Biotechnology Reports 31 (2021) e00648

Contents lists available at ScienceDirect

Biotechnology Reports

journal homepage: www.elsevier.com/locate/btre

Thermal treatment improves a process of crude glycerol valorization for the production of a heterologous enzyme by *Yarrowia lipolytica*

Monika Kubiak, Wojciech Białas, Ewelina Celińska [1,*]

Department of Biotechnology and Food Microbiology, Poznan University of Life Sciences, 48 Wojska Polskiego, 60-637 Poznań, Poland

ARTICLE INFO

Keywords:
Raw glycerol
Mathematical modeling
Design of experiments
Process design and simulation
Stress response

ABSTRACT

Valorization of crude glycerol requires a potent bifunctional biocatalyst, such as *Yarrowia lipolytica*, capable of high-density growth on this substrate, and having *i.a.* high propensity for heterologous protein synthesis. Increasing evidence suggests that controlled administration of stress, *i.a.* thermal treatment, has a positive impact on bioprocess performance. In this study, we systematically adjusted thermal treatment conditions (20 to 42 °C) in order to maximize heterologous protein production by *Y. lipolytica* growing in crude glycerol-based medium. Our results showed nearly 30% enhancement in the enzyme production triggered by temporary exposure to decreased temperature. Here developed mathematical model indicated optimal treatment conditions (20 °C, 153′) that were later applied to a process with biodiesel-derived glycerol and technical substrates. Techno-economic analysis of a pilot-scale-waste-free process was conducted. Quantitative description of the associated costs and economic gain due to exploitation of industrial substrates, as well as indication of current bottlenecks of the process, are also provided.

Background

Decarbonisation of road transport, electricity, heating and cooling is amongst most urgent priorities of governmental actions worldwide [1]. According to Renewable Energy Progress report from 2019 (COM2019–225), biodiesel represents over 80% of the biofuels consumed in the EU [2]. It is commonly known that per every 100 units of produced biodiesel, 10 units of crude glycerol are being generated [3]. To be used in pharma or cosmetics industry, glycerine must be first purified, which is an expensive and demanding process. Therefore, the biodiesel producers must seek for alternative methods of its disposal and valorization.

Yarrowia lipolytica is a non-conventional yeast species of great industrial interest, known to grow equally well on glycerol as on glucose [4]. Many studies have demonstrated its great potential in valorization of crude glycerol to high value-added products, like citric acid (CA) [5] or erythritol (ERY) [6]. *Y. lipolytica* has been also proved to efficiently produce and secrete heterologous proteins [7]. While the production of proteins for therapy and diagnostics must meet specific rigorous criteria, (thus, usage of waste streams as feedstocks in such processes is limited), bulk enzymatic preparations, used for example in bioenergy, paper and pulp processing, leather processing and agriculture, are subjected to less strict regulations. From amongst several key types of enzymes, carbohydrate hydrolases account for the largest market share. Indeed, valorization of waste streams into enzymatic preparations of relatively high market price (when compared to ethanol, CA or lactic acid etc.) is an attractive option, calling for a potent bifunctional biocatalyst. Having the two characteristics (efficient growth in crude glycerol and high level production of enzymes) *Y. lipolytica* holds a promise for meeting this bifunctionality requirement.

Enhanced production of heterologous proteins in microbial host can be achieved by different approaches*, i.a.* by manipulation with environmental factors during the production process. Factors having confirmed, significant impact on heterologous protein synthesis in *Y. lipolytica* comprise *i.a.* temperature [8], pH [9] and osmolarity [10] of the culture medium, or dissolved oxygen concentration [11]. The adverse effects of the external stress factors on the target biomolecule production are commonly known, however, increasing evidence suggests that intentional exposure to stress conditions at pre-specified intensity and period may enhance its production. Such controlled administration of the stress globally fine-tunes numerous molecular events, including general stress response and stress-specific signaling

* Corresponding author.
E-mail address: ewelina.celinska@up.poznan.pl (E. Celińska).
[1] ORCID: 0000–0001–8372–8459.

https://doi.org/10.1016/j.btre.2021.e00648
Received 11 April 2021; Received in revised form 14 June 2021; Accepted 15 June 2021
Available online 19 June 2021
2215-017X/© 2021 The Author(s). Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

pathways, to preserve integrity and functionality of the cell [12]. Typically, stress response activation is hallmarked by massive alternations in the cell physiology. Under heat stress, over 850 genes were found transcriptionally responsive in *S. cerevisiae* [13], with heat-shock protein (HSPs)-encoding genes constituting a large group of the up-regulated genes. HSPs represent molecular chaperones associated with protein folding, stabilization and degradation. Such stress-induced overrepresentation of HSPs can be "hijacked" by the ongoing, synthetically forced overproduction of heterologous protein [14,15]. It was shown that induction of a heat shock response in *S. cerevisiae* by continuous overexpression of the major heat-shock transcription factor (*HSF1* gene) enhanced yield of endogenous invertase [14].

Growth and protein production conditions for *Y. lipolytica* correspond to typical optima for the other yeast species. Due to its high resistance to different environmental stress factors, *i.a.* hyperosmotic conditions (up to 12.5% of NaCl) or very low pH (2.0–3.0) [16], *Y. lipolytica* is presumed to bear efficient stress response mechanisms. Studies on its adaptive response to heat shock demonstrated that *Y. lipolytica*, when pre-adapted to the following shock stage, is able to survive even 30 min at 45 °C (60% of the population survived) [17]. Controlled implementation of environmental stress factors was previously shown advantageous in production of several biomolecules by *Y. lipolytica*. For example, oxygen limitation exerted positive effect on production of CA [18] and succinic acid [19]. Likewise, increased osmolarity of the culture medium is commonly known to promote production of ERY [20,21]. Correspondingly, the osmotic stress, stimulated by the addition of different osmoactive compounds, increased synthesis of heterologous secretory invertase [22] and glucoamylase [10]. Controlled manipulation with the culturing temperature (elevated or decreased) was also shown to promote production of several valuable biomolecules by *Y. lipolytica*, including alpha-linolenic acid [23], CA [24], or native lipase [25].

In this study, we used a recombinant *Y. lipolytica* strain overproducing a heterologous raw starch digesting alpha-amylase (SoA) and subjected it to optimization of thermal treatment conditions in a glycerol-based medium, with the aim to develop a bioprocess of crude glycerol valorization. We adopted statistical design of experiments (DoE) approach to systematically optimize the thermal treatment conditions to maximize the heterologous protein production. We conducted a series of pre-designed batch bioreactor cultivations implementing periods of exposure to various temperatures (20–42 °C) applied for 5 to 300 min. Further statistical analysis was conducted using Response Surface Methodology (RSM), which, based on mathematical methods and statistical inference, allows explaining combined effects of all the factors covered by the design (here time and temperature). To get a deeper insight into the background behind the observed improvement, we analyzed the heterologous gene's expression under the treatment conditions. Finally, the process was conducted using industrial, technical substrates and crude glycerol derived from biodiesel production plant. Market-relevant economic gain, due to exploitation of the technical substrates, was supported by simulations of the process on a pilot scale and specific calculations. To the best of our knowledge, this is the first research into the effects of thermal treatment conditions on heterologous enzyme production by *Y. lipolytica*, which was further developed into a bioprocess of crude glycerol valorization to enzymatic preparation.

Methods

Recombinant strain and development of inoculum

Yarrowia lipolytica recombinant strain GGY237 bearing a heterologous gene encoding alpha-amylase SoA (*Sitophilus oryzae* alpha-amylase; GenBank: KP027641.1) was used in this study [26]. This Po1h-derived strain (MatA, ura3–302, xpr2–322, axp1–2) was constructed using Golden Gate modular cloning strategy, as described previously [27]. *SoA* gene was transcriptionally fused to a signal peptide of YALI0B03564g gene for efficient secretion of the protein [28] and flanked with 4UASpTEF promoter and tLip2 terminator.

Precultures of *Y. lipolytica* GGY237 were developed from 15% glycerol stocks stored at −80 °C, prior to each culture run. The biomass was spread on YPD agar medium containing (g L^{-1}): yeast extract (YE; Merck, USA), 10; bactopeptone (BP; Merck, USA), 20; glucose (POCH, Poland), 20; agar (BIOCORP, Poland), 20. Subsequently, 24-h biomass was transferred into 1-L Erlenmeyer flasks containing 100 ml of YPG_{20} medium and incubated at 30 °C with 250 rpm agitation in a rotary shaker (BIOSAN, Latvia) for 23 h.

Batch culture and thermal treatment conditions

Bioreactor cultivations were carried out in BIOSTAT® B plus (Sartorius Stedim Biotech GmbH; Germany) stirred tank bioreactors, with a total volume of 5 L and culture medium volume of 1 L, in batch mode. The culture medium YPG (labYPG), used for optimization plan cultivations, was composed as follows (g L^{-1}): YE, 10 (Merck, USA); BP, 20 (Merck, USA); glycerol, 120 (POCH, Poland). Starting glycerol concentration was chosen based on preliminary experiments (Additional file 1: Figure S1). Glycerol used in labYPG medium was of laboratory grade. For cultivations conducted in low-cost technical culture medium (techYPG), the proportions of basic YPG medium components remained unchanged, but the ingredients were exchanged to cheaper counterparts. YE was purchased from Kaczmarek komponenty (Poland). The substrate is typically used in food industry as a food additive and a natural flavoring agent. BP was purchased from BTL (Poland). Crude glycerol was obtained from ORLEN Południe (Poland). The crude glycerol used in this medium was a by-product from biodiesel production process. It contained 86% of pure glycerol (w^{-1}), as determined by HPLC analysis. Amount of the crude glycerol added to the technical culture medium was adjusted to preserve 120 g L^{-1} of glycerol content in the final medium.

The main cultures were inoculated at 10% (v^{-1}) with the preculture. pH was adjusted to 5.5 by automatic addition of basic and acidic regulators (30–40% NaOH and 10% H_2SO_4). The temperature was maintained at 28 °C throughout the culture except for the time of the thermal treatment. Stirring and aeration were automatically adjusted to 20% oxygen saturation (2 vvm was applied). To minimize foam formation, Antifoam 204 (Sigma-Aldrich) was used. The thermal stress period was executed at 20 h of culturing. At this time-point, the culture was at the end of exponential – early stationary phase of growth, which was established based on growth and glycerol consumption kinetics (Additional file 1: Figure S1). Thermal treatments were executed by pumping the cultures from a bioreactor vessel using a peristaltic pump (Watson-Marlow 323) to a sterile 2-L bottle placed in a water bath with pre-set temperature (according to Table 1), stirring and aeration (250 rpm, 2 vvm). The cultures were continued until full glycerol consumption.

Table 1
Adopted experimental design for the thermal treatments executed in this study.

Run number	Factor 1 A:Temp[°C]	Factor 2 B:Time[min]
1	31	152.5
2	31	152.5
3	31	152.5
4	42	300
5	42	5
6	31	300
7	20	300
8	31	5
9	31	152.5
10	20	5
11	31	152.5
12	42	152.5
13	20	152.5

Samples were collected throughout the culturing, centrifuged (13,400 rpm, 3 min; Eppendorf MiniSpin), and subsequently, the biomass and the supernatant were stored separately at −20 °C. All the results were analyzed in two technical replicates. The samples were analyzed for: biomass accumulation, glycerol consumption, synthesis of metabolites and the target protein.

Experimental design

Design of experiments (DoE) and the following analyses were conducted using Design-Expert 11 software (Stat-Ease, USA). A single-step optimization strategy was employed to optimize the amylase production under different thermal treatments. Significant variables were selected based on the literature data, demonstrating that both temperature [17,23] and time of the treatment [29] are important variables in terms of the stress response induction. Ranges of the variables (20–42 °C and 5–300 min, respectively) were chosen based on previous findings [20]. Importantly the adopted temperature range could not affect activity of the target enzyme itself, as shown by previous findings on biochemical properties of the amylase (max. activity at 40–45 °C) [30]. Next, DoE and RSM were used to optimize the levels of the significant variables. A three level face centered (−1, 0, 1) two-factor central composite design was used to enable statistical analysis of possible interactions between the components. The experimental design and the variables levels are shown in Table 1. Exposure to 31 °C, over respective time periods, was considered a control variant. Five replications represented the center point; four of them were axial and factorial points, altogether giving 13 individual experiments. Specific activity [AU $gDCW^{-1}$] (DCW – dry cellular weight) was used as the primary experimental response. The responses were used to develop experimental model of the response surface, in which each dependent variable was shown as a sum of contributions. For the two-factor design the model equation is as follows:

$$y = b_0 + b_1A + b_2B + b_{12}AB + b_{11}A^2 + b_{22}B^2 \quad (1)$$

where y: predicted response; b_0: intercept; b_1, b_2: linear coefficients; b_{22}: quadratic coefficients, and b_{12}: interaction coefficient.

Additionally, concentration of ERY, mannitol (MAN), CA, α-ketoglutaric acid (αKGA) and DCW [g L^{-1}] was analyzed interdependently. The cultures were conducted in labYPG medium.

Pilot scale processes simulations and calculation of associated costs

Based on experimental data obtained from the bioreactor cultivations conducted on laboratory and technical substrates, two simulations of the alpha-amylase production processes at pilot scale were prepared. The first process model (labYPG) was developed based on the results of cultivations in labYPG medium composed of laboratory reagents – laboratory grade glycerol and high quality laboratory substrates (YE, BP; Merck, USA). The second variant of a process model, techYPG, was developed using data from bioreactor cultivations conducted in techYPG medium composed of technical reagents and crude glycerol – a by-product of biodiesel production. The substrate prices are all based on the providers' quotes, accounting for annual demand for the substrate; thus the estimations reflect actual market conditions. The amount of the heterologous enzyme in grams was calculated using previously determined relationship of AU and mg of the purified SoA protein [30], and direct comparison of different enzymatic assays for the data recalculation [31]. Data from the end-points of the bioreactor batch cultivations in labYPG and techYPG were used for the processes simulations.

All the simulations were conducted using SuperProDesigner software v10 (Intelligen, USA). The key presumptions were as follows: i) funding of the process comes exclusively from private investment and no loans were taken into account, ii) the final production stage will be carried out at 10 m^3, iii) the final product (amylolytic preparation) will be formulated as a liquid concentrate, obtained after diafiltration (DF), iv) the water after DF will be recirculated for media preparation, v) yeast biomass after separation (P-13, DC-101) will be dried (P12, DDR-101) and sold for fodder purposes, vi) annual operating time will be 7920 h (330 days). The overall batch time was 186.57 h, but the cycle time could be shortened to 80.82 h due to incorporation of an additional vessel for post-culturing liquid storage (P-9, V-101). Therefore, the number of complete batches per year raised to 96.

Conducted simulations rendered detailed data on capital investment costs associated with the pilot installation construction, operating costs including costs of labor, consumables, and utilities (power, steam, chilled water, compressed air). Costs of the latter were estimated based on data from large industrial plants operating in the country, exploiting these media in production. Equipment purchase costs were estimated using SuperPro Designer v10 database, except for fermenters and blending tanks, for which the prices were obtained from local suppliers.

All the prices were expressed in US dollars ($ = USD).

Statistical analysis

Statistical importance of the differences between compared sets of data was analyzed using one-way analysis of variance (ANOVA) and Tukey's multiple comparison tests. Distributional assumptions for applying ANOVA analyses were assessed by the Shapiro-Wilk test, while homogeneity of variances between the subjects was assessed using Levene's tests. Statistical analyses were performed with the STATISTICA data analysis software system (StatSoft, Inc., Tulsa, OK, USA). The results were considered to be statistically different at a p-value of 0.05 or less. The results were expressed as mean ± standard deviation (±SD) of the replicates, as indicated for a specific analysis.

Analytical methods

Amylolytic activity assay

The extracellular alpha-amylase (SoA) activity was analyzed according to a microSIT protocol described previously [31]. One activity unit (AU) corresponds to the amount of an enzyme that contributes to decrease in starch-iodine staining value equivalent to 1 mg of starch per 1 mL, during 1 min at pH 5.0 and 40 °C, under applied experimental conditions.

Viability of the cells upon thermal treatment and light microscopy

Viability of *Y. lipolytica* cells upon the applied thermal treatment was tracked by decimal dilutions of samples withdrawn from the cultures and surface spreading on solid YPD medium. The colonies were counted after 48 h incubation at 30 °C. The analyses were conducted in biological duplicate and technical triplicate. The results were expressed as percentage values of cfu mL^{-1} counted for samples subjected to thermal treatment (20 °C and 42 °C) vs samples from control conditions (31 °C) over specified time. Additionally, the withdrawn biomass was stained with crystal violet and observed under 1000x magnification using light microscope (Primo Star, ZEISS; Oberkochen, Germany) to visualize the cells' morphology.

Concentration of chemical compounds and biomass in the cultures

Concentration of metabolites including ERY, MAN, CA, αKGA and the carbon source (glycerol) in the culturing medium was measured by HPLC. Collected samples were defrosted and passed through 0.45 μm membrane syringe filters (Millipore). The chromatograph (Elite LaChrom, VWR-Hitachi) was equipped with two detectors (RI L-2490 and UV L-2400) and a Rezex ROA 300×7.8 mm column (Phenomenex). The analyses were conducted at 40 °C, under isocratic conditions, with a flow rate of 0.6 ml min^{-1}; 10 mM H_2SO_4 was used as the mobile phase. Standard solutions of the chemicals were all purchased from Sigma Aldrich Co. The compounds in the samples were quantitatively analyzed in reference to the standard solutions (peak area) using EZChrom Elite

(Agilent) software. Biomass accumulation was assessed using a standard gravimetric method. Defrosted cellular pellets were washed in distilled water and dried at 105 °C for 48 h.

Gene expression analysis through RTqPCR

Total RNA isolation, reverse transcription (RT) reaction and real-time quantitative PCR (RTqPCR) were conducted as described previously [32]. Briefly, total RNA was extracted from *Y. lipolytica* biomass using Bead-Beat Total RNA Mini kit (A&A Biotechnology, Gdynia, Poland) and MixerMill MM400 (Retsch GmbH, Haan, Germany) according to the manufacturer's protocol. The first cDNA strand synthesis was conducted using TranScriba Kit (A&A Biotechnology, Gdynia, Poland) following the manufacturer's instruction in a Veriti Thermal Cycler (Applied Biosystems, Waltham, MA, USA). RTqPCR was conducted using SYBR®Green B PCR MasterMix (A&A Biotechnology, Gdynia, Poland) in a 7500 Real-time PCR Thermalcycler (Applied Biosystems, Waltham, MA, USA). The reaction mix was composed as indicated by the MasterMix manufacturer. The heterologous gene encoding raw starch digesting amylase SoAMY was targeted using the following primers: r-t_SoA_F: GTAACAACGTGGGAATCCGAAT, r-t_SoA_R: CCCTGGCCGTTCGAAGTAG. Ninety six-well plate (4titude, Wotton, UK) with black spacers and opaque wells were used to limit interference with adjacent wells. The results were processed according to a ΔΔCt method [33]. Sec*62* gene was used as internal calibrator based on its previously demonstrated suitability [32]. The internal reference gene was targeted using the following primers: r-t_Sec62_F: CTACCTGCGGAGCCACAAG, r-t_Sec62_R: TCGGATCGCTTACCGTTGAG. Gene expression under thermal treatment (20 °C and 42 °C) was normalized vs incubation at 31 °C over specified time.

Results and discussion

Effect of the thermal treatment on Yarrowia lipolytica cultures performance

The initial course of the cultures (prior to the thermal treatment) was corresponding in all the thirteen experiments (Fig. 1). At this stage, rapid consumption of glycerol (at an average rate of 2.94 g L^{-1} h^{-1}) was accompanied by a sharp increase in the extracellular activity of the target protein (up to 561.14±86.67 at 4 h, and 734.73±80.5 at 20 h AU L^{-1}±SD). At 20 h of culturing, the biomass concentration reached on average 24.29±4.12 gDCW L^{-1}±SD. From amongst metabolites typical for *Y. lipolytica*, ERY predominated, reaching 7.58±3.13 g L^{-1}±SD, followed by MAN (5.58±1.33 g L^{-1}±SD) and organic acids (approx. 2.6 g L^{-1} for aKGA and CA). Comparable results were observed previously in the initial batch stage of recombinant *Y. lipolytica* cultures in a similar medium [10,26]. In the previous studies, depending on the strain and starting composition of culturing medium, GLY was consumed at 1.4 to 3.9 g L^{-1} h^{-1} rate, and the biomass concentration reached ~ 28 to 34 gDCW L^{-1}. The profile of metabolites was dominated by ERY (~ 13 g L^{-1}) in [34], while in [10], CA was the primary metabolite, followed by smaller amounts of the polyols. Altogether, during the initial 20 h, the cultivations proceeded typically and comparably with the previous reports.

The executed thermal treatment caused significant differentiation in the culture course between the thirteen culture variants (Fig. 1).

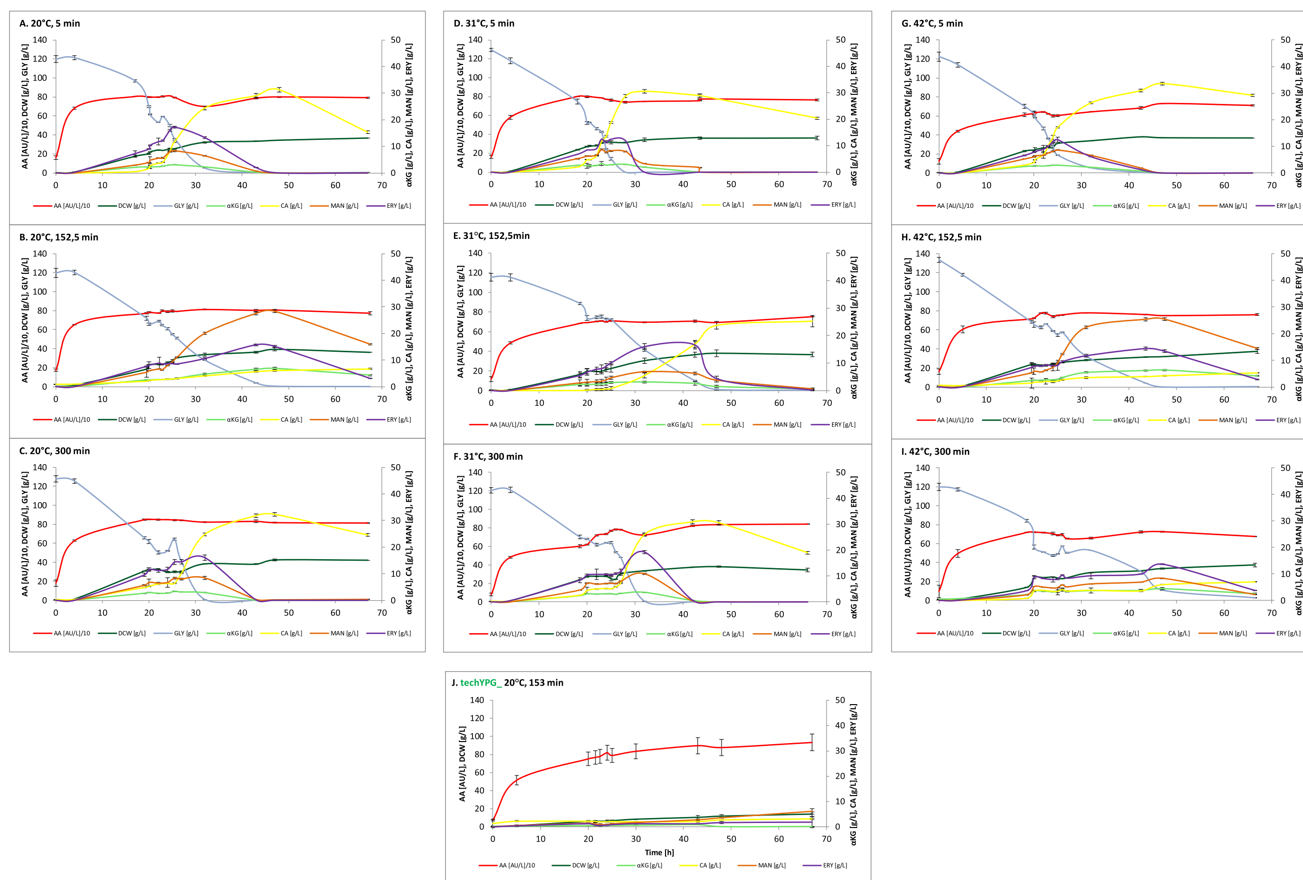

Fig. 1. Course of *Y. lipolytica* GGY237 cultures implementing different thermal treatments. The treatment conditions (temperature [°C] and time [min]) are indicated in a respective scheme's heading. Y axis concentration [g L-1] of biomass (DCW), glycerol (GLY) or metabolite (CA, MAN, ERY, αKG), amount of the target enzyme activity (AA; the activity amount values were given in 10-fold lowered values to improve clarity of the schemes). X axis: time in [h]. Cultures A – I were conducted in labYPG. Culture J was conducted in techYPG.

Depending on the experimental variant, the culture reached its maximum rate at 42 to 48 h. After that time, the concentration of metabolites and oxygen demand started to decline, which was a direct result of the main carbon source depletion. The executed thermal treatments were within the survival range of *Y. lipolytica*, as after the treatment the cells could further produce/utilize metabolites until the end of the culturing time. *Y. lipolytica* is typically cultured within the range of 28–30 °C [35], and its optimal growth temperature is described as "below 32–34 °C". However, several studies reported a wider range of the tolerated growth temperatures (from 5 °C up to even 37 °C) which was indicated as a strongly strain-dependent trait [36]. Survival rate after thermal-treatment was also dependent on the growth phase of the culture, which was studied in detail previously [17]. As found there, exponentially growing cells were more susceptible to heat-shock at 45 °C, which decreased the cells population by 96% and 99.9% after exposure for 5 and 10 min, respectively. Stationary phase cells, were more resistant, as 30 min exposure to 45 °C decreased the population's viability by only 40% [17]. In the present study, the cells were subjected to the thermal treatment at the end of exponential/early stationary phase of growth. The moment at which the treatment was executed most probably contributed to the differences between our results and the previously reported data. Expectedly, the most severe impairment in the metabolic activity and viability of the cells was observed in the variant subjected to treatment at 42 °C for 300 min (Fig. 1.I; Fig. 2). Following the heat shock, the amount of viable cells decreased to 2.48%. This was the only case where glycerol was not fully consumed at the end of culture (10.93 and 2.75 g L^{-1} at 48 h and 67 h, respectively). Interestingly, upon incubation at 20 °C the cells exhibited higher viability (and growth, as discussed in the following section) than in the control conditions (31 °C) (Fig. 2). Obviously, the *Y. lipolytica* strain used in this study possess an innate trait to propagate and maintain high metabolic activity at decreased temperatures, which, as stated previously [25,36], is a strain-dependent quality.

Effect of the thermal treatment on the biomass accumulation and metabolites production

Considering DCW evolution in the thirteen culture variants, no straightforward effect of the treatment temperature on the biomass accumulation was observed (Table 2, Table 3 – lack of A term significance in DCW model). As shown in (Fig. 1), even upon prolonged exposure to 42 °C, which severely affected the viability of the cells (Fig. 2), the biomass concentration continued to increase steadily until the end of the cultures. Probably, after the exposure to stress, a small population (~ 2%) of the surviving cells started to populate the empty niche *de novo*. On the other hand, short thermal shock (42 °C for 5 min) did not impact biomass accumulation vs the control variant of 5 min at 31 °C (Table 2). Surprisingly, no growth cessation was observed in the culture subjected to 20 °C for 300 min. In fact, upon prolonged incubation at 20 °C, the biomass concentration was increased by 20% vs 31 °C, and by 27.5% vs 42 °C. This observation is consistent with the viable cell counts discussed above (Fig. 2). Correspondingly, when WT *Y. lipolytica* strain was cultured at temperatures ranging from 24 to 34 °C, biomass accumulation increased with decreasing temperature (at any pH 3.0 – 6.0) [25]. Similarly, Li et al. [15] observed higher biomass accumulation at 23 °C than at 30 °C. It could be speculated that since decreased temperature induces formation of filaments [37], the overall biomass of a specific number of cells contained in a volume of liquid culture increases. Our microscopic observations of the cells subjected to the thermal treatments, however, did not give any straightforward indication of the occurrence of this phenomenon, and more investigation into the issue is required (Additional file 2: Figure S2).

The best model describing the relationship between the biomass production and the adopted thermal treatments was a reduced quadratic model presented in Table 3. Considering the terms in the model equation, only the time (B, B^2) and the interaction with temperature (AB) had a significant contribution, while sole temperature (A) was not a significant term. This lack of significance of temperature contrasts previously published data [25]. This discrepancy, however, might have been a result of methodological differences in the approach to thermal treatment. In the present study, the thermal treatment period lasted maximally 5 h (per 68-h culture) and the optimal thermal conditions were reestablished afterwards, while in those cited studies, the cultivation was actually conducted at decreased temperatures throughout its duration. Additionally, conducted correlation analysis showed that biomass formation was strongly correlated with the production of CA ($r = 0.74$), while the production of AU was decoupled from biomass propagation ($r = -0.17$ to $r = 0.05$). The relationship between CA and DCW formation was significant, and lasted throughout the culture. The biological sense of this observation seems to be straightforward, as CA is the major product of the TCA cycle (fed by glycerol or lipids re-utilization), strongly enhanced by propagating cells under aerobic conditions [38]. In contrast, a weak positive correlation between production of polyols (ERY $r = 0.57$, MAN $r = 0.46$) observed at the 25 h of culturing, was inverted to a negative correlation at the terminal point of the cultures (42 h; ERY $r = -0.33$; MAN $r = -0.77$). Such a switch in the type of the DCW-polyols relationship results from the different roles of ERY and MAN at different stages of the cultures – from products at the proximal points to substrates at distal stages [38,39].

Among the metabolites synthesized by *Y. lipolytica* in the analyzed

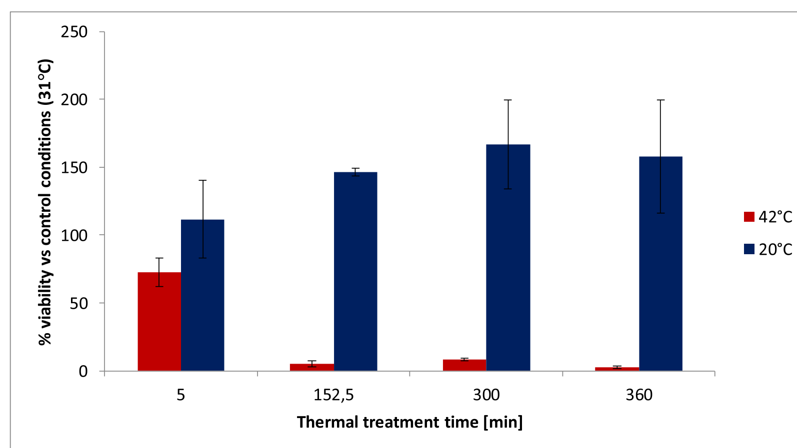

Fig. 2. Viability of *Y. lipolytica* GGY237 strain followed by thermal treatments. The results were expressed as percentage values of cfu mL^{-1} counted for samples subjected to thermal treatment (20 °C and 42 °C) vs samples from control conditions (31 °C) over specified time. Condition 360 min denotes 300 min of thermal treatment and 60 min of recovery in 28 °C.

Table 2
Experimental responses for the main parameters following different thermal treatments in labYPG and techYPG medium.

Medium		Variant	SoA Amount [AU L^{-1}] ± SD	SoA Specific productivity [AU gDCW^{-1} h^{-1}] ± SD	DCW [g L^{-1}] ± SD	CA [g L^{-1}]	ERY [g L^{-1}]	MAN [g L^{-1}]
labYPG	A	20 °C, 5 min	791.70 ± 6.05	5.63 ± 0.08	25.55 ± 0.21	11.77	17.26	8.34
	B	20 °C, 152.5 min	**790.26 ± 14.69** **802.67 ± 4.91** (43 h) **772.75 ± 16.23** (end)*	8.13 ± 0.16	24.43 ± 0.18	2.81	8.65	9.34
	C	20 °C, 300 min	839.158 ± 13.99	5.60 ± 0.15	29.98 ± 0.88	23.12	14.40	8.35
	D	31 °C, 5 min	754.63 ± 20.29	4.76 ± 0.31	31.75 ± 1.56	18.77	12.48	8.14
	E	31 °C, 152.5 min	704.98 ± 7.05	6.44 ± 0.34	22.45 ± 1.64	1.13	8.65	4.64
	F	31 °C, 300 min	769.64 ± 8.03	6.18 ± 0.38	25.00 ± 2.19	21.74	12.05	7.29
	G	42 °C, 5 min	598.78 ± 14.36	3.87 ± 0.09	31.15 ± 0.00	17.16	12.51	8.61
	H	42 °C, 152.5 min	759.43 ± 14.31	6.22 ± 0.14	24.43 ± 0.18	2.24	8.13	9.07
	I	42 °C, 300 min	688.50 ± 6.84	5.84 ± 0.08	23.58 ± 0.11	3.42	8.08	4.87
techYPG	**J**	20 °C, 153 min	**790.18 ± 65.35** **898.89 ± 10.32** (43 h) **933.89 ± 7.82** (end)*	23.24 ± 0.80	6.81 ± 0.80	2.12	0.89	1.22

Culture in techYPG (variant J) was conducted only for the optimized thermal treatment condition (B). All the values represent means from at least two replicates. For HPLC data ±SD was <10%.
* Data used for the process model development in SuperProDesigner.

Table 3
Models and ANOVA analysis for parameters studied under thermal treatments.

Response	Model	Significant components	F-value	R2 coefficient
DCW	DCW [g/L] = 21.97 + 0.6958A - 1.65B - 3.00AB + 5.86B^2	B, AB, B^2	44.33	0.8048
CA	CA [g/L] Sqrt(2 + 0.03) = 1.05 - 0.4037A - 0.0971B - 0.9166AB + 2.83B^2	AB, B^2	53.39	0.9003
ERY	ERY [g/L] = 8.58 - 1.93A −1.29B + 4.22B^2	A, B^2	7.87	0.7239
MAN	MAN [g/L] = +5.52 - 0.5801A + 2.58A^2	A^2	3.95	0.4411
SoA Specific productivity	**Final Equation in Terms of Coded Factors** [AU/gDCW*xh*]) ^−1.33 = 0.0798 + 0.0143A −0.0177B - 0.0177AB + 0.0332B^2 **Final Equation in Terms of Actual Factors** [AU/gDCW*xh*]) ^−1.33 = 0.041606 + 0.002966 Temp. - 0.000247 Time −0.000011 Temp.* Time + 1.52572E-06 Time^2	A, B, AB, B^2	83.77	0.8816

* A – temperature, B – time, AB – interaction, B^2 / A^2 – quadratic term; Actual factors are given in °C (Temp.) and min (Time).

cultures, CA was the primary product, reaching a peak level of approx. 30 g L^{-1} in the majority of studied variants (Fig. 1). According to statistical analysis, CA synthesis relied on the time of treatment rather than on the temperature (see the model shown in Table 3 and details of statistical analysis in Additional file 3: Table S1). Prolonged thermal treatment (>152.5 min) at 42 °C caused a severe decrease in CA synthesis, which was not recovered until the end of the cultures (Fig. 1.H.I). In those cases (and in 152.5 min at 20 °C), CA concentration was close to 4 g L^{-1} throughout the cultures. Previous studies on the impact of cultivation temperature (within the range 20–35 °C) on CA production by *Y. lipolytica* unambiguously indicated that CA synthesis is the highest at temperatures close to 30 °C [40,41]. However, in those studies the indicated temperature was implemented throughout cultivation of the strains, and time of the treatment was not an investigated variable. In this way, those previous reports and our current findings (time of the treatment has a higher impact on CA amounts than the temperature) do not remain in opposition. Interestingly, exposure of the culture to the thermal treatment (either 20 °C or 42 °C) was followed by a very low production of CA and a very high production of polyols. In those two specific cases, the concentration of ERY reached on average 14 g L^{-1}, but more interestingly, the amount of MAN was nearly two-fold higher (approx. 27 g L^{-1}) (Fig. 1.B.H). In all the remaining variants studied here, ERY concentrations were higher than those of MAN. Such high predomination of MAN over ERY is not typical for the majority of *Y. lipolytica* cultures, but was observed in several specific cases [21,26]. High level production of polyols is a known marker of stress response in yeast. *Y. lipolytica* responds with enhanced synthesis of ERY and MAN to elevated osmotic pressure [20,42–44]. This phenomenon is employed to promote production of ERY [21]. Moreover, exposure to oxidative stress [39] or decreased pH [6] were shown to stimulate polyols synthesis by *Y. lipolytica*. Typically, ERY synthesis dominates at pH close to 3.0, but when the pH raises >4.5, CA becomes the main metabolite [6]. In the present study, pH was stably maintained at 5.5, suggesting that CA should predominate. So it can be speculated that predominance of polyols is a marker of physiological stress (42 °C for 152.5 and 300 min, 20 °C for 152.5 min). In high relevance to the stress factor studied here, Hackenschmidt et al. [45] showed that *Y. lipolytica* specifically responded with elevated production of MAN when the cultivation temperature was increased from 28 to 35 °C. A corresponding observation was made for a related species, *Candida magnoliae*, which synthesized MAN at an elevated rate under temperatures >37 °C (studied range 28–48 °C; [46]). Based on those previous studies and our current results it could be speculated that MAN is a stress response molecule, overproduced by *Y. lipolytica* encountering specific stress conditions. Likewise, our previous study demonstrated that the production of a large and complex protein triggered cellular stress response, accompanied by elevated MAN formation (1.75-fold higher MAN than ERY; [26]). On the other hand, it is not yet clear why this phenomenon was observed in the variant treated at 20 °C for 152.5 min, and not in the one at the same temperature but with a longer treatment time (300 min). Definitely, this issue requires further in-depth studies.

Effect of the thermal treatment on the target protein production and heterologous gene expression

As summarized in Table 2, the highest enzyme amounts were recorded in cultures exposed to 20 °C, which stays valid for all the tested incubation periods. Interestingly, only in these cultures a clear, positive relationship between the time of the treatment and the enzyme amount was observed. Prolonged incubation at 20 °C (up to 300 min) allowed to increase the enzyme amount by approx. 48 AU L^{-1} (vs the 5 min variant). Although the DCW L^{-1} parameter was also increased under these conditions, no direct straightforward correlation between the biomass accumulation and the SoA protein production was observed (as mentioned above). Similar conclusion on the positive impact of prolonged incubation at reduced temperature on protein production was drawn in a study on native lipase synthesis by *Y. lipolytica* temporarily exposed to 24 °C [25]. In contrast, no such correlation could be seen for the other two thermal treatments studied here (31 and 42 °C). Under high-temperature incubation (at 42 °C) the enzyme amount increased along with the treatment time extending from 5 min to 152 min (by approx. 76 AU L^{-1}), but further prolongation to 300 min caused a decrease in this parameter (by ~ 40 AU L^{-1} *vs* the 152.5 min variant). These observations were reflected in volumetric productivity values (Table 2). Most probably, high mortality of the cells upon prolonged incubation at 42 °C was the reason for these observations (Fig. 2). Adverse effects of prolonged incubation at elevated temperature (34 °C) on the native lipase production by WT *Y. lipolytica* were previously reported [25].

The positive impact of decreased temperature on proteins production by different yeast species was also reported previously. The amount of the native lipase was 2-fold increased along with decreasing the temperature by 10 °C (34 to 24 °C) [25]. Furthermore, about 10-fold increase in the amount of a recombinant protein was reached in *K. phaffii* cultures conducted at 23 °C vs 30 °C [15]. As experimentally revealed, enhanced protein folding capacity and prolonged viability of the cells under lower temperature underlie the increased production of the proteins. Likewise, over 2-fold improvement in a chimeric protein amount was observed in *K. phaffii* cultures when the temperature was decreased from 30 °C to 22 °C [47]. On the other hand, Raimondi et al. [8] showed that the optimal temperature of the production process was protein-dependent in *Kluyveromyces marxianus*, and, while a native enzyme was produced in abundance at 30 °C, a recombinant enzyme was more efficiently produced at elevated temperature (40 °C; tested range from 5 to 40 °C).

To get an insight into the molecular bases behind observed changes in the amylase synthesis, we analyzed the expression level of the heterologous gene (Fig. 3). Expectedly, upon prolonged incubation (≥ 152.5 min) at 42 °C the expression was significantly decreased ($p<0.05$) when compared to 31 °C and 20 °C variants. On the other hand, a brief exposure to the high temperature led to a slightly elevated expression of the heterologous gene. As can be further inferred from Fig. 3, incubation at decreased temperature enhanced the expression of the gene, which was positively correlated with time of the treatment. It is presumed that the artificially forced transcription of the heterologous gene could be continued for a longer time under the decreased temperature, while it started to decline under the control (and the elevated) temperatures. The promoter governing expression of the heterologous gene is phase-dependent and most active in the stationary phase of growth; which could at least partly explain this observation and support presumptions. Importantly, the observed transcription pattern of the heterologous gene under thermal treatment well explained the observed variation in the extracellular activity of the heterologous enzyme.

The best model for maximizing the heterologous protein production was a reduced quadratic model (Table 3; for details on statistical analysis please go to accompanying Additional file 3: Table S1). Importantly, all the terms were shown to significantly contribute to the enzyme's productivity ($p<0.0001$ for all, A, B, AB, B^2). The developed model was characterized by high significance ($p<0.0001$) and accurate fit, so could be used to navigate the design space. Based on the model indications, the predicted optimum temperature and time of thermal treatment maximizing the target protein productivity were: temperature 20 °C, time of thermal treatment: 153.494 min, which was predicted to yield 9.46 [AU gDCW^{-1} h^{-1}]. Corzo and Revah [25] found 29.5 °C as the temperature maximizing production of native lipase by *Y. lipolytica*. However, in those previous studies, the Authors actually cultured the yeast under specified thermal conditions (24, 30 and 34 °C for 48 h or 60 h) rather than executed a thermal treatment period, as in the present study. In that former report, it was found that the optimal temperature for *Y. lipolytica* growth, was also the best for the native enzyme production. In the current research, we focused more on studying the impact of stress factor-induced response on production of heterologous enzyme by *Y. lipolytica*.

Production of the enzyme under optimized thermal treatment conditions in crude glycerol-based medium

In the final stage of this research, we assessed the industrial relevance of knowledge gained from the optimization plan and the process modeling. Therefore the optimized treatment conditions (20 °C, 153

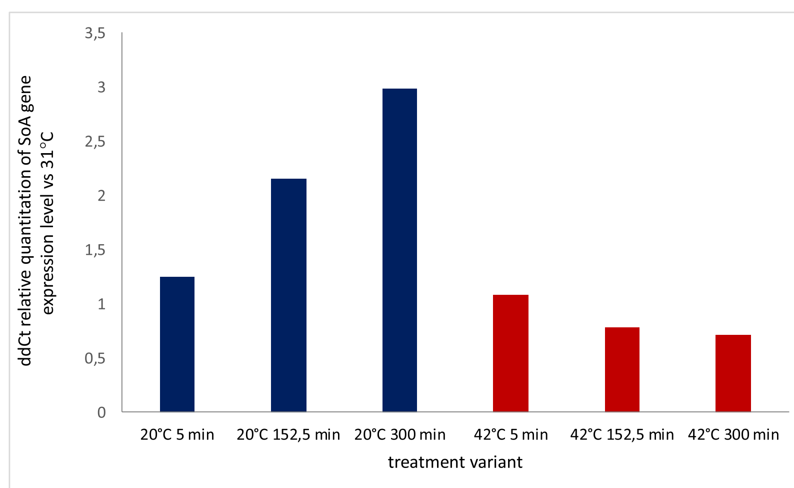

Fig. 3. Heterologous gene expression in *Y. lipolytica* GGY237 strain followed by thermal treatments. The results were expressed as relative quantitation values (ddCt) for samples subjected to thermal treatment (20 °C and 42 °C) vs samples from control conditions (31 °C) over specified time.

min) were implemented in a production process. This process was conducted with crude glycerol obtained from a biodiesel production plant and technical substrates (BP, YE; medium techYPG). Comparison of the processes conducted with the laboratory reagents (labYPG) and the technical substrates (techYPG) is presented in Table 2 (variants B and J) and Fig. 1.B.J. Details on process parameters and statistical analysis are given in accompanying Additional file 4: Table S2. To our surprise, the process conducted in techYPG was either equally efficient or slightly better in terms of the enzyme production, depending on the time point considered. Until 32 h of culturing, SoA amount and volumetric productivity were not significantly different for the two compared processes (at $p<0.05$). In the following time-point (43 h), all the parameters, SoA amount, volumetric productivity and specific productivity were higher for the process conducted in techYPG ($p<0.005$). At that time-point, the biomass in techYPG was 3-fold lower than in labYPG (Fig. 1.B.J). Also, all the main metabolites, typical for *Y. lipolytica* (ERY, MAN, CA) were produced at marginal level in techYPG when compared to labYPG (Fig. 1.B.J). The reason for such unexpected beneficial impact of the technical substrates on the enzyme production, at the expense of biomass and metabolites, is not currently known. Further investigation into this issue is definitely required and should be focused on determination of elementary composition of all the substrates used, and their individual impact on heterologous enzymes production in *Y. lipolytica*.

Pilot scale process simulation and costs estimation

To quantitatively express the economic gain due to exploitation of technical substrates, and to identify current bottlenecks of the process, we developed two pilot scale processes of the enzyme production using our experimental data from the processes run in labYPG and techYPG. A common flowsheet for a complete process is depicted in Fig. 4. and complementary calculations concerning the input materials consumption and costs, overall mass balance analysis, and summary of equipment and associated costs are shown in Table 4. and accompanying Additional file 5: Table S3. and Additional file 6: Table S4. In general, the developed process consists of initial scale-up through subsequent subculturing up to ~10 m3 (from P-1 to P-8 in FR-101), separation of the post-culturing medium by centrifugation on a sedicanter (P-13, DC-101), drying of the yeast biomass (P-12, DDR-101), and formulation of the enzymatic preparation by microfiltration (P-10, MF-101) and diafiltration (P-11, DF-101), to finally obtain a liquid concentrate of high amylolytic activity (stream “Amylase” in Fig. 4). The proposed combination of membrane separation processes ensures satisfactory level of the enzyme purification, considering its projected applications. Any residual contaminants from peptone or yeast extract (typically < 10 kDa), which in majority are utilized by the yeast strain over cultivation, should be separated though diafiltration. The simulated processes are well balanced in terms of input and output mass. Some minor (by 2%) differences in the estimated cost of equipment are related to the differences in the streams volumes in labYPG (more biomass) and techYPG (slightly more enzyme) processes.

As presented in Table 4, the economic gain from running the process with alternative substrates would result in 2.35-fold reduction in the annual costs of the production process, in addition to streaming of problematic waste by-product from biodiesel production plant. Indeed, the exchange of laboratory grade glycerol with waste glycerol would in fact decrease the annual cost of this specific material by half, but it is not the main driver of the overall expenses. The largest fraction of the annual cost is associated with the prices of BP and YE. Exploitation of technical YE allowed to reduce the percentage contribution (%) brought by this component to the annual cost by over 2-fold (nearly 13-fold

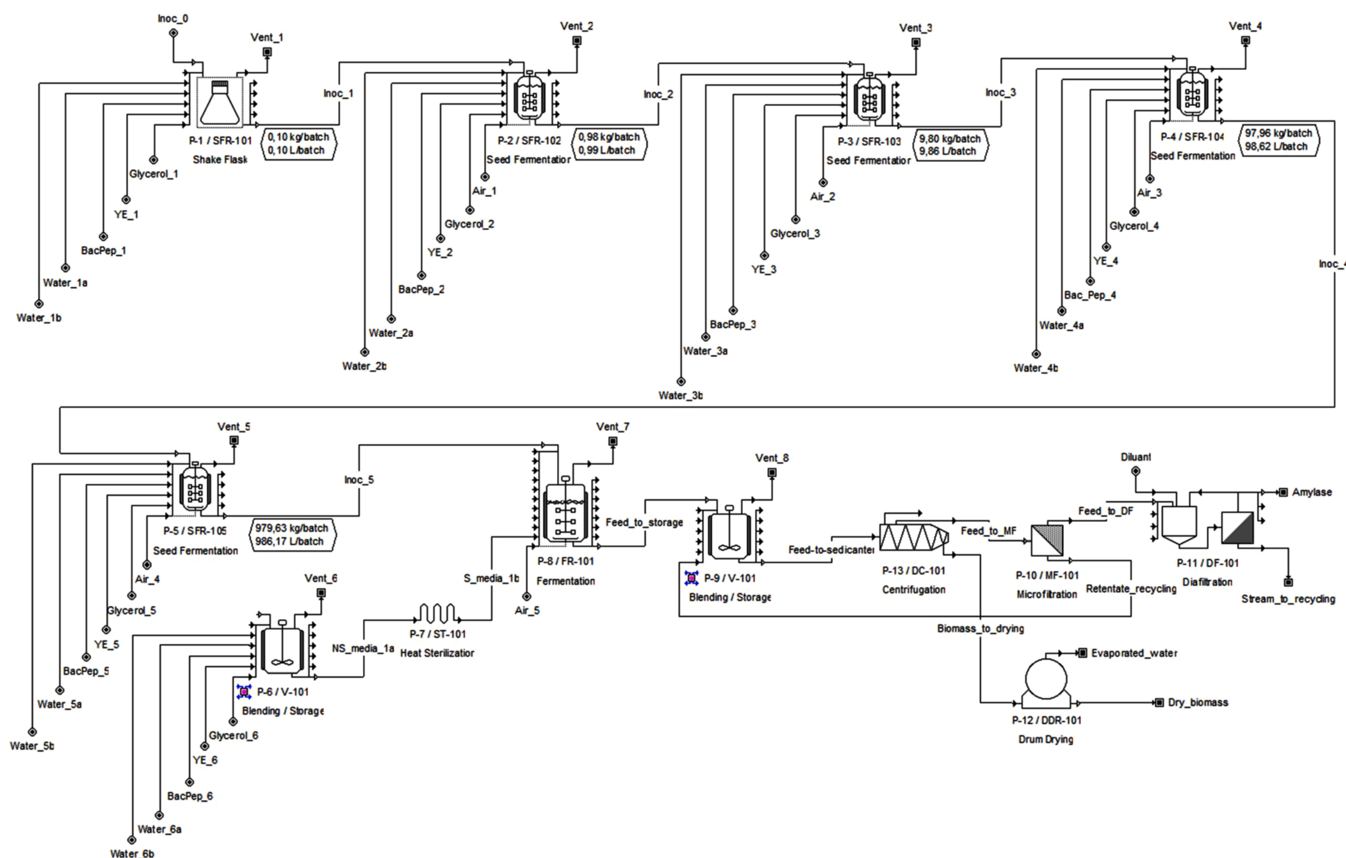

Fig. 4. Schematic representation of a model process of the enzymatic preparation production from glycerol by *Y. lipolytica*. The Flowsheet of the Model processes and all the calculations were prepared with SuperPro Designer software.

Table 4
Material and utilities cost for simulated pilot-scale model processes in labYPG and techYPG.

		labYPG Batch Size 79.76 kg MP						techYPG Batch Size 85.31 kg MP					
	Units	UnitCost ($)	AnnualAmount	AnnualCost ($)	%	kg/kg MP	Unit production cost($/kg MP)	UnitCost ($)	AnnualAmount	AnnualCost ($)	%	kg/kg MP	Unit production cost($/kg MP)
Bulk Material							*Material Cost*						
Air	m3 (STP)	0.02	8029,013	145,982	2.65	1331.96	19.065	0.02	7874,278	143,169	6.08	1221.38	17.48
BP	kg	224.00	21,333	4778,662	86.65	2.79	624.96	100.25	21,333	2138,665	90.78	2.60	260.65
Glycerol	MT	366.23	117,33	42,971	0.78	15.32	5.61	143.00	133,760	19,128	0.81	16.33	2.18
Water	MT	2.25	1057,134	2383	0.04	138.05	0.31	2.25	1036,931	2338	0.10	126.61	0.285
YE	kg	50.02	10,666	533,546	9.67	1.39	69.68	3.89	10,666	41,536	1.76	1.30	5.057
Medium							*Utilities Cost*						
Steam	MT	32.21	186.85	6018.16	24.80	0.024	0.79	32.21	117	3762.55	17.35	0.014	0.46
Chilled water	MT	0.36	20,143.72	7272.67	29.97	2.63	0.95	0.36	19,761	7134.61	32.90	2.41	0.87
Voltage	kW-h	0.12	93,886.84	10,973.79	45.23	12.26	1.43	0.12	92,316	10,790.28	49.75	11.27	1.32
TOTAL				**5503,544**			**735.29**			**2344,834**			**301.43**

reduction of the annual cost of this material – Table 4), which was not the case for BP (only 2.2-fold reduction in the annual cost due to usage of technical substrate). Limitation of BP usage is of fundamental importance for further research on adjusting the techYPG process to industrial realities. Works on exploitation of defined inorganic nitrogen sources combined with provision of cheap, specific amino acids for efficient production of the protein are being pursued. Finding an alternative substrate is essential, as with the current market prices of BP, and its input amounts, the process is not economically feasible. Nevertheless, the exchange of labYPG to techYPG would have already allowed decreasing the unit production cost of the enzymatic concentrate by nearly 2.5-fold (Table 4).

As mentioned above and depicted in Fig. 4, thanks to implementation of the presumptions iv) and v) (section Pilot scale processes simulations and calculation of associated costs) the process is nearly completely waste-free. All introduced water is recycled for the preparation of the culture media, and the dried yeast biomass is further used for animal feed, provided that specific legislative conditions on GMM regulations are fulfilled. Safety and beneficial impact of Y. lipolytica biomass intake on human and animal wellness and health has been already firmly established and supported by adequate data and expertise issued by EFSA [4,48–50]. Recycled water gives additionally a small economic benefit (close to 2400 $/yr; comparable for both processes) to the production process. The same relates to small revenues from selling dried yeast biomass (33,351 $/yr for labYPG and 2.5-fold lower income for techYPG, due to limited biomass propagation). Still, it is worth considering that instead of channeling the whole separated biomass from each cycle to the drying process, some portion / one-in-several batches could be recycled for inoculation of a new process. In that way, the number of subcultures at the scale-up stage could be minimized, improving efficiency and overall performance of the whole installation.

Conclusions

We provide evidence for positive impact of temporary exposure to lowered temperature on production of heterologous proteins by *Y. lipolytica*. Both time and temperature were found significant for the process productivity. Production of the protein was decoupled from biomass or metabolites synthesis. The optimized treatment was adopted in a production process conducted with technical substrates and raw glycerol. No loss in the enzyme production was observed due to exploitation of the alternative substrates. Development of the pilot-scale waste-free process models allowed to assess actual economic gain from the exchange of substrates, and to identify current bottlenecks.

Author statement

MK conducted laboratory work (bioreactor cultivations and the following analyses), collected and prepared the data for analyses, wrote the manuscript and secured financial support. WB designed the experiments, conducted statistical analysis, developed mathematical models and simulated process models. EC conceived the study, analyzed the results and wrote the manuscript.

Funding

This study was financially supported by the Ministry of Science and Higher Education (currently, Ministry of Education and Science) project Diamentowy Grant VII number: DI 2017 001047.

Additional files

Additional file 1.

Figure S1. Kinetics of glycerol production (GLY) and biomass formation (DCW) at different starting concentrations of glycerol.

Declaration of Competing Interest

None.

Data Availability: All data accompanying this research are presented directly in the manuscript and supplementary materials.

Acknowledgements

The Authors would like to thank DBFM's chromatography team for great help in HPLC analyses. Piotr Kubiak is greatly acknowledged for valuable discussions and critical reading of the manuscript.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.btre.2021.e00648.

References

[1] Directive (EU) 2018/2001 of the European Parliament and of the Council of 11 December 2018 on the promotion of the use of energy from renewable sources, 2018.
[2] P. Dusser, The European Energy Policy for 2020–2030 RED II: what future for vegetable oil as a source of bioenergy? Ocl 26 (2019) 51, https://doi.org/10.1051/ocl/2019040.
[3] N. Vivek, R. Sindhu, A. Madhavan, A. Jose, E. Castro, V. Faraco, A. Pandey, P. Binod, Recent advances in the production of value added chemicals and lipids utilizing biodiesel industry generated crude glycerol as a substrate – Metabolic aspects, challenges and possibilities: an overview, Bioresour. Technol. 239 (2017) 507–517, https://doi.org/10.1016/j.biortech.2017.05.056.
[4] M. Groenewald, T. Boekhout, C. Neuvéglise, C. Gaillardin, P.W.M. Van Dijck, M. Wyss, Yarrowia lipolytica, Safety assessment of an oleaginous yeast with a great industrial potential, Crit. Rev. Microbiol. 40 (2014) 187–206, https://doi.org/10.3109/1040841X.2013.770386.
[5] S. Papanikolaou, L. Muniglia, I. Chevalot, G. Aggelis, I. Marc, *Yarrowia lipolytica* as a potential producer of citric acid from raw glycerol, J. Appl. Microbiol. 92 (2002) 737–744, https://doi.org/10.1046/j.1365-2672.2002.01577.x.
[6] W. Rymowicz, A. Rywińska, M. Marcinkiewicz, High-yield production of erythritol from raw glycerol in fed-batch cultures of *Yarrowia lipolytica*, Biotechnol. Lett. 31 (2009) 377–380, https://doi.org/10.1007/s10529-008-9884-1.
[7] C. Madzak, *Yarrowia lipolytica*: recent achievements in heterologous protein expression and pathway engineering, Appl. Microbiol. Biotechnol. 99 (2015) 4559–4577, https://doi.org/10.1007/s00253-015-6624-z.
[8] S. Raimondi, E. Zanni, A. Amaretti, C. Palleschi, D. Uccelletti, M. Rossi, Thermal adaptability of *Kluyveromyces marxianus* in recombinant protein production, Microb. Cell Fact. 12 (2013) 1–7, https://doi.org/10.1186/1475-2859-12-34.
[9] S. Curvers, P. Brixius, T. Klauser, D. Weuster-Botz, R. Takors, C. Wandrey, Human Chymotrypsinogen B Production with *Pichia pastoris* by Integrated Development of Fermentation and Downstream Processing. Part 1. Fermentation, Biotechnol. Prog. 17 (2001) 495–502, https://doi.org/10.1021/bp000164j.
[10] M. Kubiak, M. Borkowska, W. Białas, P. Korpys, E. Celińska, Feeding strategy impacts heterologous protein production in *Yarrowia lipolytica* fed-batch cultures—Insight into the role of osmolarity, Yeast 36 (2019) 305–318, https://doi.org/10.1002/yea.3384.
[11] M. Gorczyca, J. Kaźmierczak, S. Steels, P. Fickers, E. Celińska, Impact of oxygen availability on heterologous gene expression and polypeptide secretion dynamics in *Yarrowia lipolytica*-based protein production platforms, Yeast (2020) 37, https://doi.org/10.1002/yea.3499.
[12] D. Mattanovich, B. Gasser, H. Hohenblum, M. Sauer, Stress in recombinant protein producing yeasts, J. Biotechnol. 113 (2004) 121–135, https://doi.org/10.1016/j.jbiotec.2004.04.035.
[13] H.C. Causton, B. Ren, S.S. Koh, T. Christopher, E. Kanin, E.G. Jennings, I. Lee, L. Heather, E.S. Lander, R.A. Young, Remodeling of Yeast Genome Expression in Response to Environmental Changes, Mol. Biol. Cell. 12 (2001) 323–337, https://doi.org/10.1091/mbc.12.2.323.
[14] J. Hou, T. Österlund, Z. Liu, D. Petranovic, J. Nielsen, Heat shock response improves heterologous protein secretion in *Saccharomyces cerevisiae*, Appl. Microbiol. Biotechnol. 97 (2013) 3559–3568, https://doi.org/10.1007/s00253-012-4596-9.
[15] Z. Li, F. Xiong, Q. Lin, M. Anjou, A.J. Daugulis, D.S.C. Yang, C.L. Hew, Low-Temperature Increases the Yield of Biologically Active Herring Antifreeze Protein in *Pichia pastoris*, Protein Expr. Purif. 445 (2001) 438–445, https://doi.org/10.1006/prep.2001.1395.
[16] W. Praphailong, G.H. Fleet, The effect of pH, sodium chloride, sucrose, sorbate and benzoate on the growth of food spoilage yeasts, Food Microbiol 14 (1997) 459–468, https://doi.org/10.1006/fmic.1997.0106.
[17] E.N. Biryukova, A.G. Medentsev, A.Y. Arinbasarova, V.K. Akimenko, Adaptation of the Yeast *Yarrowia lipolytica* to Heat Shock, Microbiology 76 (2007) 158–163, https://doi.org/10.1134/S0026261707020051.
[18] S.V. Kamzolova, N.V. Shishkanova, I.G. Morgunov, T.V. Finogenova, Oxygen requirements for growth and citric acid production of *Yarrowia lipolytica*, FEMS Yeast Res 3 (2003) 217–222, https://doi.org/10.1016/S1567-1356(02)00188-5.
[19] B. Jost, M. Holz, A. Aurich, G. Barth, T. Bley, R.A. Müller, The influence of oxygen limitation for the production of succinic acid with recombinant strains of *Yarrowia lipolytica*, (2015) 1675–1686. https://doi.org/10.1007/s00253-014-6252-z.
[20] L. Tomaszewska, A. Rywińska, W. Gladkowski, Production of erythritol and mannitol by *Yarrowia lipolytica* yeast in media containing glycerol, J. Ind. Microbiol. Biotechnol. 39 (2012) 1333–1343, https://doi.org/10.1007/s10295-012-1145-6.
[21] M. L. Yang, X. Zhan, Z. Zheng, J. Wu, C.L. Gao, A novel osmotic pressure control fed-batch fermentation strategy for improvement of erythritol production by *Yarrowia lipolytica* from glycerol Bioresour. Technol. 151 (2013) 120–127, https://doi.org/10.1016/j.biortech.2013.10.031.
[22] Z. Lazar, E. Walczak, M. Robak, Simultaneous production of citric acid and invertase by *Yarrowia lipolytica* SUC+ transformants, Bioresour. Technol. 102 (2011) 6982–6989, https://doi.org/10.1016/j.biortech.2011.04.032.
[23] L.T. Cordova, H.S. Alper, Production of α-linolenic acid in *Yarrowia lipolytica* using low-temperature fermentation, Appl. Microbiol. Biotechnol. 102 (2018) 8809–8816, https://doi.org/10.1007/s00253-018-9349-y.
[24] A. Crolla, K.J. Kennedy, Optimization of citric acid production from *Candida lipolytica* Y-1095 using n-paraffin, J. Biotechnol. 89 (2001) 27–40, https://doi.org/10.1016/S0168-1656(01)00278-4.
[25] G. Corzo, S. Revah, Production and characteristics of the lipase from *Yarrowia lipolytica* 681, Bioresour. Technol. 70 (1999) 173–180, https://doi.org/10.1016/S0960-8524(99)00024-3.
[26] P. Korpys-Woźniak, P. Kubiak, W. Białas, E. Celińska, Impact of overproduced heterologous protein characteristics on physiological response in *Yarrowia lipolytica* steady-state-maintained continuous cultures, Appl. Microbiol. Biotechnol. 104 (2020) 9785–9800, https://doi.org/10.1007/s00253-020-10937-w.
[27] E. Celińska, R. Ledesma-Amaro, M. Larroude, T. Rossignol, C. Pauthenier, J. M. Nicaud, Golden Gate Assembly system dedicated to complex pathway manipulation in *Yarrowia lipolytica*, Microb. Biotechnol. 10 (2017) 450–455, https://doi.org/10.1111/1751-7915.12605.
[28] E. Celińska, M. Borkowska, W. Białas, P. Korpys, J.M. Nicaud, Robust signal peptides for protein secretion in *Yarrowia lipolytica*: identification and characterization of novel secretory tags, Appl. Microbiol. Biotechnol. 102 (2018) 5221–5233, https://doi.org/10.1007/s00253-018-8966-9.
[29] M. Mühlhofer, E. Berchtold, C.G. Stratil, G. Csaba, E. Kunold, N.C. Bach, S. A. Sieber, M. Haslbeck, R. Zimmer, J. Buchner, The Heat Shock Response in Yeast Maintains Protein Homeostasis by Chaperoning and Replenishing Proteins, Cell Rep. 29 (2019) 4593–4607, https://doi.org/10.1016/j.celrep.2019.11.109.
[30] E. Celińska, W. Białas, M. Borkowska, W. Grajek, Cloning, expression, and purification of insect (Sitophilus oryzae) alpha-amylase, able to digest granular starch, in *Yarrowia lipolytica* host, Appl. Microbiol. Biotechnol. 99 (2015) 2727–2739, https://doi.org/10.1007/s00253-014-6314-2.
[31] M. Borkowska, W. Białas, M. Kubiak, E. Celińska, Rapid micro-assays for amylolytic activities determination: customization and validation of the tests, Appl. Microbiol. Biotechnol. (2019) 1–13, https://doi.org/10.1007/s00253-018-09610-0.
[32] M. Borkowska, W. Białas, E. Celińska, A new set of reference genes for comparative gene expression analyses in *Yarrowia lipolytica*, FEMS Yeast Res. 20 (2020), https://doi.org/10.1093/femsyr/foaa059.
[33] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method, Methods 25 (2001) 402–408, https://doi.org/10.1006/meth.2001.1262.
[34] E. Celińska, M. Borkowska, W. Białas, Enhanced production of insect raw-starch-digesting alpha-amylase accompanied by high erythritol synthesis in recombinant *Yarrowia lipolytica* fed-batch cultures at high-cell-densities, Process Biochem. 52 (2017) 78–85, https://doi.org/10.1016/j.procbio.2016.10.022.
[35] G. Barth, H. Weber, Improvement of sporulation in the yeast *Yarrowia lipolytica*, Antonie Van Leeuwenhoek 51 (1985) 167–177, https://doi.org/10.1007/BF02310010.
[36] J.B. Sutherland, *Yarrowia Lipolytica* (*Candida Lipolytica*), Second Edi, Elsevier, 2014, https://doi.org/10.1016/B978-0-12-384730-0.00056-2.
[37] J. Ruiz-Herrera, R. Sentandreu, Different effectors of dimorphism in *Yarrowia lipolytica*, Arch. Microbiol. 178 (2002) 477–483, https://doi.org/10.1007/s00203-002-0478-3.
[38] A. Makri, S. Fakas, G. Aggelis, Metabolic activities of biotechnological interest in *Yarrowia lipolytica* grown on glycerol in repeated batch cultures, Bioresour. Technol. 101 (2010) 2351–2358, https://doi.org/10.1016/j.biortech.2009.11.024.
[39] Y. Kobayashi, H. Iwata, J. Yoshida, J. Ogihara, J. Kato, T. Kasumi, Metabolic correlation between polyol and energy-storing carbohydrate under osmotic and oxidative stress condition in *Moniliella megachiliensis*, J. Biosci. Bioeng. 120 (2015) 405–410, https://doi.org/10.1016/j.jbiosc.2015.02.014.
[40] E. Cavallo, H. Charreau, P. Cerrutti, M.L. Foresti, *Yarrowia lipolytica*: a model yeast for citric acid production, FEMS Yeast Res 17 (2017) 1–16, https://doi.org/10.1093/femsyr/fox084.

[41] L. Moeller, B. Strehlitz, A. Aurich, A. Zehnsdort, T. Bley, Optimization of citric acid production from glucose by *Yarrowia lipolytica*, Eng. Life Sci. 7 (2007) 504–511, https://doi.org/10.1002/elsc.200620207.
[42] S. Hohmann, Osmotic Stress Signaling and Osmoadaptation in Yeasts, Microbiol, Mol. Biol. Rev. 66 (2002) 300–372, https://doi.org/10.1128/mmbr.66.2.300-372.2002.
[43] S. Karlgren, N. Pettersson, B. Nordlander, J.C. Mathai, J.L. Brodsky, M.L. Zeidel, R. M. Bill, S. Hohmann, Conditional osmotic stress in yeast: a system to study transport through aquaglyceroporins and osmostress signaling, J. Biol. Chem. 280 (2004) 7186–7193, https://doi.org/10.1074/jbc.M413210200.
[44] D.A. Rzechonek, A.M. Day, J. Quinn, A.M. Mirończuk, Influence of ylHog1 MAPK kinase on *Yarrowia lipolytica* stress response and erythritol production, Sci. Rep. 8 (2018) 1–12, https://doi.org/10.1038/s41598-018-33168-6.
[45] S. Hackenschmidt, S. Bruder, F. Bracharz, R. Daniel, A. Thürmer, J. Kabisch, Effects of a high cultivation temperature on the physiology of three different *Yarrowia lipolytica* strains, FEMS Yeast Res. 19 (2019), https://doi.org/10.1093/femsyr/foz068.
[46] L.S. Savergave, R.V. Gadre, B.K. Vaidya, V.V. Jogdand, Two-stage fermentation process for enhanced mannitol production using *Candida magnoliae* mutant R9, Bioprocess Biosyst. Eng. 36 (2013) 193–203, https://doi.org/10.1007/s00449-012-0775-4.
[47] M. Jahic, M. Gustavsson, A.K. Jansen, M. Martinelle, S.O. Enfors, Analysis and control of proteolysis of a fusion protein in *Pichia pastoris* fed-batch processes, J. Biotechnol. 102 (2003) 45–53, https://doi.org/10.1016/S0168-1656(03)00003-8.
[48] D. Turck, J. Castenmiller, S. De Henauw, K.I. Hirsch-Ernst, J. Kearney, A. Maciuk, I. Mangelsdorf, H.J. McArdle, A. Naska, C. Pelaez, K. Pentieva, A. Siani, F. Thies, S. Tsabouri, M. Vinceti, F. Cubadda, K.H. Engel, T. Frenzel, M. Heinonen, R. Marchelli, M. Neuhäuser-Berthold, M. Poulsen, Y. Sanz, J.R. Schlatter, H. van Loveren, R. Ackerl, H.K. Knutsen, Safety of selenium-enriched biomass of *Yarrowia lipolytica* as a novel food pursuant to Regulation (EU) 2015/2283, EFSA J 18 (2020) 1–13, https://doi.org/10.2903/j.efsa.2020.5992.
[49] D. Turck, J. Castenmiller, S. De Henauw, K.I. Hirsch-Ernst, J. Kearney, A. Maciuk, I. Mangelsdorf, H.J. McArdle, A. Naska, C. Pelaez, K. Pentieva, A. Siani, F. Thies, S. Tsabouri, M. Vinceti, F. Cubadda, K.H. Engel, T. Frenzel, M. Heinonen, R. Marchelli, M. Neuhäuser-Berthold, M. Poulsen, Y. Sanz, J.R. Schlatter, H. van Loveren, R. Ackerl, H.K. Knutsen, Safety of chromium-enriched biomass of *Yarrowia lipolytica* as a novel food pursuant to Regulation (EU) 2015/2283, EFSA J. 18 (2020), https://doi.org/10.2903/j.efsa.2020.6005.
[50] A. Czech, A. Smolczyk, K. Ognik, M. Kiesz, Nutritional Value of *Yarrowia Lipolytica* Yeast and its Effect on Growth Performance Indicators in Piglets, Ann. Anim. Sci. 16 (2016) 1091–1100, https://doi.org/10.1515/aoas-2016-0034.