Chapter 2
Sustainable Production of Omega-3 Eicosapentaenoic Acid by Fermentation of Metabolically Engineered *Yarrowia lipolytica*

Dongming Xie, Edward Miller, Bjorn Tyreus, Ethel N. Jackson and Quinn Zhu

Abstract The omega-3 fatty acid, *cis*-5,8,11,14,17-eicosapentaenoic acid (C20:5; EPA) has wide-ranging benefits in improving heart health, immune function, and mental health. Currently, the major source for EPA is from fish oil, which is subject to challenges in its availability and sustainability due to the concerns of overfishing and contamination in the ocean environment. DuPont has developed a sustainable source of the omega-3 EPA through fermentation using metabolically engineered strains of *Yarrowia lipolytica*. EPA biosynthesis and supporting pathways have been engineered to accumulate high EPA content in *Yarrowia* biomass under fermentation conditions. Many production strain candidates were generated in the molecular biology group, and fermentation research was conducted to (1) identify the best production strains by high throughput fermentation screening; (2) optimize the fermentation medium and process conditions for the selected production strains to achieve the highest EPA titer, rate, and yield; and (3) scale-up the developed fermentation process for commercialization. This chapter summarizes the major fermentation engineering work that has been accomplished at DuPont to achieve large-scale production of *Yarrowia* biomass with high EPA content. This work led to two commercial products, New Harvest™ EPA oil and Verlasso® sustainably farmed salmon.

Keywords *Yarrowia lipolytica* · Omega-3 fatty acid · Eicosapentaenoic acid (EPA) · Fermentation engineering

D. Xie (✉) · E. Miller · B. Tyreus · E.N. Jackson · Q. Zhu
Industrial Biosciences, E.I. Du Pont de Nemours and Company,
Wilmington, DE, USA
e-mail: Dongming_Xie@uml.edu

Q. Zhu
e-mail: Quinn.Zhu@dupont.com

D. Xie
Department of Chemical Engineering, University of Massachusetts-Lowell,
One University Avenue, 01854 Lowell, MA, USA

© Springer-Verlag GmbH Germany 2016

P.C.K. Lau (ed.), *Quality Living Through Chemurgy and Green Chemistry*,
Green Chemistry and Sustainable Technology,
DOI 10.1007/978-3-662-53704-6_2

2.1 Introduction

Fatty acids have two ends, the carboxylic acid (−COOH) end, which is considered the beginning (alpha) of the chain, and the methyl ($-CH_3$) end, which is considered the tail (omega) of the chain. Omega-3 fatty acids refer to the long-chain polyunsaturated fatty acids (LCPUFA) that contain the first C=C double bond at the third carbon from the methyl (omega) end of the carbon chain. Many clinical studies have shown a wide range of health benefits from two major omega-3 fatty acids, the eicosapentaenoic acid (C20:5; EPA) and docosahexaenoic acid (C22:6; DHA) [1, 2]. While most omega-3 products on the current market address only the "omega-3" concepts in the labels, there are significant differences in health benefits among different omega-3 fatty acids, even between EPA and DHA. Based on the clinical studies, it is believed that EPA is able to improve cardiovascular health, mental health, and immune function, while DHA tends to be better in improving mental health and cognitive development in infants [2]. The Japanese EPA Lipid Intervention Study (JELIS) suggests that EPA is a promising treatment for prevention of major coronary events for people at risk of heart health issues [3]. In addition, the AMR101 study by Amarin Pharma Inc. showed that pure EPA ethyl ester significantly reduced triglyceride levels in adult patients with severe hypertriglyceridemia [4].

EPA and DHA are essential fatty acids in human health. However, the human body cannot efficiently synthesize EPA and DHA de novo and as such, these fatty acids are typically obtained from protein sources within the diet [2], especially the cold water oceanic fishes [1]. Certain sea fishes (e.g., wild salmon, Pacific sardine) accumulate significant amounts of EPA and DHA by consuming microalgae cells in the ocean, which are capable of synthesizing EPA and DHA de novo.

Fish oil is the main source of EPA and DHA on the market. Farm raised salmon accumulate DHA and EPA in their flesh and muscle when the oil from wild caught sea fishes is included in the salmon feed (Fig. 2.1). Usually, 4 kg of sea fish are required to raise 1 kg of salmon. The future availability and sustainability of current fish oil sources of omega-3 essential oils have been questioned due to potential overfishing and contamination issues in the ocean environment. To overcome these challenges, biotechnology companies such as Martek (now a part of DSM) started to produce DHA-enriched oil directly from microalgae in large-scale fermentation processes [5]. There is still no large-scale land-based EPA production from wild-type organisms due to the challenges in achieving high EPA productivity and low cost targets essential for commercial viability.

To address this need, DuPont initiated a research program to develop a sustainable EPA source by metabolic engineering of *Yarrowia lipolytica*, an industrial fungal work horse (Figs. 2.1, 2.2). The first targeted product for commercialization was EPA due to its unique health benefits and the lack of a land-based sustainable supply. *Yarrowia* cells have been metabolically engineered to accumulate EPA up

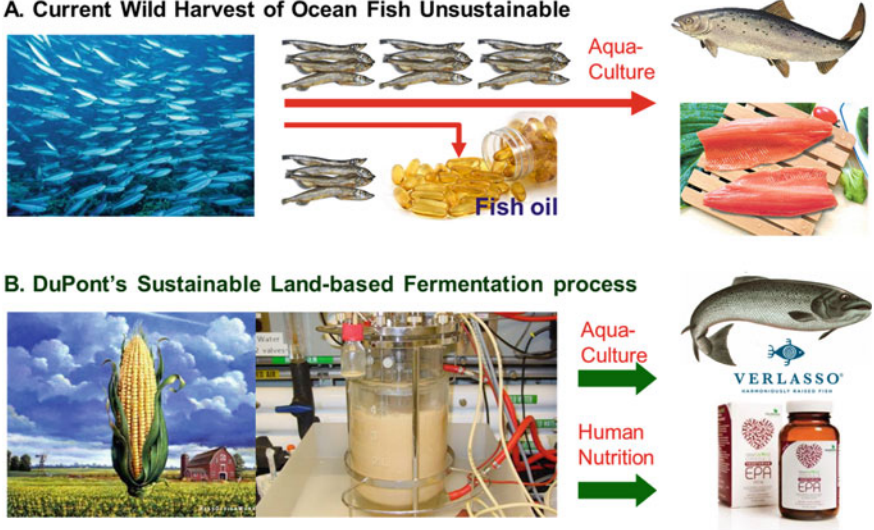

Fig. 2.1 Current major source of omega-3 from wild harvested ocean fishes and DuPont's sustainable land-based source of omega-3 from fermentation of *Yarrowia lipolytica*

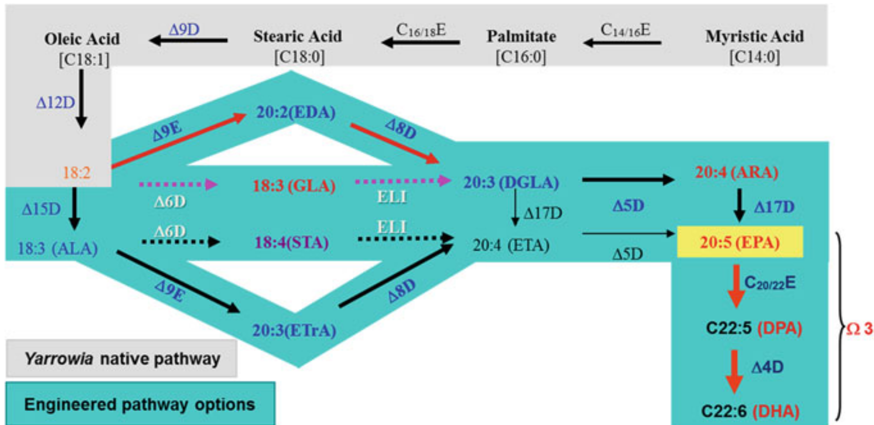

Fig. 2.2 EPA and DHA production by *Yarrowia lipolytica* through both Δ6 (the Δ6-desaturase) and Δ9 (Δ9-elongase and Δ8-desaturase) pathway engineering [7]

to 25 % of total biomass [6–8]. The purified lipids from these EPA producing strains have been used to develop New Harvest™ EPA oil, a commercial human nutritional supplement. The high EPA biomass of *Y. lipolytica* yeast has also been used to raise sustainably farmed salmon, Verlasso®. Sustainability being driven by the fact that only one kilogram sea fish is required to raise one kilogram of salmon due to the use of the yeast biomass in the salmon feed (Fig. 2.1). In addition, the *Y.*

lipolytica metabolic engineering technology has been sufficiently developed such that engineered strains were constructed to produce a variety of omega-3 and omega-6 fatty acids [7, 9].

This chapter summarizes the fermentation engineering research efforts of the *Y. lipolytica* metabolic engineering program to achieve successful commercialization of omega-3 EPA production using this technology. The work has paved the way for further improvement in omega-3 and omega-6 production for more specific applications in future. The advanced *Y. lipolytica* biotechnology platform can also be used for production of other high-value products.

2.2 Metabolic Engineering in *Yarrowia lipolytica* for EPA Production

The nonconventional yeast *Y. lipolytica* was selected as the host for metabolic engineering for a variety of reasons. *Y. lipolytica* is found primarily in foods such as dairy products and meat. *Y. lipolytica* was first used to produce single cell protein using cheap and abundant *n*-paraffins as the sole carbon source for animal feed [10]. It has also been classified as "Generally Recognized as Safe (GRAS)" for commercial production of food grade citric acid (U.S. Food and Drug Administration list of microbial-derived ingredients approved for use in food; Title 21, Part 173, Sec. 165). Other applications include production of erythritol, wax esters, 2-ketoglutaric, 2-hydroxyglutaric, and isopropylmalic acids and secretion of heterologous proteins, including several food enzymes [10, 11]. Forty different *Y. lipolytica* strains from various public depositories all over the world have been collected and screened as part of this effort. American Type Culture Collection (ATCC) #20362 was selected as the starting point of the metabolic engineering program largely based on its good fermentation performance: dry cell weight (DCW) greater than 100 g/L and lipid productivity greater than 1 g/L/h.

Wild type *Y. lipolytica* strains such as ATCC 20362, do not make omega-3 fatty acids naturally. The following strategies to engineer EPA producing strains were employed:

1. Build an efficient EPA biosynthetic pathway by (a) using strong promoters; (b) codon optimization of heterologous genes; (c) increasing/optimizing the copy numbers of structural genes; (d) focusing on limiting steps in the EPA pathway; and (e) pushing and pulling carbon flux.
2. Screen various strain libraries for high lipid and EPA productivities.
3. Generate mutants with increased lipid content by modification of the peroxisome and by disrupting certain genes in the ß-oxidation pathway.
4. Control fatty acid transport.
5. Manipulate global regulators.

The fatty acid profile of the wild-type strain ATCC #20362 shows that it can synthesize the omega-6 fatty acid, linoleic acid (LA, C18:2 n-6), but not any of the omega-3 fatty acids [9, 12, 13]. There are different biosynthetic routes to make EPA, the anaerobic polyketide synthase pathway [14] or an aerobic desaturase and elongase pathway [15]. However the rate, titer, and yield from these organisms could not meet the requirement for commercial production [16]. Recently, the aerobic pathways including both Δ6 (the Δ6-desaturase pathway, found in algae, mosses, fungi, and others) and Δ9 pathways (Δ9-elongase and Δ8-desaturase pathway) [17, 18] have been well studied (Fig. 2.2). In the Δ6 pathway, the LA and/or alpha-linolenic acid (ALA, 18:3n-6) is converted to gamma-linolenic acid (GLA, 18:3 n-6) and/or steridonic acid (STA, 18:4 n-3) by the Δ6-desaturase. Subsequently, the GLA and/or STA is converted to dihomo-gamma-linoleic acid (DGLA, 20:3n-6) and/or eicosatetraenoic acid (ETA, 20:4 n-3) by a $C_{18/20}$-elongase. In the Δ9 pathway, the LA and/or ALA is converted to eicosadienoic acid (EDA, 20:2n-6) and/or eicosatrienoic acid (ETrA, 20:3n-3) by a Δ9 elongase. The EDA and/or EtrA is then converted to DGLA and/or ETA by a Δ8-desaturase. The last two steps leading to EPA are the same for both pathways (Fig. 2.2) employing Δ15- and Δ17-desaturases. The desaturation and elongation enzymes carry out their reactions in the endoplasmic reticulum membrane [12, 15, 18].

Promoters for *Y. lipolytica* genes are no exception among various microbial hosts in controlling the expression of foreign. A set of promoters from genes expressing enzymes involved in glucose central metabolism that are at least as strong as the TEF (translation elongation factor) promoter have been isolated [19]. In screening studies, each individual promoter was placed in front of the ß-glucuronidase (GUS) reporter and the signal strength recorded in quantitative fluorometric assays [20]. Results showed that the *FBA in* promoter was the strongest among the first set of six promoters. The *GPM1* promoter was as strong as the *TEF* promoter, a benchmark comparison; the *GPD1* promoter was 2.5 times stronger than the *GPM1* promoter; and the *FBAin* promoter activity was 5.5 and 2.2 times stronger than the *GPD1* and *GPM1* promoters, respectively [21]. In addition, the YAT1 promoter activity increased by 35-fold when the fermentation was switched from nitrogen-rich to nitrogen-limiting conditions [22], which provides a unique advantage for application in the two-stage omega-3 fermentation process (see Fig. 2.6). The relative strength of the promoters examined under nitrogen-limiting conditions is as follows: FBA_{in} > YAT1 > FBA > GPD, EXP > GPAT > GPM = TEF. In addition, other new *Y. lipolytica* promoters could be generated by random mutagenesis using a specific promoter as template [23].

Codon optimization is also critical for further improving the expression of foreign genes in *Y. lipolytica*. Hence, all genes used in the construction of various strains which include the Δ6-desaturase, $C_{18/20}$-elongase, Δ5-desaturase and Δ17-desaturase, were codon optimized according to the codon usage pattern and GC content of highly expressed genes of *Y. lipolytica*, which typically have a GC content 52–54 % GC. In addition, they contain the consensus sequence (5′-ACCATGG-3′) around the 'ATG' translation initiation codon with a built-in *Nco*I site and a *Not*I site after its stop codon [7, 12] to facilitate cloning in conventional

vectors. It was found that the substrate conversion was increased in almost all of the codon-optimized genes except the Δ5-desaturase gene derived from *M. alpine* [12].

Another strategy to further improve EPA pathway activity is by increasing the copy number of the overexpressed genes. In early engineering of the Δ6 pathway, strain Y9027 was generated to produce EPA at 40 % of the total lipid fraction [12] by increasing the copy number of the pathway genes. The "pushing" of carbon flux into the engineered pathway was accomplished by overexpression of the $C_{16/18}$-elongase gene [24] and the Δ12-desaturase gene of *Fusarium moniliforme* [25]. The in vivo substrate conversion catalyzed by the overexpressed Δ6-, Δ5-, and Δ17-desaturases in strain Y9027 were about 86, 90 and 97 %, respectively, of the total fatty acids entering the triglyceride pool. The strategy of increasing the overexpressed gene copy number was further applied in the Δ9 pathway engineering for generating more advanced EPA production strains.

One bottleneck to achieving high carbon flux in the initial engineering of the Δ6 pathway was the low efficiency of the $C_{16/18}$-elongase, which led to high GLA content in the lipid profile. To reduce GLA accumulation, genes encoding the Δ9-elongases [26] and Δ8-desaturases [27] (Fig. 2.2) were isolated and characterized from *Euglena gracilis, E. anabaena,* and *Eutreptiella,* sp. CCMP389. Three Δ9-elongase and Δ8-desaturase bifunctional fusion genes were also created [28], which almost doubled the Δ8-desaturase activity while keeping similar Δ9-elongase activity. In addition, three genes encoding Δ5-desaturases from *E. gracilis, E. anabaena,* and *Eutreptiella,* sp. CCMP626 [29] and three genes encoding Δ17-desaturases from *Pythium aphanidermatum*, *Phytophthora ramorum,* and *Phytophthora sojae* were also isolated and studied for their activities and substrate selectivity [13]. Several genes encoding different acyltransferases were also isolated and used to improve fatty acid traffic in the endoplasmic reticulum [30]. "Pushing" and "pulling" the carbon flux into the engineered Δ-9 pathway was achieved by overexpression of the $C_{16/18}$-elongase gene and the Δ12-desaturase gene [25] and by using multiple copies of Δ17-desaturase genes [13].

One critical finding during engineering of the Δ9 pathway was the mutation of PEX encoding genes that are involved in peroxisome biogenesis and matrix protein import, such as PEX3 or PEX10. Mutation of these genes improved the EPA content in the lipid fraction by two fold [9, 31]. Deletion of the *pex10* gene in engineered strains producing DGLA and ARA were also discovered to double DGLA and ARA titers in the lipid fraction compared to the parent strains with a wild-type *pex*10 gene [9]. In *pex10* mutation strains, ß-oxidation was greatly reduced and unidentified membrane-like structures, possibly deformed nonfunctional peroxisomes, were also observed. By combining the above Δ9 pathway engineering strategies, strain Z5567 [20] was generated that contains 41 copies of 19 different genes. Z5567 produced increased lipid fraction as high as 50 % of dry cell weight (DCW) with an EPA content as high as 25 % of DCW under typical fed-batch fermentation conditions.

2.3 Selection of High EPA Production Strains

Fermentation research plays a critical role in converting the omega-3 metabolic engineering research to commercial application. Both fermentation and strain engineering research were conducted in concert from the beginning of the program. The major fermentation research activities included (1) establishing high throughput strain screening protocols at various scales of fermentation and selecting the top producing strains using these protocols, (2) optimizing fermentation conditions for selected promising candidate strains, and (3) fermentation process scale-up to achieve high EPA production at pilot and commercial scales.

Figure 2.3 summarizes a typical process workflow for the omega-3 strain screening. Tens of thousands of new candidate strains were generated yearly by the metabolic engineering program. Candidate strains were first tested in 24-well plates to identify the top strains based on the EPA content in the lipid fraction as determined by GC analysis [32]. Among them, thousands of high EPA strains were selected for testing under shake flask conditions. Those strains with both high lipid titer in biomass and high EPA content in the lipid fraction were selected for further testing in the Micro-24 bioreactor system [33]. In the Micro-24 bioreactor experiments, the titer and yield of both lipid and EPA were obtained to better evaluate each candidate strain's performance. Dozens of top strains identified from the Micro-24 bioreactor experiments were further evaluated in lab-scale fermentors (2–10 L), where the titer, rate, and yield of cell density (DCW or dry cell weight), lipid, and EPA were carefully compared to identify the very top production strain candidates. Some of the top strains (e.g., Y4305) were described previously in

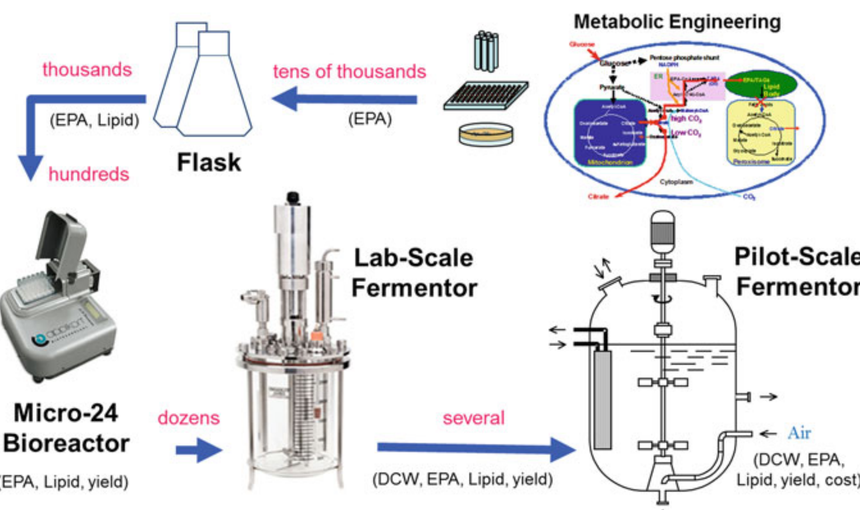

Fig. 2.3 Typical process flow for screening and selection of high EPA production strains under various bioreactor conditions

details [9]. Those top candidates from the lab-scale fermentation experiments were evaluated in pilot-scale fermentors using commercial medium and process conditions. Then the cost to produce EPA by each candidate strain was evaluated to determine the final production strain.

A key step in the strain screening process is evaluation in small scale bioreactors. It is desirable to have a multi-bioreactor system with small working volumes to allow testing of hundreds of candidate strains, yet each small bioreactor system must have high-quality process controls so that the data obtained from these small bioreactors predict the performance in lab- and pilot-scale fermentors. There are many bioreactor tools available for strain screening. The 24-well blocks and test tubes are the simplest bioreactors and can be easily operated. However, the data is much less reliable due to the low controllability and insufficient sample volumes available for both lipid and cell density analysis [34, 35]. Shake flasks are also simple and easy to run at larger working volumes (10–100 mL), but they still do not monitor and control pH values and dissolved oxygen (DO) levels. Besides that, they are more labor intensive, especially for the two-stage omega-3 process [36]. Recently, some Microtiter plates/bioreactors such as the BioLector system have been developed, which have a large number of small reaction wells (1 mL or less) with each well's pH value and DO level monitored [37, 38], but precise control of pH and DO levels are still not available. The small working volume of each well also limited the microtiter bioreactor's application in the omega-3 project due to the large sample size needed for DCW and lipid analysis.

Recently, EPA strain screening and fermentation optimization was performed in a Micro-24 Bioreactor system [33]. The Micro-24's ability to monitor and control each reactor's temperature, dissolved oxygen level, pH values, and potential for real-time data acquisition made it amenable for performing scale-down experiments

Bioreactor	Reactor/ working vol.	Controllability	Experimental Data	Work Capacity
	10~50 flasks/shaker, 25-50 mL	T	Final point – titer, rate, yield	2000 individual experiments /year/person
	24 reactors, 3-7 mL	Individual T, pH, pO_2	Online process- T, pH, pO2 Final point – titer, rate, yield	1000 individual experiments /year/person
	Single reactor, 2-10 L	T, pH, pO_2 feeding	Online process- T, pH, pO2, feeding Time course – titer, rate, yield	40 individual experiments /year/person

Fig. 2.4 Comparison of process controllability, data quality, and work capacity between shake flasks, Micro-24 bioreactor, and lab-scale fermentors

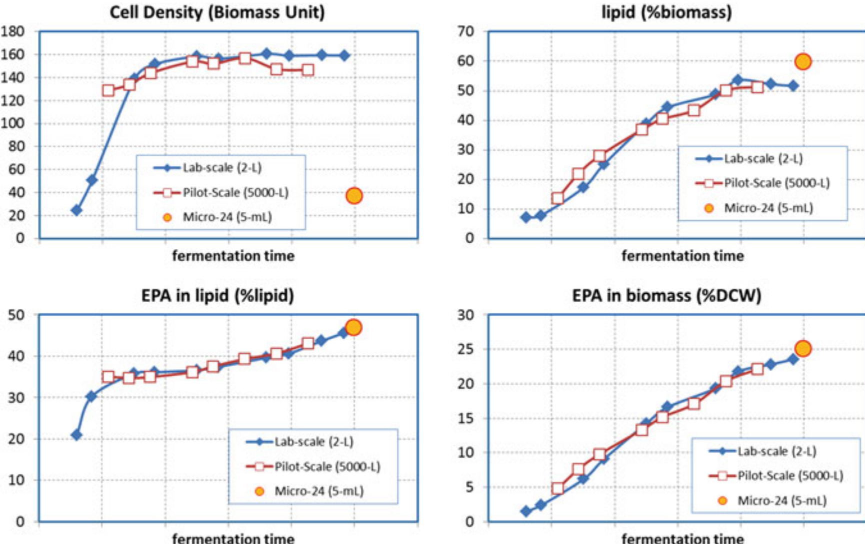

Fig. 2.5 Comparison of omega-3 fermentation data between the Micro-24 Bioreactor, 2-L fermentor, and 5000-L fermentor experiments

of larger lab-scale fermentations in a high throughput manner (Fig. 2.4). Using this system environmental conditions (T, pH, pO_2, medium…) could be varied across a wide range to examine impacts on end of run fermentation performance metrics, including byproduct analysis, DCW, lipid content, EPA content in lipid, and EPA conversion yield. These data significantly increased the predictability of each individual strain's performance in lab- or large-scale fermentation. As can be seen in Fig. 2.5, the lipid and EPA data from the Micro-24 bioreactor experiments agree well to those from the 2-L and 5000-L experiments though were operated at much lower cell densities in the Micro-24 bioreactor experiments.

2.4 Two-Stage Fermentation Process for EPA Production

Optimal fermentation conditions are often different for production strains with different genetic backgrounds. After selection of a top production strain from thousands of candidate strains (Fig. 2.3), fermentation conditions including seed culture, inoculation process, fermentation medium, substrate feeding and control, other process parameters (e.g., T, pH, DO, etc.) were optimized for the given strain. The goal of fermentation optimization is to maximize rate, titer, and yield of EPA production and minimize by-product formation (e.g., organic acids). The omega-3 fatty acids are an intracellular product, thus maintaining both high cell density and high EPA content in biomass is critical for fermentation optimization.

The omega-3 fermentation is a typical two-stage fermentation process (Fig. 2.6) with oil and EPA produced under nitrogen-limiting conditions that is largely non-growth associated. In the first stage of the fermentation, the *Yarrowia* cells are grown on the carbohydrate substrate with nitrogen provided by both an organic source (e.g., yeast extract) and an inorganic source (e.g., $(NH_4)_2SO_4$) in the initial medium. An alternative approach is to supply inorganic nitrogen in the form of NH_4OH used for pH control in the growth phase. After a certain period of time, the residual nitrogen in the fermentation medium is consumed, cell growth stops, and the *Yarrowia* cells start to accumulate lipids by converting the carbohydrate continuously supplied to the nitrogen limited culture.

While flasks and microfermenters gave general guidance around optimization of the fermentation conditions, they are not sufficient for further optimizing the rate, titer, and yield of a complete fermentation run due to limitations in process controllability and low capacities for mixing and mass transfer that do not mimic larger scale fermentor capabilities. For that purpose, the optimization work for a selected production strain was mainly conducted in lab-scale fermenters. The optimized fermentation conditions were further examined in pilot- or commercial scale fermentors to test their scalability. Scale down and re-optimization experiments are sometimes required when significant difference in fermentation performance is seen between lab-scale and large-scale fermentors.

Growth Stage

- To build up enough biomass;
- To provide enough nutrient (e.g. nitrogen source) for cell growth;
- To optimize growth conditions.

Lipid Production (Oleaginous) Stage

- To build up lipid and EPA/DHA by limiting nitrogen;
- To feed substrate for energy and carbon source;
- To optimize oleaginous conditions.

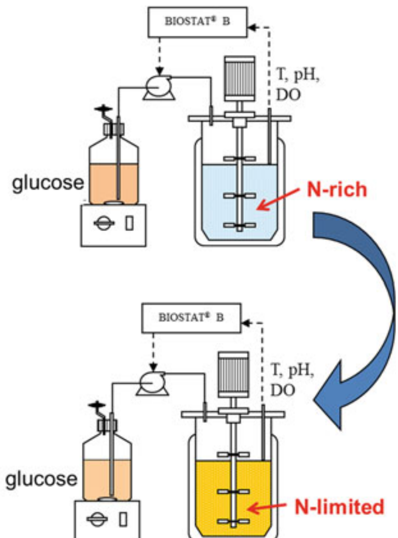

Fig. 2.6 Two-stage (growth and production) fed-batch fermentation process for EPA production by metabolically engineered *Yarrowia lipolytica*

2.5 Using Modeling to Guide Fermentation Optimization and Scale-up

Optimization of lab-scale and large-scale fermentation is critical to achieve target EPA production and guide decisions as to further strain engineering. In practice, it was always a challenge to complete optimization experiments for each strain due to the multitude of strains to be screened opposite time and resource limitations. Our strategy was to use mathematical models to help analyze fermentation performance under different conditions and then to design and predict optimal conditions. Dynamic models were developed to describe fermentation behavior based on the significant amount of fermentation data available and on the understanding of both the strain and the process that had been accumulated. The model was established by fitting to old experimental data, validated and modified by matching with more experimental results under new conditions, and then used to predict new conditions for improved fermentation performance (Fig. 2.7).

A set of unstructured kinetic equations were built from first principles, which included the equations of cell growth, substrate consumption, nitrogen utilization, oxygen uptake, lipid and EPA formation, and byproduct accumulation. The models

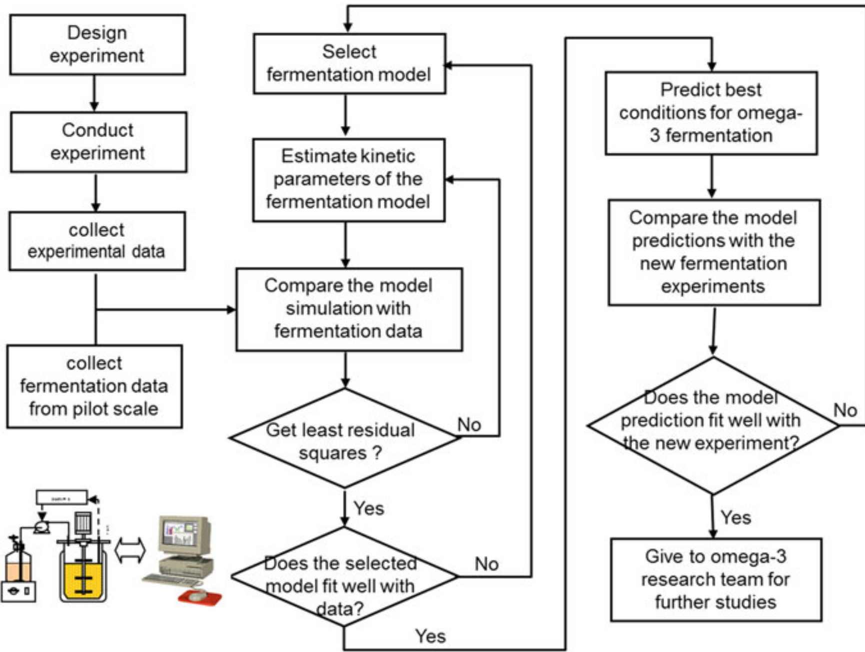

Fig. 2.7 A schematic diagram of establishing a mathematical model for omega-3 fermentation based on experimental data. The model is then used to help guide experiments under new fermentation conditions

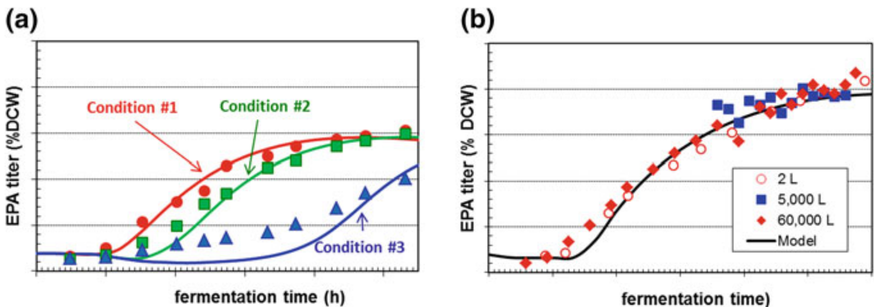

Fig. 2.8 Examples of using mathematical model for process optimization (**a**) and scale-up (**b**). In graph **a**, the model (*solid lines*) guided the fermentation experiments (*symbols*) to improve EPA titers by changing conditions from #3 to #1 and #2. In graph **b**, the model was used to guide the fermentation scale-up from 2L to pilot and commercial scale

could predict cell growth, DCW, DO level, oxygen uptake rate (OUR), CO_2 evolution rate (CER), and other process parameters that were also measured during the fermentation as a function of various medium and process conditions. When the production strain or key process conditions were changed, only a few model parameters needed to be adjusted to maintain the model's predictability. The changes were often associated with rate constants that were used to describe phenotypic changes as the result of certain genetic constructs. The dynamic models were able to predict the key performance parameters (e.g., titer, rate, and yield of a product) before and during the run, and further help to guide the fermentation optimization and process scale-up. Examples of the dynamic model's capability are shown in Fig. 2.8.

2.6 Fermentation Scale-Up for Commercial Production

Achieving high titer, rate, and yield of EPA production in lab-scale fermentation experiments marked the beginning of fermentation research efforts. The ultimate, more challenging goal was to demonstrate similar or better fermentation performance at large scale. Scale-up studies were necessary and became extremely important in the latter stage of the R&D program. The general criteria used for fermentation scale-up included key factors of geometry/size, power input per volume, mass transfer coefficient K_{La}, etc. [39]. Selected production strains were tested in pilot-scale facilities to determine the best criteria for fermentation scale-up, and to gather more information on strain performance, dynamic behavior, and scale-up effects that could influence EPA rate, titer, and yield. During scale-up the set of measured variables expanded beyond temperature, pH, feed rate, and DO levels, and included agitation, aeration rate, and mass transfer characteristics. The latter became specific focal points for scale-up.

The benefits of highly predictive dynamic models became evident as the process moved from lab scale to pilot plant and commercial production. The models were used to study the mass transfer characteristics of the broth as a function of superficial gas velocity and agitation power. The dynamic models were used to predict the commercial scale fermentation performance taking into account the mass transfer characteristics obtained from the pilot trials which were used to set specific agitation and aeration rates. With the aid of the models a successful process scale-up to commercial production was obtained using the least number of pilot trials (Fig. 2.8b).

The major challenges encountered during the scale-up process were limitations in either the medium/feed components or in the achievable process controls in large-scale fermentors. For example, most commercial scale fermentation uses complex media with commercially available raw materials. However, the complex media may have some other minor components affecting the fermentation performance. In addition, fluid dynamic behavior in large-scale fermentors is significantly different from that in lab scale. When significant difference/inconsistency between the fermentation performance in lab-scale and large-scale fermentors was observed, a series of lab-scale "scale-down" experiments were designed to mimic the fermentation medium and/or process conditions at commercial scale [40, 41]. The scale-down studies elucidated a few important factors that affect the scale-up. The identified scale-up issues were fixed by further engineering of the strain or by modifying fermentation protocols to better follow lab-scale and pilot-scale experiments.

2.7 Omega-3 EPA Oil Produced by *Yarrowia lipolytica*

The end result of this effort is the production of an omega-3 oil with a lipid profile that has certain heart health advantages over other omega-3 oils on the market (Fig. 2.9). The *Yarrowia* yeast oil has more than 50 % EPA as a fraction of total lipid and around 5 % saturated fatty acids making the EPA oil unique among omega-3 containing oils that have not been derivatized or fractionated. Saturated fatty acids (C14:0, C16:0 & C18:0) are relatively high in most other oils, which was believed to increase low-density lipoprotein (LDL) cholesterol, a risk factor for heart disease [42]. Fish oil contains roughly 30 % omega-3 content largely as EPA and DHA in an 18:12 ratio and is less desirable from a heart health point of view. Algae oil has close to 40 % DHA content, and is also high in saturated fatty acids. Flax oil has about 40 % ALA, but ALA has very low efficiency (5–10 %) for further conversion to EPA and DHA in the human body. The *Y. lipolytica* technology platform provides an alternative route to high omega-3 content oil with low saturated fatty acids levels that is obtainable without the need for further concentration and purification using conventional distillation technology. The consistent supply and high targeted specific fatty acid content also provides for downstream purification advantages in applications where a single fatty acid is required. In this

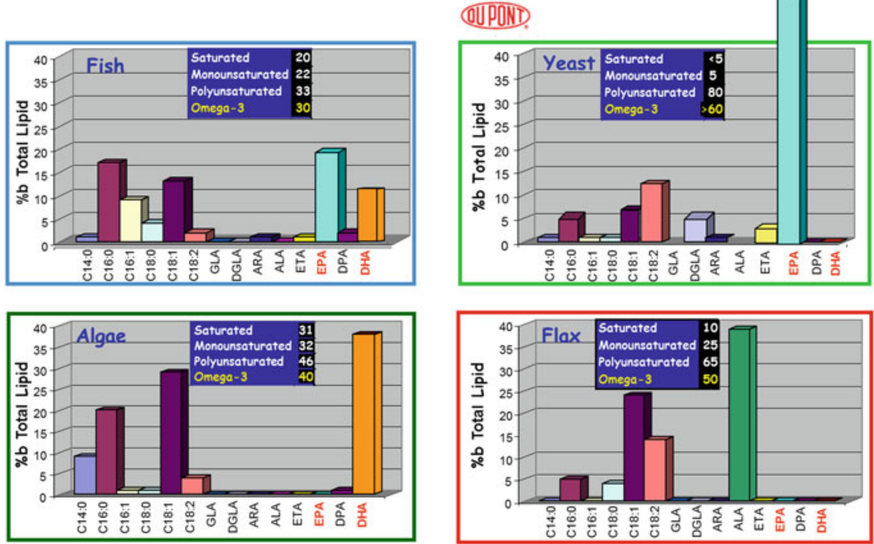

Fig. 2.9 Comparison of the lipid profiles between DuPont's *Yarrowia lipolytica* oil and other major omega-3 oil products on market

latter case, the technology platform developed by DuPont is amenable to produce tailored omega-3 (EPA, DHA) and/or omega-6 (ARA, GLA) fatty acid mixtures in the cellular lipid profiles, which can meet more technical or customer needs in the future [7, 9].

2.8 Conclusion

The nonconventional yeast *Y. lipolytica* was engineered in DuPont to produce a high content of omega-3 EPA (C20:5) in biomass under commercial-scale fermentation conditions. A combination of enzymes were overexpressed to synthesize EPA via the Δ9/Δ8 pathway. The lipid metabolism was optimized to maximize total lipid and EPA production. The expression levels of all other pathway enzymes were also carefully balanced. A key finding during pathway engineering and evaluation was that disruption of peroxisome biogenesis had a major positive impact on the production of EPA and the metabolism of storage lipid, as well as reduction of the major by-products. The result of the metabolic engineering effort produced a high EPA production strain, Z5567, which is capable of producing a single fatty acid, in this case EPA, to levels as high as 25 % of dry biomass.

Fermentation engineering played a critical role in helping to transition the omega-3 research to commercial production. Research in both strain engineering

and fermentation process development was initiated at the same time and carried out in parallel. Close collaboration between strain engineering and fermentation research was essential to success. An advanced microfermentor system with well-controlled process parameters significantly improved the efficiency of strain screening and the predictability of the selected strain's performance in larger scale fermentation. The omega-3 fed-batch fermentation process consisted of a growth phase to maximize biomass production and a production (oleaginous) phase to maximize EPA production while minimizing byproduct formation. Mathematical modeling of the fermentation process was developed and used to guide fermentation optimization and scale-up. Two commercial products, NewHarvest™ EPA oil and Verlasso® salmon were developed by using the sustainable EPA source from *Yarrowia lipolytica*. The road to commercial success was made possible by the power of modern biotechnology to combine both fundamental scientific research and industrial engineering.

Acknowledgments We would like to thank Dr. Henry Bryndza and Dr. Michael Saltzberg for their strong support. We also thank all members in the omega-3 technical, manufacturing, and business teams for their collaboration and contributions.

References

1. Martins DA, Custódio L, Barreira L, Pereira H, Ben-Hamadou R, Varela J, Abu-Salah KM (2013) Alternative sources of n-3 long-chain polyunsaturated fatty acids in marine microalgae. Mar Drugs 11:2259–2281
2. Kapoor R, Patil UK (2011) Importance and production of omega-3 fatty acids from natural sources. Int Food Res J 18:493–499
3. Yokoyama M, Origasa H, Matsuzaki M, Matsuzawa Y, Saito Y, Ishikawa Y, Oikawa S, Sasaki J, Hishida H, Itakura H, Kita T, Kitabatake A, Nakaya N, Sakata T, Shimada K, Shirato K (2007) Effects of eicosapentaenoic acid on major coronary events in hypercholesterolaemic patients (JELIS): a randomised open-label, blinded endpoint analysis. Lancet 369:1090–1098
4. Ballantyne CM, Bays HE, Kastelein JJ, Stein E, Isaacsohn JL, Braeckman RA, Soni PN (2012) Efficacy and safety of eicosapentaenoic acid ethyl ester (AMR101) Therapy in statin-treated patients with persistent high triglycerides (from the ANCHOR Study). Am J Cardiol 110:984–992
5. Kyle DJ (2001) The large-scale production and use of single-cell highly enriched oil in docosahexaenoic acid. In: Shahidi F, Finley J (eds) Omega-3 fatty acids: chemistry, nutrition, and health effects. Oxford University Press, Oxford, pp 92–107
6. Hong S-Y, Seip J, Sharpe PL, Xue Z, Yadav NS, Zhang H, Zhu QQ (2011) Recombinant microbial host cells for high eicosapentaenoic acid production. U.S. Patent Application 20120052537
7. Xie D, Jackson E, Zhu Q (2015) Sustainable source of omega-3 eicosapentaenoic acid from metabolically engineered *Yarrowia lipolytica*: from fundamental research to commercial production. Appl Microbiol Biotechnol 99:1599–1610
8. Zhu Q, Jackson E (2015) Metabolic engineering of *Yarrowia lipolytica* for industrial application. Curr Opin Biotechnol 36:65–72

9. Xue Z, Sharpe PL, Hong SP, Yadav NS, Xie D, Short DR, Damude HG, Rupert RA, Seip JE, Wang J, Pollak DW, Bostick MW, Bosak MD, Macool DJ, Hollerbach DH, Zhang H, Arcilla DM, Bledsoe SA, Croker K, McCord EF, Tyreus BD, Jackson EN, Zhu Q (2013) Production of omega-3 eicosapentaenoic acid by metabolic engineering of *Yarrowia lipolytica*. Nat Biotech 31:734–740
10. Ratledge C (2005) Single cell oils for the 21st century. In: Cohen Z, Ratledge C (eds) Single cell oils. AOCS Press, Champaign, pp 1–20
11. Groenewald M, Boekhout T, Neuvéglise C, Gaillardin C, van Dijck PW, Wyss M (2014) *Yarrowia lipolytica*: safety assessment of an oleaginous yeast with a great industrial potential. Crit Rev Microbiol 40:187–206
12. Zhu Q, Xue Z, Yadav N, Damude H, Pollak D, Rupert R, Seip J, Hollerbach D, Macool D, Zhang H (2010) Metabolic engineering of an oleaginous yeast for the production of omega-3 fatty acids. In: Cohen Z, Ratledge C (eds) Single cell oils, 2nd edn. AOCS Press, Urbana, pp 51–73
13. Xue Z, He H, Hollerbach D, Macool DJ, Yadav NS, Zhang H, Szostek B, Zhu Q (2013) Identification and characterization of new Δ-17 fatty acid desaturases. Appl Microbiol Biotechnol 97:1973–1985
14. Metz JG, Roessler P, Facciotti D, Levering C, Dittrich F, Lassner M, Valentine R, Lardizabal K, Domergue F, Yamada A, Yazawa K, Knauf V, Browse J (2001) Production of polyunsaturated fatty acids by polyketide synthases in both prokaryotes and eukaryotes. Science 293:290–293
15. Meesapyodsuk D, Qiu X (2012) The front-end desaturase: structure, function, evolution and biotechnological use. Lipids 47:227–237
16. Napier (2004) The production of long chain polyunsaturated fatty acids in transgenic plants by reverse-engineering. Biochimie 86(11):785–792
17. Wallis JG, Browse J (1999) The Delta8-desaturase of *Euglena gracilis*: an alternate pathway for synthesis of 20-carbon polyunsaturated fatty acids. Arch Biochem Biophys 365:307–316
18. Sayanova O, Napier JA (2004) Eicosapentaenoic acid: biosynthetic routes and the potential for synthesis in transgenic plants. Phytochemical 65:147–158
19. Muller S, Sandal T, Kamp-Hansen P, Dalboge H (1998) Comparison of expression systems in the yeasts *Saccharomyces cerevisiae*, *Hansenula polymorpha*, *Klyveromyces lactis*, *Schizosaccharomyces pombe* and *Yarrowia lipolytica*. Cloning of two novel promoters from *Yarrowia lipolytica*. Yeast 14:1267–1283
20. Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6:3901–3907
21. Hong S-Y, Seip J, Walters-Pollak D, Rupert R, Jackson R, Xue Z, Zhu Q (2011) Engineering *Yarrowia lipolytica* to express secretory invertase with strong FBA1IN promoter. Yeast 29:59–72
22. Xue Z, Zhu QQ (2012) Ammonium transporter promoter for gene expression in oleaginous yeast. US Patent 8323960
23. Alper H, Fischer C, Nevoigt E, Stephanopoulos G (2005) Turning genetic control through promoter engineering. Proc Natl Acad Sci USA 102:12678–12683
24. Macool, DJ, Xue Z, Zhu Q (2008) A *Mortierellaalpina* C16/18 fatty acid elongase. US Patent 7470532
25. Yadav NS, Zhu Q, Zhang H (2009) Δ12 desaturases suitable for altering levels of polyunsaturated fatty acids in oleaginous yeast. US Patent 7504259
26. Damude HG, Zhu Q (2007) Delta-8 desaturase and its use in making polyunsaturated fatty acids. US Patent US 7256033
27. Damude HG, McGonigle B, Zhu Q, Zhixiong X (2007) Delta-9 elongases and their use in making polyunsaturated fatty acids. US Patent Application US20070118929
28. Damude HG, Kinney AJ, Ripp KG, Zhu QQ (2008) Multizymes and their use in making polyunsaturated fatty acids. US Patent Application 20080254191

29. Pollak DW, Bostick MW, Yoon H, Wang J, Hollerbach DH, He H, Damude HG, Zhang H, Yadav NS, Hong SP, Sharpe P, Xue Z, Zhu Q (2012) Isolation of a delta 5 desaturase gene from *Euglena gracilis* and functional dissection of its HPGG and HDASH motifs. Lipids 47:913–926
30. Zhang H, Damude HG, Yadav NS (2012) Three diacylglycerolacyl transferases contribute to oil biosynthesis and normal growth in *Yarrowia lipolytica*. Yeast 29:25–38
31. Hong SP, Sharpe PL, Xue Z, Yadav NS, Zhu QQ (2009) Peroxisome biogenesis factor protein (pex) disruptions for altering polyunsaturated fatty acids and total lipid content in oleaginous eukaryotic organisms. US Patent Application 20090117253
32. Cahoon EB, Ripp KG, Hall SE, Kinney AL (2001) Formation of conjugated Δ8, Δ10-double bonds by Δ12-oleic acid desaturase-related enzymes: biosynthetic origin of calendic acid. J Biol Chem 276:2637–2643
33. Xie D (2012) Using an advanced microfermentor system for strain screening and fermentation optimization. In: Cheng Q (ed) Methods in molecular biology, 834 (microbial metabolic engineering). Humana Press, New York, pp 217–231
34. Danielson PB, Büchs J, Stockmann C, Fogleman JC (2004) Maximizing cell densities in miniprep-scale cultures with H15 medium and improved oxygen transfer. Biochem Eng J 17:175–180
35. Stockmann C, Losen M, Dahlems U, Knocke C, Gellissen G, Büchs J (2003) Effect of oxygen supply on passaging, stabilizing and screening of recombinant *Hansenula polymorpha* production strains in test tube cultures. FEMS Yeast Res 4:195–205
36. Büchs J (2001) Introduction to advantages and problems of shaken cultures. Biochem Eng J 7:91–98
37. Amanullah A, Otero JM, Mikola M, Hsu A, Zhang J, Aunins J, Schreyer HB, Hope JA, Russo AP (2010) Novel micro-bioreactor high throughput technology for cell culture process development: Reproducibility and scalability assessment of fed-batch CHO cultures. Biotechnol Bioeng 106:57–67
38. Duetz WA, Ruedi L, Hermann R, O'Connor K, Büchs J, Witholt B (2000) Methods for intense aeration, growth, storage and replication of bacterial strains in microtiter plates. Appl Environ Microbiol 66:2641–2646
39. Shuler ML, Kargi F (2002) Bioprocess Engineering, 2nd edn. Prentice Hall PTR, Upper Saddle River, pp 286–306
40. Nienow AW, Nordkvist M, Boulton CA (2011) Scale-down/scale-up studies leading to improved commercial beer fermentation. Biotechnol J 6:911–925
41. Ozbek B (1997) Mathematical modeling and simulation studies of scale-down fermentation systems. Chem Eng Technol 20:259–267
42. Katan M, Zock P, Mensink R (1994) Effects of fats and fatty acids on blood lipids in humans: an overview. Am J Clin Nutr 60(Suppl):1017S–1022S

http://www.springer.com/978-3-662-53702-2

Quality Living Through Chemurgy and Green Chemistry
C.K. Lau, P. (Ed.)
2016, XX, 369 p. 95 illus., 52 illus. in color., Hardcover
ISBN: 978-3-662-53702-2