucosidase inhibitory activities of, 346–351

- biological activities of, 332–333

- encapsulation of, 332–336

- Eucalyptus staigeriana*, antimicrobial activity of, 334–335

- interactions of, 336–341, 336*f*, 339–341*t*
- oregano, 333

- sea fennel, 333–334

- xanthine oxidase inhibitory activities of, 351–357

Estrogen receptors (ER), 96

*Eucalyptus staigeriana* essential oils, antimicrobial activity of, 334–335

*Euphorbia fischeriana*, 50

*Euphorbia fischeriana* Steud, 55

*Euphorbia kopetdaghi*, 61

*Euphorbia lathyris*, 63

*Euphorbia prolifera*, 63

*Euphorbia sororia*, 62–63

Exoribonuclease (Exo/nsp14), 447–448, 451–452*t*

Exosomes, 396–397

Extracellular matrix (ECM), 368, 370–372

- native polymers, 378–379

- structure and topography, 378–379

Extrusion printing, wound dressings, 405*f*, 406–407

- 3D bioprinting, 407

## F

Fabricated nanofibers, 395–396

Fabricating complex skin architectures, 403

Fabrication, natural polymer in dressing, 368

Fabrication of wound dressings

- natural polymers, 368, 381–394, 382*f*
- polysaccharide-based, 382–390, 383*f*, 386*f*

- protein-based polymers, 390–394

- novel strategies in, 398–413

FAS. *See* Fatty acid synthase (FAS)

Fatty acids, free, 309

$\omega$ -3 Fatty acids of fish, 308, 309*f*

Fatty acid synthase (FAS), 200

Feline infectious peritonitis (FIP), 448

Fibrinogen, 370, 394

Fibrinolytic enzymes, 397

Fibroin, silk, 392

Films wound dressings, 374–377*t*

FIP. *See* Feline infectious peritonitis (FIP)

Fisetin, 85, 87*f*

## Fish

- bioactive characteristics of fish components, 308, 309*f*

- bioactive component of fish and liquid smoked fish after novel LTHV-assisted cold process, 309–312, 310–311*t*

- LTHV-assisted drying of raw and liquid smoked, 307, 308*f*

- novel low-temperature unit operations in, 306–312, 307*f*

Flavonoids, anticancer activity and

- classification of

- anthocyanidins, 88–89, 89*f*

- aurones, 90

- biflavonoids, 90

- biosynthesis, 84–85

- chalcones, 90

- chalcone synthase, 84–85

- cinnamic acid derivatives, 84

- flavanols, 86–88, 88*f*

- flavanones, 88, 89*f*

- flavones, 85, 86*f*

- flavonols, 85, 87*f*

- isoflavones, 86, 87*f*

- natural flavonoids, 85

- ortho*-hydroxy acetophenone, 84

- phenylalanine, 84

- phenylpropanoid chain (C6-C3-C6), 85

- tyrosine amino acids, 84

DNA damage, 82

epigenetic changes, reversibility of, 82

general aspects of, 84

molecular mechanism of action, 82, 83*f*

polyphenols, 82

SAR studies of, 85*f*

anthocyanidins, 101  
 aurones, 91*f*, 102–103  
 biflavonoids, 103  
 chalcones, 90*f*, 101–102  
 epigenetic modifications in, 103–104  
 flavanols, 98–99  
 flavanones, 99–101  
 flavones, 92–94  
 flavonols, 94–96  
 isoflavones, 96–98  
 multidrug resistance (MDR), 104–105  
 three-dimensional quantitative  
   structure–activity relationship  
   (3D-QSAR) studies, 105–106  
 in vivo studies, 82  
 Fluidization drying method, 313–314  
 Fluidized bed-drying technique, 313–314  
 Foams wound dressings, 374–377*t*  
 Food derived active peptides, 290  
 Food-derived peptides, antigout activities of,  
   279–280  
 Fourier transform (FT) algorithm, 175–176  
 Fractional inhibitory concentration (FIC)  
   index, 336  
 Free fatty acids, 309  
 Free induction decay (FID), 175–176  
 Freeze-drying, 297  
 Fucoxanthin, 181–182  
 Furin, 450, 451–452*t*

## G

Gas chromatography-mass spectrometry  
   (GC-MS), 165–166  
 Gelatin, 391–392  
 Gelatine, 407  
   nanofibers, 395–396  
 Genetically modified organisms (GMOs),  
   202–203  
 Genistein, 97  
 Genome-scale models (GSMs), of *Yarrowia*  
   *lipolytica*, 203–204  
 Ginkgetin structure, 90, 91*f*  
 Glucans, 2–7  
   biosynthesis of, 3–7  
 $\alpha\beta$ -D-Glucans, assembly of linear, 24*s*, 25–27  
 $\alpha,\beta$ -Glucans structure, 14–15, 15*s*  
 $\alpha$ -D-Glucans  
   assembly of linear, 24–25, 24*s*  
   maltoligosaccharide structures, 27*s*, 28  
 $\beta$ -D-Glucans  
   assembly of linear, 23, 24*s*, 25, 26*s*  
   cellooligosaccharide structures, 27–28, 27*s*

D-Glucans, 2–3, 4*f*  
   cellooligosaccharide structures, 27–28, 27*s*  
   core structure of branched, 5*f*  
   linear and branched linkages of, 6*t*  
   2-*O*- TAB group for stereoselective  
     glycosylation the synthesis of, 10–12, 10*s*  
   stereoselective glycosylation for synthesis  
     of, 7–9  
   structure of nonbranched, 4*f*  
   synthesis  
     bimodal approach, 10–15  
     using 2-*O*- TAB approach, 23–29, 23*f*  
 $\alpha$ -Glucopyranosides, 1,2-*cis* glycosylations for  
   synthesis of, 8–9, 8*f*, 9*s*  
 $\beta$ -Glucopyranosides, 1,2-*trans* glycosylations  
   for synthesis of, 7–8, 7*s*  
 $\alpha$ -Glucose-branched- $\alpha$ -D-glucan, assembly of,  
   27*s*, 28  
 $\alpha$ -Glucose-branched-D-glucan, assembly of,  
   28–29, 28*s*  
 $\beta$ -Glucosidase, inhibitory activities of essential  
   oils (EOs), 346–351, 348–350*t*  
 $\beta$ -Glucosylated disaccharide products, 10–12  
 Glucosylations  
   condition, screening, 11*t*  
   effect of 2-*O*- TAB group on for  $\alpha$ - and  
      $\beta$ -selective, 12–13, 13*s*  
 Glucuronidation, 98, 98*f*  
 Glycerol, 213  
 Glycosylation  
   nonclassical NGP strategies for  
     stereoselective, 9  
   reactions, 2  
 Glycyrrhetic acid (GA)-1, 105–106  
 GMOs. *See* Genetically modified organisms  
   (GMOs)  
 Gout, 279–280, 287–288, 332  
 Growth factors, 396

## H

HCoV-19, 443–444  
 Hemagglutinin esterase (HE), 445–447  
 Heterocyclic aromatic amines (HAAs), 99  
 Heterologous enzymes, 212–213  
 Heterologous glycerol dehydratase, 213  
 High-performance liquid chromatography  
   (HPLC), 170  
 High-resolution mass spectrometers (HRMS),  
   170  
 High-resolution mass spectrometry, 168  
 Homologous recombination repair-defective  
   cancer (HRR), 60

Honey, 397  
 Huperxanthones, 241–242  
 Hyaluronic acid (HA), 387  
     biological properties of, 387–388  
 Hydrocolloids wound dressings, 374–377*t*  
 Hydrofibers wound dressings, 374–377*t*  
 Hydrogels wound dressings, 374–377*t*  
 Hydrogen-bond-mediated aglycone delivery  
     (HAD) approach, 16–17  
 Hydrolysis, enzymatic, 281–284  
 Hypertension, 342  
 Hyperuricemia, 279–280, 287  
     long-term, 279–280

## I

IBV. *See* Infectious bronchitis virus (IBV)  
*Icacina trichantha*, 51  
 Indole alkaloid, 255–256  
 Infectious bronchitis virus (IBV), 443–444  
 Inflammation, 152  
 Inhibitors  
     angiotensin-converting enzyme (ACE), 332  
     synthetic, 332  
 Inkjet printing  
     piezoelectric, 404–406  
     thermal, 404–406  
     wound dressings, 404–406, 405*f*  
 In situ skin bioprinting, wound dressings,  
     409–410  
 Interpenetrating polymer network (IPN)  
     architectures, 394  
 Intramolecular aglycon delivery (IAD), 16–17  
 Iranian propolis, 185  
 Isocoumarins, 259–262  
*Isodon eriocalyx* var. *laxiflora*, 56–57  
 Isorhamnetin, 85, 87*f*

## J

*Jatropha gossypifolia*, 61–62  
 Jatrophanes, 62–63  
 Jatrophane, 61–62  
 Jolkinolide B (JB), 55  
*Juncus effusus*, effusol and juncusol from,  
     117–118

## K

*Kaempferia pulchra*, 53  
 Kaempferol, 85, 87*f*  
 Kahweol, 61  
 Keratin, 393  
 Keratinocytes, 407  
 Kopetdaghinane, 61

## L

LA. *See* Linoleic acid (LA)  
 Laser-assisted printing, wound dressings, 405*f*,  
     408  
 Lignocellulosic biomass, 217  
 Linoleic acid (LA), 197–200  
 Lipid compounds, *Yarrowia lipolytica* for  
     production of, 214–215  
 Lipoxygenase (LOX) inhibitory activity, of  
     anthocyanins, 101  
 Liquid chromatography-mass spectrometry  
     (LC-MS), 165–166  
 Liquid smoke (LS), 306–307  
 Lithography, 398–400, 399*f*  
     centrifugal, 400  
     soft, 399–400  
 LM-pectin, 389–390  
 Long-term hyperuricemia, 279–280  
 Lotus pollen, 304–305  
 Low-temperature high velocity  
     (LTHV)-assisted cold process  
     bioactive component of fish and liquid  
     smoked fish after novel, 309–312,  
     310–311*t*  
 Low-temperature high velocity (LTHV)  
     fluidized bed (LTHV<sub>fb</sub>) drying  
     technology, 298–322, 300–301*f*  
     on bioactive content of bee pollen, 303–305,  
     305–306*t*  
     effect of, 303–304  
 LS. *See* Liquid smoke (LS)  
 LTHV. *See* Low-temperature high velocity  
     (LTHV)  
 Luteolin, 85, 86*f*  
 Lyophilization, 334  
 Lytic enzymes, 397

## M

Main protease (Mpro/3CL-protease), 451–452*r*  
 Marfey's method, 246–249, 266–267  
 Matrix metalloproteinase (MMP) levels,  
     390–391  
 Meggitt–Wagner wound classification system,  
     369  
 Membrane protein (M protein), 447–448  
 MERS-CoV. *See* Middle East respiratory  
     syndrome coronavirus (MERS-CoV)  
 Metabolic engineering  
     of nutraceutical products, 202  
     in *Yarrowia lipolytica* for omega-3 EPA and  
     DHA production, 205, 205*f*  
 Metabolic pathway, of uric acid formation,  
     280*f*

## Metabolites

## isolation of secondary

- alkaloids, 253–259, 253–259*f*
- anthraquinones, 226–241, 240–241*f*
- diketopiperazines, 246–252, 249–252*f*
- peptides, 246–252, 246–248*f*
- terpenes, 244–246, 244–246*f*
- xanthenes, 241–244, 241–244*f*

secondary, biological activity classification of, 269, 270*f*

## Metabolomics, bioactive pharmaceuticals in

## complex matrices

## analytical methods, 169–174

## GC-MS and LC-MS, 169–172

## NMR spectroscopy, 172–174

## analytical techniques, 165–166

## bioinformatics tools

## data (pre)processing, 174–177

software and databases, 177–178, 179*t*

## biological models

## algae, 180–183

maize (*Z. mays* L.), 183–184

## propolis, 184–186

## biomedicines, 166

## chemometrics, 166

## chromatographic and spectroscopic

## techniques, 166

## complex mixtures, analytical challenges on, 167–169

## functional genomics, 165

## natural products, 166

## nontargeted metabolomics, 166

## synthetic drugs, 166

## target metabolites, 166

workflow of, 167*f*Methylation, 98, 98*f*

## Microalgae, 181–182

Microbial PUFAs, biosynthesis, 204–208, 205*f*, 206–208*t*

## Microorganisms, recombinant, 203

## Middle East respiratory syndrome coronavirus (MERS-CoV), 443–444, 448

## Monosodium urate (MSU) crystals, 279–280

## Multidrug resistance (MDR), 62–63

## Mutant haploid, coexpression in, 214–215

Myricetin, 85, 87*f*

## N

## Nanocapsules, 332–333

## Nanofibers

## dressings, 402

## electrospun silk fibroin, 392

## fabricated, 395–396

## PVA/gelatine, 395–396

## Nanoparticles, antibacterial, 397

## Nanostructured lipid carrier (NLC), 66

## Natural dihydrophenanthrenes, 118

## Natural Eggshell Membrane (NEM®), 393

## Natural foods, 295–296

## Natural polymers

## advantages of, 368

## synthetic polymers in combination with, 394–396

wound dressings by, 413, 414–416*t*

## Natural products, 444–445

anticoronaviral, 450–453, 454–456*t*bioactive, against coronaviruses, 457*f*

## stereoconformers of, 453

## Nature-derived materials, 368–369

*Nicotiana tabacum*, 56

## Nitric oxide (NO), 152

## NMR spectroscopy, 168, 172–174

## Noise filtration, 175

## Nonstructural proteins (nsps), 447–448, 450

## Normalization, 175

## Nsp13, 445–447

## Nuclear magnetic resonance (NMR), 165–166

## Nuclear overhauser effect (NOE) experiment, 226–241

## Nutraceutical products, metabolic engineering of, 202

## O

## Oils, vegetable, 200

## Oleaginous yeasts, 204, 209

## Oligopeptides synthesis, 2

## OrCel™, 411

## Oregano essential oils, 333

## Oridonin (7,20-epoxy-ent-kauranes), 59

*Oryza Sativa*, 2812-*O*- TAB groupdonor for stereoselective mannosylation, 17–19, 18–19*t*, 20*s*for further elongation, deprotection, 13–15, 14*s*stereoselective galactosylation, application, 13, 14*s*stereoselective mannosylation, application, 16–17, 16*s*

## Ovatodiolide (Ova), 53

## Overhauser Nuclear Effect (NOE), 173–174

## Oxidation

## of antioxidant components, 315

## related diseases, 154

**P**

- Papain-like protease (PLpro), 451–452*t*
- Parvifloron D (ParvD, 3), 58
- Parvifloron F, 54
- Pectin, 388
- Peppermint oil, 333–334
- Peptides, 246–252, 246–248*f*  
   arginine-glycine-aspartic acid (RGD), 385  
   bioactive, 281, 290  
   food derived active, 290  
   and peptide-rich fractions, inhibitory  
     properties against XOD of  
     purified, 285–286*t*
- P-glycoprotein (P-gp), 62–63
- Phenolic compounds, bioactive, 317
- Phochinenin, 152
- Phoenix roebelenii*, 345
- Photolithography, 398–400
- Phytoalexins, 153
- Piezoelectric inkjet printing, 404–406
- Pigmacopremna herbacea*, 54
- Plant-derived diterpenes, for breast cancer  
   treatment  
     antiproliferative activity, 53–54  
     basic structure of, 43*f*  
     cell death mechanisms, induction of,  
       57–62  
     diterpenes, 42–44  
     diterpenic quinones, 60  
     drug-resistant cell lineages, therapeutic  
       option in, 62–64  
     effects, in breast cell lines, 45*f*  
     metastasis by, 54–57  
     nanotechnology, 64–66  
     new diterpenes identification, with  
       anti-breast cancer activity, 45–53,  
       46–47*t*  
     secondary metabolites, 42  
     sites of interaction and structure–activity  
       relationship, 66–72, 67–70*t*  
     taxol (C<sub>47</sub>H<sub>51</sub>NO<sub>14</sub>), 44  
     terpenes, 42  
     tumor angiogenesis, inhibition of, 54–57
- Plectranthus*, 58
- Plectranthus amboinicus* (Lour.) Spreng, 50
- Plinabulin of diketoperazine, 226
- Podocarpus nagi* (Podocarpaceae), 50
- Pollen  
   bee, 296, 302–303  
   bioactive characteristics of, 303, 304*f*  
   LTHV fluidized bed (LTHV<sub>fb</sub>) drying on  
     bioactive content of, 303–305,  
     305–306*t*  
   novel low-temperature unit operation  
     applications in, 302–305
- Poly(dimethylsiloxane) (PDMS)  
   hydrophobic surface of, 399–400  
   liquid prepolymer of, 399–400  
   soft lithography on, 400
- Polycyclic aromatic hydrocarbon (PAH)  
   formation, 299
- Polyelectrolyte complexes (PECs), 394
- Poly(lactic acid) (PLA), 395–396
- Polymers. *See also specific types*  
   natural  
     advantages of, 368  
     synthetic polymers in combination with,  
       394–396  
     wound dressings by, 413, 414–416*t*  
   protein-based, 390–394  
     collagen, 390–391  
     eggshell membrane (EgM), 393  
     fibrinogen, 394  
     gelatin, 391–392  
     keratin, 393  
     silk fibroin, 392  
     silk sericin, 392–393  
   synthetic  
     advantages of, 399  
     in combination with natural polymers,  
       394–396
- Poly (ADP-ribose) polymerase (PARP), 60
- Polyprotein open-reading frames (ORFs), in  
   coronaviruses, 445–447
- Polysaccharide-based natural polymers,  
   fabrication of wound dressings,  
   382–390, 383*f*, 386*f*
- Polyunsaturated fatty acids (PUFAs), 197–200,  
   198*t*, 296  
   biosynthesis, 200  
   of microbial PUFAs, 204–208, 205*f*,  
   206–208*t*  
   sources of, 201*t*  
   structures of, 199*f*  
   workflow of fermentation of, 214*f*
- Polyunsaturated fatty acids (PUFAs)  
   production  
   in engineered *Yarrowia lipolytica*, 210*t*  
   genetic engineering strategy inducing PUFA  
     production, 202–204  
   metabolic engineering in *Yarrowia*  
     *lipolytica* for, 202*f*  
   natural and atypical, 214–215  
   pilot—scale process for microbial PUFA  
     production, 213–214, 214*f*  
   using different carbon sources, 212–213

*Yarrowia lipolytica* for, 202*f*  
 Polyvinyl alcohol (PVA)-based dressings,  
   advantage of, 395  
 Prenylated diphenyl ethers, 266–267  
 Prenylated indole diketopiperazines, 251  
 Prenyl-substituted dihydrophenanthrenes, 118  
 Principal component analysis (PCA), 176–177  
 Protein-based polymers, 390–394  
   collagen, 390–391  
   eggshell membrane (EgM), 393  
   fibrinogen, 394  
   gelatin, 391–392  
   keratin, 393  
   silk fibroin, 392  
   silk sericin, 392–393  
 Proteolytic enzymes, 397  
 Purine catabolism, 279–280  
 Pyruvate, 208

## Q

Quantitative structure–activity relationship  
   (QSAR) model, 101–102  
 Quantitative structure–permeability  
   relationship (QSPR) study, 97–98  
 Quercetin, 85, 87*f*

## R

*Rabdosia rubescens* oridonin, 56  
 RBDs. *See* Receptor-binding domains (RBDs)  
 Reactive oxygen species (ROS), 55–56, 380  
 Receptor-binding domains (RBDs), 447–448  
 Recombinant microorganisms, 203  
 Remdesivir, 450–453  
*Rhodospiridium toruloides*, oleaginous strain  
   of, 217  
 Rhubarb (Polygonaceae)  
   effect of LT drying method on bioactive  
   compounds of, 320, 321*t*  
 Ricinoleic acid, 214–215  
 RNA-dependent RNA polymerase (RdRp/  
   nsp12), 447–448, 451–452*t*

## S

*Salmonella* Enteritidis, 334–335  
*Salvia corrugata*, 59  
*Salvia leriifolia*, 49–50  
*Salvia sahendica*, 52  
*Salvia somalensis*, 63–64  
 SARS-CoV, 443–444  
   C-terminus in, 447–448  
   infection, 448

SARS-CoV-2, 443–444  
   contagion of, 461  
   genome organization of, 445–447  
 Sclareol (SC), 65  
*Scutellaria barbata*, 48  
 Sea fennel essential oils, 333–334  
 Secondary metabolites  
   biological activity classification of, 269,  
   270*f*  
   isolation of, 226–241  
   structural diversity of, 264–265  
*Selaginella moellendorffii*, 48  
 $\alpha$ - and  $\beta$ -selective mannosylations, effect of  
   2-*O*- TAB group on, 20–21, 21*s*  
 Sericin, silk, 392–393  
 Sesquiterpenoid  
   aromatic bisabolene-type, 244–245  
 Shear-thinning bioinks, 403–404  
*Siegesbeckia pubescens*, 49–50  
 Silk fibroin, 392  
 Silk sericin, 392–393  
 Sirtuin 1 (SIRT1), 103–104  
 Skin-derived decellularized skin ECM  
   (dECM), 412–413  
 Soft lithography, 399–400  
 Solid lipid nanoparticles (SLN), 65  
 Spice (*Coriandrum sativum*)  
   bioactive component content of spice after  
   novel LTLH-assisted drying of,  
   320–322  
   novel low-temperature unit operations in,  
   319–322  
 STAT3 signaling pathway, 55–56  
 Stemanthrene, 152  
 Stereoconformers, of natural products, 453  
 Stereolithography (SLA), 405*f*, 408–409  
 Stereoselective glycosylations, nonclassical  
   NGP strategies for, 9  
 Stereoselective mannosylation  
   application of 2-*O*- TAB group, 16–17, 16*s*  
   2-*O*- TAB group donor for, 17–19, 18–19*t*,  
   20*s*  
 Stereoselective *O*-glycosylation, 2, 7  
 Sterigmatocystin derivatives, 242  
 Stimuli-responsive drug carriers, 373–378  
 Surface patterning, 398  
 Synergism, 337  
 Synthetic antihypertensive drugs, 342  
 Synthetic inhibitors, 332  
 Synthetic polymers  
   advantages of, 399  
   in combination with natural polymers,  
   394–396

## T

- TAGs. *See* Triacylglycerols (TAGs)
- Taichunamide, 255–256
- Taiwania cryptomerioides*, 45–48
- Taxus brevifolia*, 44
- Taxus wallichiana* var. *mairei*, 49
- TBARS. *See* Thiobarbituric acid reactive substances (TBARS)
- Tea (*Camellia sinensis*), 296
- bioactive content of, 299
- Tea, white
- bioactive characteristics of, 316–318, 316f
- bioactive component in white tea after novel LT process and, 319
- freeze withering (LTw), 318, 318t
- low-temperature white tea withering, curling and brewing, 315–316
- LTHV-assisted fluidized bed drying, 318–319, 319t
- novel low-temperature unit operations in white, 312–319, 313f
- Terpenes, 244–246, 244–246f
- Terpenoids, 71
- Tetrahydroxanthone dimers, 242–244
- Thermal inkjet printing, 404–406
- Thiobarbituric acid reactive substances (TBARS), 309
- Three-dimensional quantitative structure–activity relationship (3D-QSAR) studies, 105–106
- Tinospora cordifolia*, 61
- Tissue engineered skin substitutes, wound dressings, 410–412
- Total phenol content (TPC), 320–322
- Traditional wound dressings, 374–377t
- 1,2-*Trans* glycosylations, for synthesis of  $\beta$ -glucopyranosides, 7–8, 7s
- Translation elongation factor (TEF) promoter, 209
- 1,2-*Trans* manno-type pyranoside, 16
- Transmembrane serine protease 2 (TMPRSS2), 450, 451–452t
- Triacylglycerols (TAGs), 212
- synthesis in *Yarrowia lipolytica*, 216–217, 216f
- Triepoxide (TP), 64
- Triple-negative breast cancers (TNBC), 53–54
- Triptolide, 59
- Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), 58–59

## U

- Ulothrix flacca*, 181
- Uric acid formation, metabolic pathway of, 280f

## V

- Vacuolar-type H<sup>+</sup> ATPase (V-ATPase), 451–452t
- Vasoconstriction of vessels, 370
- Vegetable oils, 200
- Versicamides, 251
- Versicolols, 259–260
- Versicoloritides, 247
- Versicone, 241–242
- Versicoumarins, 259–260
- Versiquinazolines, 256–257
- Versispiroketal, 263–264
- Versixanthones, 242
- Vinyl-substituted dihydrophenanthrenes, 118
- Viral proteases, 450
- Vitamins, 397–398

## W

- Water vapor transmission rate (WVTR), 380
- Wound care, parameters to consider during, 372
- Wound dressings, 368–369, 372–378
- antimicrobial activity to, 385
- classification of, 374–377t
- decellularized skin ECM (dECM), 412–413
- design, functions of natural materials on, 378–381
- extracellular matrix (ECM) structure and topography, 378–379
- mechanical properties, 380–381
- porosity, 379–380
- water uptake ability (swelling) and hydrophilicity (wettability), 379
- water vapor transmission rate (WVTR) and oxygen permeation, 380
- in market fabricated using natural polymers, 414–416t
- by natural polymers, 413
- novel strategies in fabrication of, 398–413
- bioprinting, 403–410, 405f
- electrospinning, 401–403, 401f
- extrusion-based printing, 405f, 406–407
- inkjet printing, 404–406, 405f
- laser-assisted printing, 405f, 408
- lithography, 398–400, 399f
- in situ skin bioprinting, 409–410

- stereolithography (SLA), 405*f*, 408–409
- tissue engineered skin substitutes, 410–412
- traditional, 372
- Young's modulus of, 380–381
- Wound healing, 368
  - advancements in, 378
  - chronic, 395–396
  - cycles of normal wounds and chronic wounds, 369, 370*f*
  - phases, 370–372, 371*f*
- Wounding and wound types, 369, 370–371*f*
- Wound management, 368
  - multidrug-resistant (MDR) microorganisms on, 397

## X

- Xanthine oxidase (XOD), 279–280
  - enzyme, 332
  - inhibitory activities of essential oils (EOs), 351–357, 353–357*t*
- interactions between XOD and antihyperuricemic peptides, 287–290
- Xanthone–chromanone dimers, 242
- Xanthones, 241–244, 241–244*f*

## Y

- Yarrowia lipolytica*, 202–203
  - for biodiesel production, 215–217
  - biosynthesis of lipids in modified, 209–212
  - genome-scale models (GSMs) of, 203–204
  - for omega-3 EPA and DHA production, metabolic engineering, 205, 205*f*
  - for production of lipid compounds, 214–215
  - production of PUFAs in engineered, 210*t*
  - for PUFA production, 202*f*
  - TAG synthesis in, 216–217, 216*f*
- Yeasts, oleaginous, 204, 209
- Young's modulus, of wound dressings, 380–381
- Yuanhuatane, 60

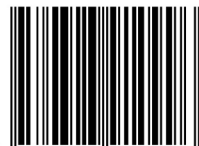
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