

Article

Optimizing the Production of Recombinant Hydroperoxide Lyase in *Escherichia coli* Using Statistical Design

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Abstract: Hydroperoxide lyase (HPL) catalyzes the synthesis of volatiles C6 or C9 aldehydes from fatty acid hydroperoxides. These short carbon chain aldehydes, known as green leaf volatiles (GLV), are widely used in cosmetic industries and as food additives because of their “fresh green” aroma. To meet the growing demand for natural GLVs, the use of recombinant HPL as a biocatalyst in enzyme-catalyzed processes appears to be an interesting application. Previously, we cloned and expressed a 13-HPL from olive fruit in *Escherichia coli* and showed high conversion rates (up to 94%) during the synthesis of C6 aldehydes. To consider a scale-up of this process, optimization of the recombinant enzyme production is necessary. In this study, four host-vector combinations were tested. Experimental design and response surface methodology (RSM) were used to optimize the expression conditions. Three factors were considered, i.e., temperature, inducer concentration and induction duration. The Box–Behnken design consisted of 45 assays for each expression system performed in deep-well microplates. The regression models were built and fitted well to the experimental data (R^2 coefficient > 97%). The best response (production level of the soluble enzyme) was obtained with *E. coli* BL21 DE3 cells. Using the optimal conditions, 2277 U L^{−1} of culture of the soluble enzyme was produced in microliter plates and 21,920 U L^{−1} of culture in an Erlenmeyer flask, which represents a 79-fold increase compared to the production levels previously reported.

Keywords: hydroperoxide lyase; response surface methodology; Box–mBehnken design; protein expression optimization; *E. coli* heterologous protein production



Citation: Vincenti, S.; Mariani, M.; Croce, J.; Faillace, E.; de Caraffa, V.B.-B.; Berti, L.; Maury, J. Optimizing the Production of Recombinant Hydroperoxide Lyase in *Escherichia coli* Using Statistical Design. *Catalysts* **2021**, *11*, 176. <https://doi.org/10.3390/catal11020176>

Academic Editors: Richard Daniellou and Anwar Sunna

Received: 5 January 2021

Accepted: 25 January 2021

Published: 28 January 2021

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1. Introduction

Hydroperoxide lyase (HPL) is a membrane-bound enzyme mainly distributed in higher plants, algae, and fungi [1–3]. HPL is involved in the lipoxygenase (LOX) pathway, which is a lipid oxidation pathway activated in response to biotic or abiotic stress, or during various plant stages development, to produce phyto-oxylipins acting as defense and signaling molecules [4–12]. In this pathway, LOX first catalyzes the oxygenation of polyunsaturated fatty acids (PUFAs), such as linoleic or α -linolenic acids, to produce corresponding hydroperoxides [4,13,14]. Subsequently, HPL acts on these PUFA hydroperoxides to form volatile short-chain aldehydes (C₆ or C₉-aldehydes) and ω -oxoacids [6,15–19]. HPLs are heme enzymes belonging to the cytochrome P450 family and are divided into two subfamilies by their substrate specificities, namely CYP74B for 13-HPLs and CYP74C for 9-HPLs and 9/13-HPLs. 13-HPLs preferentially act on 13-hydroperoxide of linolenic acid (13-HPOT) and 13-hydroperoxide of linoleic acid (13-HPOD) to form (3Z)-hexenal and hexenal, respectively [20–25], while 9-HPLs transform 9-hydroperoxide of linolenic acid (9-HPOT) and 9-hydroperoxide of linoleic acid (9-HPOD) into (3Z)-nonadienal and (3Z)-nonenal, respectively [16]. (3Z)-hexenal, (3Z)-nonadienal, and (3Z)-nonenal are enzymatically or spontaneously isomerized into more stable (2E)-hexenal, (2E)-nonadienal, and (2E)-nonenal. 9-/13-HPLs have both 9- and 13-HPL activities [26–28].

Volatile aldehydes produced by HPL are part of the green leaf volatiles (GLVs) compounds and are important constituents of the fresh smell of green leaves and cut grass called “green note” [29,30]. Because of their green, fresh and fruity aromas, GLVs are widely used in perfumes and fragrances. In food products, GLVs are added mainly to restore the natural fresh green aroma of fruits and vegetables, which is lost during industrial processing or sterilization processes, but they can also improve the shelf life and safety of processed foods due to their antibacterial activities [31]. The industrial production of GLVs by chemical synthesis is environmentally unfriendly, and nowadays, consumers have a strong preference for naturally synthesized additives and flavors. Given the high demand for such natural flavors and the low yield of extracting the compounds directly from plants, efficient biocatalytic processes using the enzymes of the LOX pathway are developed [19,32]. In these processes, vegetal oils are hydrolyzed by lipase, and then the PUFAs released are converted by LOX and HPL into green note aldehydes [23,33–36]. Since lipases and LOXs are commercially available, genetic engineering approaches focus on the production of recombinant HPL [19,37–41].

In previous work, we isolated a complementary DNA (cDNA) encoding for a 13-HPL from black olive fruit (*Olea europaea* L., Leccino variety) [42]. Olive recombinant 13-HPL was expressed in *Escherichia coli* (*E. coli*) M15, purified by affinity chromatography and biochemically characterized [42]. In another work, we improved the stability and increased activity of the enzyme using selected chemical additives [43]. The use of olive recombinant 13-HPL for C₆-aldehydes synthesis from 13-HPOD and 13-HPOT showed that it is an efficient and promising biocatalyst for the bioconversion process (conversion rates up to 94%) [42,43]. Its use for C₆-aldehyde production in a high-scale bioreactor requires an optimization of the heterologous expression of recombinant HPL in order to recover larger amounts of the soluble and active enzymes.

E. coli is the most commonly used host for the expression of heterologous genes, notably due to its rapid growth, relatively low culture cost, significant knowledge of genetic and metabolism and many molecular tools (such as plasmids) available to induce a strong expression of the genes coding for heterologous proteins [44,45]. Protein overproduction in *E. coli* is today very widespread [46–48] and used in large-scale industry for the production of drugs, vaccines, enzymes or modified proteins. However, the formation of insoluble protein aggregates as inclusion bodies is often a phenomenon limiting heterologous expression [49,50]. Various *E. coli* strains and/or expression vectors were specifically designed to improve protein overexpression and solubility, sometimes leading to significant success, although the improvements are highly protein-dependent [45]. Otherwise, one of the simplest means generally used to try to lower protein aggregation and to promote better folding of proteins is decreasing the culture temperature of *E. coli*.

In the present study, we proposed to test different suitable systems of *E. coli* strains/ expression vectors and to perform for each a screening of the culture conditions (by varying factors including temperature, inducer concentration and induction duration) in order to optimize the expression of the soluble and active olive recombinant 13-HPL. Since many factor combinations were to be tested to find the best conditions for enzyme expression, a statistical design of the experiments was carried out. The response surface methodology (RSM) was used to evaluate the effect of the factors on the soluble enzyme production, identify the optimum factor levels and provide reliable predictions of production yields. Improved yields of the soluble active enzyme were reached, which will greatly facilitate future studies and will allow the higher-scale use of the olive recombinant HPL in the bioproduction processes of flavoring aldehydes.

2. Results

2.1. Optimizing the Culture Conditions Using RSM

2.1.1. Model Fitting and Statistical Analysis

For each of the four *E. coli* strain/ expression vector systems (M30, I30, B19 and A19), the optimization of the production of the soluble and active olive recombinant HPL was

carried out using RSM. A Box–Behnken design was used to investigate the effects of three factors, i.e., temperature (T), inducer concentration (I) and induction duration (D), on this enzyme production estimated by measuring soluble enzyme activity (expressed in U L^{-1} of culture). The results obtained from 45 experimental runs carried out according to the Box–Behnken design are summarized in Supplementary Materials, Table S1.

Regression analysis was then performed on the experimental data. The measured values of the soluble activity were fitted to a second-order polynomial equation. For each expression system (M30, I30, B19 and A19), the predicted model can be described by the equation showed in Table 1.

Table 1. Response surface quadratic model analysis of hydroperoxide lyase (HPL) activity level obtained with M30, I30, B19 and A19 expression system, after model simplification—second-order polynomial equations and determination coefficients.

Expression System	Equation ^a	R ²	Adj R ²
M30	Soluble activity (U L^{-1} of culture) = $-5346 + 516.1 \times T + 97 \times I + 56.89 \times D - 9.929 \times T^2 + 146.7 \times I^2 - 0.3363 \times D^2 - 8.99 \times T \times I - 1.0205 \times T \times D + 1.27 \times I \times D$	0.9724	0.9653
I30	Soluble activity (U L^{-1} of culture) = $-4477 + 500.8 \times T + 185 \times I + 28.38 \times D - 10.336 \times T^2 - 32.2 \times I^2 - 0.2090 \times D^2 + 1.08 \times T \times I - 0.3006 \times T \times D - 1.090 \times I \times D$	0.9706	0.9630
B19	Soluble activity (U L^{-1} of culture) = $-3809 + 430.8 \times T + 176 \times I + 34.64 \times D - 8.573 \times T^2 + 207 \times I^2 - 0.2275 \times D^2 - 9.70 \times T \times I - 0.4994 \times T \times D - 0.724 \times I \times D$	0.9801	0.9750
A19	Soluble activity (U L^{-1} of culture) = $-3571 + 442.1 \times T - 152 \times I + 86.35 \times D - 9.566 \times T^2 + 132 \times I^2 - 0.4398 \times D^2 + 0.27 \times T \times I - 2.734 \times T \times D + 1.49 \times I \times D$	0.9665	0.9578

^a Factors coded as follows: temperature (T), inducer concentration (I) and culture duration (D).

Through regression-fitting, the second-order polynomial equations represent the relationship between the various factors and the HPL activity in the soluble fraction of bacterial lysate.

R² coefficients of the model were calculated to be 0.97, 0.97, 0.98 and 0.97 for the expression systems M30, I30, B19 and A19, respectively. They revealed a satisfactory adjustment of the model to the experimental data and indicated that, respectively, 97, 97, 98 and 97% of the variability in the response could be explained by the model. Thus, these models could be used for the analysis and prediction of the soluble HPL activity produced.

The significance of the fit of the second-order polynomial for enzyme activity is assessed by carrying out an analysis of variance (ANOVA). The results of the second-order response surface model fitting in the form of ANOVA and *p*-values of each linear, quadratic or interaction terms are reported in Supplementary Materials, Table S2. A *p*-value < 0.05 was considered to be significant. The models were simplified by keeping terms showing *p*-values lower than 0.05 (except for the first-order terms, which were kept as long as an interaction or a quadratic term including this factor was statistically significant). The simplified models are shown in Table 2.

Table 2. Response surface quadratic model analysis of HPL activity level obtained with M30, I30, B19 and A19 expression system, after model simplification—analysis of variance (ANOVA).

Source ^a	Sum of Squares				Degree of Freedom				F Value				p-Value ^b			
	M30	I30	B19	A19	M30	I30	B19	A19	M30	I30	B19	A19	M30	I30	B19	A19
Model	1,963,026	1,532,237	1,284,812	4,758,653	9	9	9	9	136.81	128.21	191.51	112.09	0.000	0.000	0.000	0.000
T	482,971	473,401	350,658	979,862	1	1	1	1	302.93	356.50	470.40	207.73	0.000	0.000	0.000	0.000
I	36,465	83,497	83,355	17,275	1	1	1	1	22.87	62.88	111.82	3.66	0.000	0.000	0.000	0.064
D	190,478	101,023	96,520	1,175,413	1	1	1	1	119.47	76.08	129.48	249.19	0.000	0.000	0.000	0.000
T ²	682,550	739,644	508,860	633,549	1	1	1	1	428.12	557.00	682.63	134.31	0.000	0.000	0.000	0.000
I ²	9777	-	19,462	-	1	-	1	-	6.13	-	26.11	-	0.018	-	0.000	-
D ²	415,704	160,588	190,188	710,970	1	1	1	1	260.74	120.93	255.13	150.73	0.000	0.000	0.000	0.000
T × I	-	-	5720	-	-	-	1	-	-	-	7.67	-	-	-	0.009	-
T × D	181,548	15,610	43,104	1,291,336	1	1	1	1	113.87	11.76	57.82	273.77	0.000	0.002	0.000	0.000
I × D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lack of fit	49,275	33,761	18,529	156,098	3	3	3	3	80.54	28.32	26.14	185.12	0.000	0.000	0.000	0.000
Pure error	6526	12,715	7561	8994	32	32	32	32	-	-	-	-	-	-	-	-
Total	2,018,827	1,578,714	1,310,903	4,923,745	44	44	44	44	-	-	-	-	-	-	-	-

^a Factors coded as follows: temperature (T), inducer concentration (I) and culture duration (D); ^b *p*-value < 0.05 indicates a statistical significance of the factor.

Analysis of RSM for the M30 expression system revealed that linear terms temperature (T), induction concentration (I), and induction duration (D) showed a *p*-value lower than 0.05, indicating that those three factors significantly influence the production of the soluble enzyme. The quadratic terms— T^2 , I^2 and D^2 —were also statistically significant. Concerning interaction terms, only temperature × induction duration ($T \times D$) exhibited a significant influence on the soluble enzyme production.

Regarding the I30 expression system, linear terms temperature T, I and D had a significant impact on the production of the soluble enzymes. The quadratic terms T^2 and D^2 were statistically significant. Likewise, only the interaction term $T \times D$ had a significant influence.

For the B19 expression system, the linear terms T, I and D as well as the associated quadratic terms T^2 , I^2 and D^2 were statistically significant. $T \times D$ and $T \times I$ were the two cross-product terms influencing the production of the soluble enzyme.

Concerning the A19 expression system, the linear terms T and D were statistically significant, as well as their quadratic terms T^2 and D^2 . Neither the linear term I nor the quadratic term I^2 was significant. Interaction term $T \times D$ had a significant influence on the soluble enzyme production.

Henry's half-lines displaying a comparison of the effects of each significant linear, square and interaction term were made for each of the expression systems (Figure 1). The adjustment line indicates where the points should be if there was no effect. All significant terms are labeled, are located on the right side of the graph, and their distance from the line highlights their relative importance.

For the M30, I30 and B19 expression systems, the temperature (T and T^2) was the variable that had the largest effect on the soluble enzyme production. The value for the normalized effect for expression systems M30, I30 and B19 was, respectively, 22, 23 and 27 for the quadratic term T^2 and 17.5, 19 and 22 for the linear term T. The order of the variables influencing the soluble enzyme production was as follows: temperature > induction duration > inducer concentration.

For the A19 expression system, the interaction term $T \times D$ had the most significant effect on the soluble activity (normalized effect with a value of 16.5) showing the importance of jointly adjusting the value of these two variables to influence enzyme production. Concordantly, T and D showed fairly close normalized effect values (respectively 14.5 and 15.7).

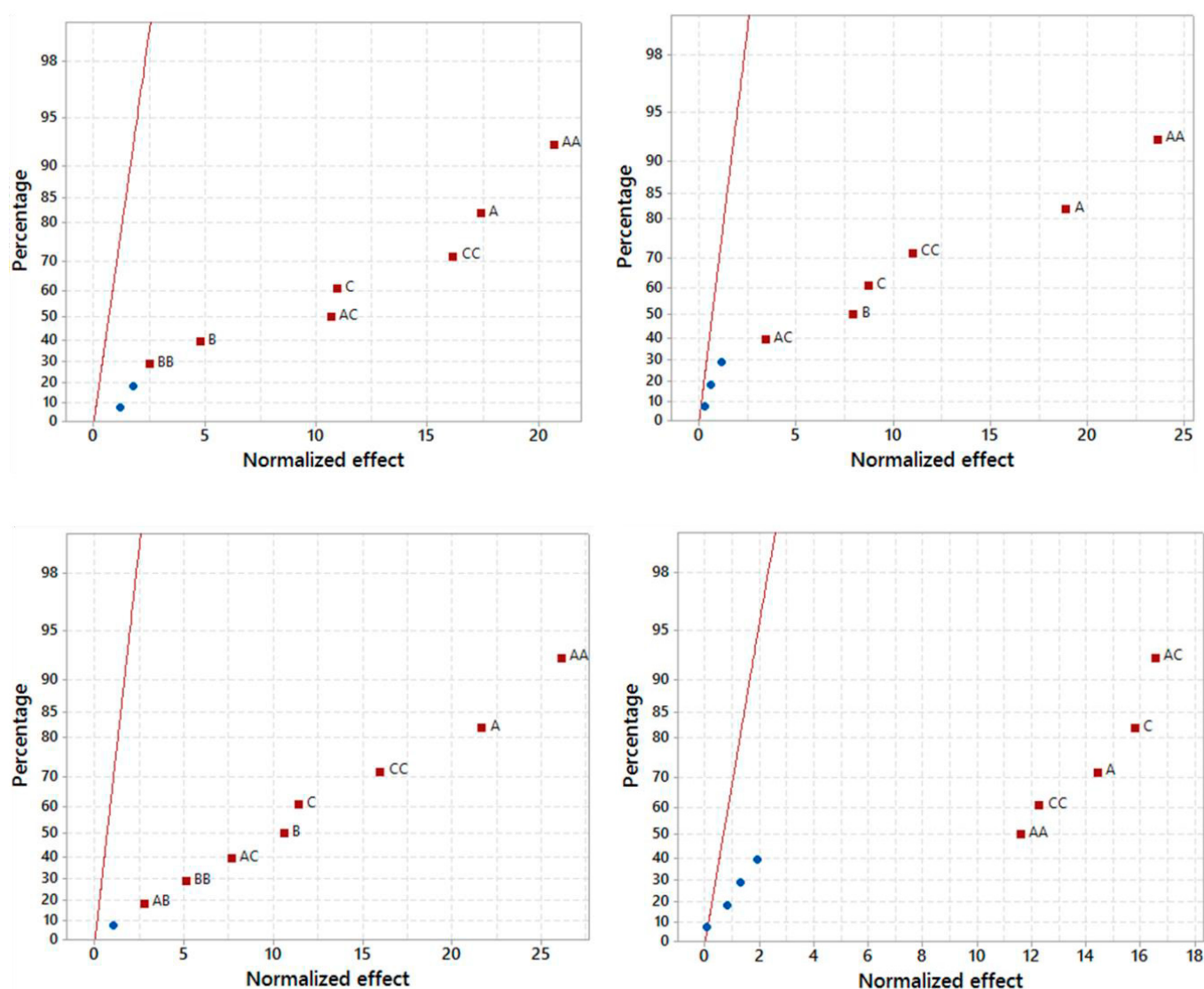


Figure 1. Henry's half-line of the normalizing effects of the temperature, inducer concentration and induction duration, for M30, I30, B19 and A19; factors are coded as follows: temperature (A), inducer concentration (B), induction duration (C); answer = enzyme activity (U L⁻¹ of culture); $\alpha = 0.05$; the adjustment line in red indicates where the points should be if there was no effect. Henry's half-line displays the absolute value of all the normalized effects, positive and negative. All terms are on the right side of the graph, and their distance from the line highlights their relative importance; significant effects are represented by red squares (■), while non-significant effects are represented by blue circles (●).

2.1.2. 3D Response Surface Plots and Experimental Validation

Effects of cross term product factors on the soluble activity level were further examined using three-dimensional (3D) response surface plots (Figure 2) or contour plots (Supplementary Materials, Figure S1). Only significant interactions were kept.

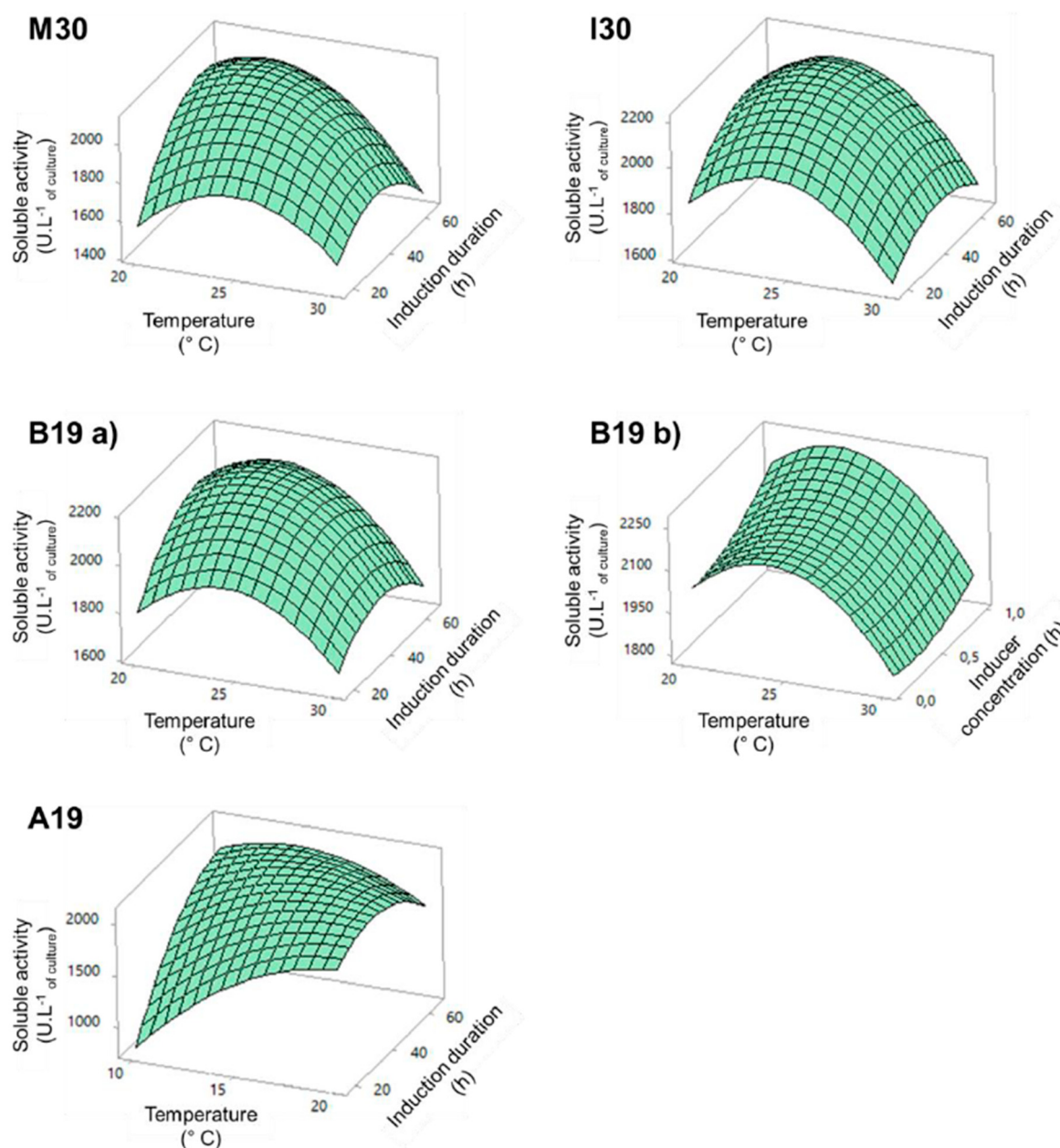


Figure 2. Three-dimensional (3D) response surface plots of the HPL activity recovered in the soluble fraction for M30 and I30 expression systems, as a function of the temperature and induction duration; for B19 expression system, as a function of (a) temperature and induction duration and (b) temperature and inducer concentration; and for A19 expression system.

The M30 and I30 expression systems, for which only the $T \times D$ interaction term was significant, displayed similar plots (Figure 2). Response surface plots as a function of the temperature and induction duration confirmed that these variables are critical factors influencing soluble enzyme production and must be tightly adjusted to reach a maximum production at 23 °C and 51.5 h for M30 and 23.5 °C and 48.5 h for I30 (Figure 2).

Regarding the B19 expression system, the $T \times D$ and $T \times I$ interaction terms were significant. Response surface plot as a function of the temperature and induction duration confirmed that these variables were closely linked, both strongly influencing soluble enzyme production, as observed for M30 and I30. A maximum of the soluble activity could be reached for 23 °C and 49 h.

For B19 as well as for M30 and I30, on the 3D response surface plot (Figure 2), for the highest temperatures like 30 °C, we observed little influence of the induction duration.

For example, for I30, the soluble enzymatic activity remained between about 1600 and 1700 U L^{-1} of culture at 30 °C whatever the induction duration. This is in agreement with Henry's half-lines results (Figure 1), indicating that temperature had a higher impact on the soluble enzymatic activity than the duration of the induction. For example, for I30, the normalized effect value of T (18.9) was 2.2-fold higher than that of D (8.7) (Figure 1).

For B19, the response surface plot as a function of the temperature and inducer concentration confirmed the value of 23 °C as the optimal temperature and showed that a maximum production could be reached with 1 mM inducer concentration. Even if the $T \times I$ interaction term exhibited a significant p -value, only a small impact of the inducer concentration was observed on the 3D response surface plot at the various temperatures (Figure 2).

Concerning the A19 expression system, the temperature and the induction duration are factors strongly affecting enzyme expression. In Figure 2, we observed that for temperatures below 15 °C, the induction duration strongly influences the soluble enzymatic activity, which increased with the duration of the induction. On the other hand, for durations of the induction less than 40 h, the temperature had an important influence, and soluble enzymatic activity increased with the increase of the temperature. Thus, temperature and induction duration must be adjusted together in order to obtain maximum soluble enzymatic activity. Henry's half-line showed that the interaction term $T \times D$ had the most important effect on the soluble activity (Figure 1). When the temperature and induction duration were tightly adjusted, a maximum production could be reached at 16 °C and 50.5 h.

The RSM approach determines relationships between the input factors and the response to provide a prediction of the optimal factor values to maximize response. Thus, this approach allowed us to predict the maximum value of the soluble HPL activity (expressed in U L^{-1} of culture) for each expression system and the optimal expression conditions (temperature, induction duration, inducer concentration) to achieve this predicted value of the enzymatic activity. The model was then subject to experimental validation by measuring the soluble HPL activity obtained in the optimal expression conditions (Table 3).

Table 3. Predicted and measured HPL soluble activity level with the optimal conditions from response surface methodology (RSM) analyses for M30, I30, B19 and A19 expression systems.

Expression System	Optimal Expression Conditions			Predicted HPL Activity ^a (U L^{-1} of culture)	Measured HPL Activity ^b (U L^{-1} of culture)
	T (°C)	I (mM)	D (h)		
M30	23	1	51.5	2197	2254 ± 19
I30	23.5	1	48.5	2250	2177 ± 20
B19	23	1	49	2277	2285 ± 12
A19	16	1	50.5	2143	2209 ± 16

Factors are coded as follows: temperature (T), inducer concentration (I), induction duration (D); ^a activity predicted by RSM analysis for 2 mL production in microplates; ^b two-milliliter production in microplates for experimental validation. Measured activity in the optimal conditions predicted by RSM analysis.

According to the model, a predicted soluble HPL activity of 2197 U L^{-1} of culture for the M30 expression system could be obtained with the optimal conditions set at a temperature of 23 °C, an inducer concentration (IPTG) of 1 mM and an induction duration of 51.5 h. Experimentally, a soluble HPL activity of 2254 ± 19 U L^{-1} of culture was recovered, which is in accordance with the predicted value, considering a standard deviation of 2.5%.

In a previous work [42], we expressed the olive recombinant HPL using the M30 expression system (pQE30/*E. coli* M15 bacteria). The bacterial culture was incubated at 37 °C until the cell density had increased to A600 0.6 to 0.8. Then, the expression of HPL was induced by the addition of 0.5 mM IPTG followed by stirring at 15 °C for 48 h. Applying this unoptimized protocol in a 2 mL expression experience in microplates, we recovered a soluble HPL activity of 399 ± 25 U L^{-1} of culture. Optimizing HPL expression in the M30 system, therefore, allows a 5.6-fold increase of the soluble enzymatic activity compared to the previous unoptimized protocol.

For the I30 expression system, the model predicted the highest value of 2250 U L^{-1} of culture for the soluble enzymatic activity with the optimal conditions set at 23.5 °C, 1 mM

ITPG and induction duration of 48.5 h. The measured experimental HPL activity was $2177 \pm 20 \text{ U L}^{-1}$ of culture and is closed to the predicted value (standard deviation of 3.2%). Otherwise, compared to the previous unoptimized studies, it represents a 5.5-fold improvement.

Concerning B19, the optimal production of 2277 U L^{-1} of culture was predicted with the optimal conditions set at 23°C , 1 mM ITPG and an induction duration of 49 h. This prediction was experimentally validated with a measured enzymatic activity of $2285 \pm 12 \text{ U L}^{-1}$ of culture, corresponding to a standard deviation value of only 0.4%. A 5.7-fold increase of the soluble enzymatic activity production was thus obtained compared to the previous assays without optimization.

Finally, regarding A19, the results of RSM showed that a maximum activity production of 2143 U L^{-1} of culture could be reached with the optimal conditions set at 16°C , 1 mM IPTG and an induction duration of 50.5 h. A soluble enzymatic activity of $2209 \pm 16 \text{ U L}^{-1}$ of culture was experimentally recovered. This result is in accordance with the predicted value, considering a 3% standard deviation. This represents a 5.5-fold increase compared to the soluble enzymatic activity production obtained with the previous unoptimized protocol.

For each expression system, the experimental results validated the model and, therefore, the optimal conditions for the production of the soluble and active HPL.

2.2. Large-Scale Production

A large-scale production (400 mL) of olive recombinant HPL was performed in a 1 L Erlenmeyer flask, using the optimized conditions determined from the model prediction. Soluble enzymatic activity reached $20,740 \pm 537$, $20,634 \pm 486$, $21,920 \pm 356$ and $20,687 \pm 980 \text{ U L}^{-1}$ of culture with M30, I30, B19 and A19 expression systems, respectively.

In a previous unoptimized work [42] using the M30 expression system, we expressed the olive recombinant HPL in 400 mL culture medium in 1 L Erlenmeyer flask, and we recovered $277 \pm 9 \text{ U L}^{-1}$ of culture. Optimization of HPL expression in M30, I30, B19 and A19 system represents, respectively, a 75, 74, 79, and 75-fold increase of the soluble enzymatic activity recovered compared to the value obtained in the previous work.

2.3. Protein Expression Analysis by SDS-PAGE and Western Blot

Identical amounts of proteins from the soluble fractions of the bacterial lysate, obtained with M30, I30, B19 and A19 expression systems, were separated by electrophoresis on 12% polyacrylamide gel in the presence of SDS and stained with Coomassie blue or transferred to membrane for Western blot analyses (Figure 3).

The Western blot allowed to specifically reveal the recombinant HPL by using a specific anti-His antibody. A band estimated around 50 kDa, corresponding to the size of an HPL subunit, can be observed on all lanes, thus confirming HPL expression (Figure 3b, lanes 1 to 4). For A19, on the gel stained with Coomassie blue, a high-intensity band around 55 kDa corresponding to chaperones (Cpn60 and Cpn10), overexpressed in Arctic Express DE3 host strain (Figure 3a, line 4) can be observed.

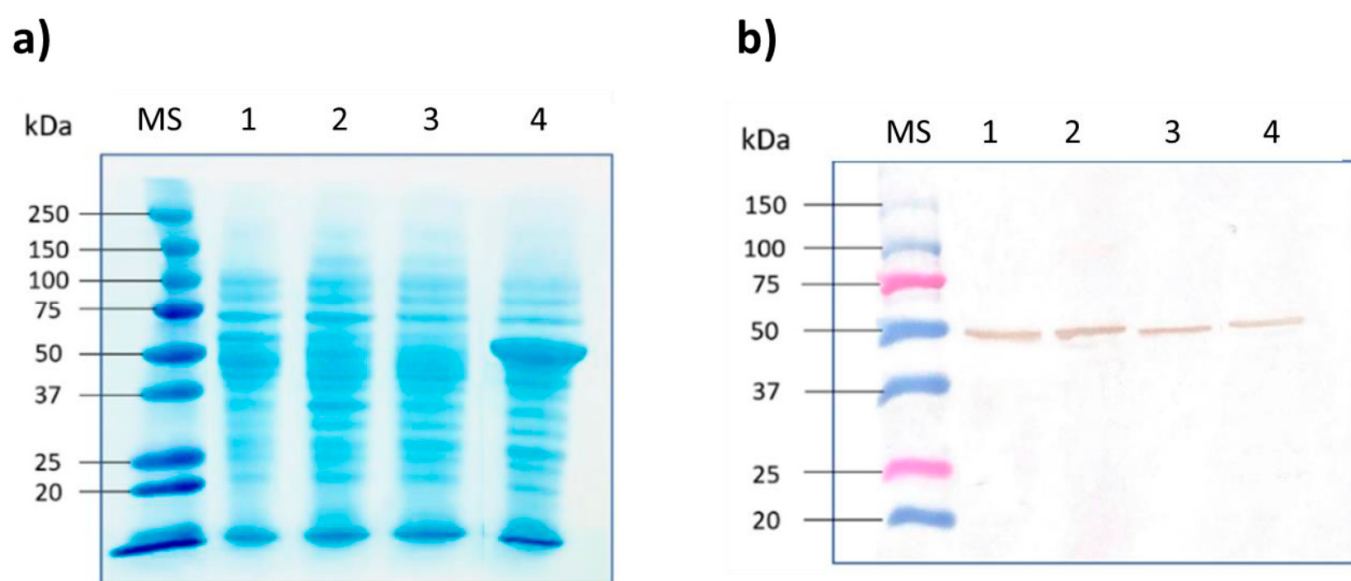


Figure 3. SDS–PAGE (12% acrylamide) analysis of proteins from the soluble fraction of cell lysate. The proteins (15 μ g for (a) and 0.5 μ g for (b)) were separated by electrophoresis then (a) stained by EZBlue gel staining reagent or (b) blotted and probed with the specific monoclonal peroxidase conjugated anti-His antibody; MS: molecular mass standard (Precision Plus protein dual-color standard, Bio-Rad, Hercules, CA, USA). Lanes 1 to 4 relate to expression, respectively, with M30, I30, B19 and A19 expression systems.

3. Discussion

In order to increase the amount of heterologous protein produced, the first approach is to test several bacterial host strains to maximize chances to produce recombinant protein in a soluble fraction. The expression vector is also a key parameter. In this study, we proposed to test four *E. coli* expression strains, M15 and IqExpress strains, based on the T5 system with pQE30 expression vector and BL21 DE3 and Arctic Express DE3 based on the T7 system with pET19b expression vector.

Besides the fact that the improvements depend on the genetic context of the recombinant cell and are protein-dependent, culture parameters that influence protein expression and folding must be systematically considered. The high concentration of nascent polypeptide chains in a recombinant cell promotes aggregation reactions at the expense of the productive reaction to the native state. The production of large amounts of the incorrectly folded protein, generating aggregates of proteins in a mostly biologically inactive state known as inclusion bodies, is the main trouble to the production of heterologous proteins [44–47,49–51]. While the aggregated protein may be easy to purify [52–54], obtaining active protein from inclusion bodies typically requires protein-specific and labor-intensive *in vitro* re-folding steps, with no guarantee of obtaining biologically active product [55]. The composition of the culture medium, the cell growth temperature, the level of transcription or translation of the recombinant gene, the overexpression of the chaperones and the inactivation of the genes coding for the proteases are all examples of parameters that can influence the amount of the soluble recombinant proteins recovered.

The option of optimizing recombinant olive HPL production by means of experimental design and response surface methodology (RSM) was attractive. The three-factor Box–Behnken design developed here in microplates was fast and efficient to optimize the soluble enzyme production. The model was well-fitted to the data, as evidenced by the high R^2 coefficients (0.97–0.98) and the small standard deviation (at most 3.2%) between the predicted activities and the measured activities.

Among the three studied factors (temperature, inducer concentration and induction duration), the temperature and induction duration were the two most important ones,

as they showed the strongest effects on the soluble enzyme activity (linear, quadratic and cross term interactions, Figure 1 and Table 2). Decreasing culture temperature slows down the cell machinery, probably diminishing protein aggregation and formation of inclusion bodies [56–59]. This method has been effective for many proteins such as luciferase, β -lactamase, rabbit glycogen phosphorylase, interferon α -2, or α -(1 $\rightarrow$ 2) branching sucrase [60,61].

In this study, the temperature of choice for maximal production of active olive recombinant HPL was 23 °C for M30 and B19 expression systems and 23.5 °C for the I30 expression system (Figure 2 and Table 3). The results showed that temperatures above this optimal temperature and especially temperatures above 25 °C resulted in a huge decrease of the soluble and active enzyme production (Figure 2). As a general rule, the rise in the temperature increases the exposure of the hydrophobic regions of the proteins, thus increasing the rate of aggregation, which participates in the formation of the inclusion bodies [62]. By reducing the culture temperature, the correct folding of the protein should be favored [62]. Indeed, the drop in the temperature has the effect of slowing down cellular metabolism with the consequence of lower protein production, which can be an advantage for better folding of the proteins produced. Noordermeer et al. [37] reported that the optimal temperature for expression of active alfalfa HPL in M15 *E. coli* cells appeared to be 25 °C. They showed that at higher temperatures, most of the expressed HPL was inactive and probably located in inclusion bodies. Delcarte et al. [38] reported that the temperature required for the expression of HPL from immature bell peppers in M15 *E. coli* cells is 20 °C and observed a 500 times lower expression at 37 °C. Vuillemin et al. [61] using RSM to optimize the production of an α -(1 $\rightarrow$ 2) branching sucrase in *E. coli* showed that the optimal temperature for the production of the enzyme in Arctic Express and BL21 strains was 23 °C. They reported that a drastic decrease of the culture temperature (down to 18 °C) resulted in a decrease in the soluble enzyme production [61].

Moreover, the temperature is tightly linked to the induction duration (Figures 1 and 2). For all considered expression systems, at the lowest temperatures (20 °C for M30, I30 and B19 or 10 °C for A19), the highest soluble activities were observed for the highest induction duration (66 h) (Figure 2). This highlights the importance of using experimental design and RSM that consider cross term interactions to establish the best compromise. For all expression systems tested, among the cross term interactions, the interaction between temperature and induction duration has the highest normalized effect (Figure 1), and is the only cross term interaction that is significant for M30, I30 and A19 expression systems (Table 2). The tight link between temperature and induction duration is most marked for the A19 system.

A less intense effect of the inducer concentration was observed for all expression systems (Figure 1), and the interaction between temperature and inducer concentration was only significant for the B19 expression system (Table 2). Even so, for all considered expression systems, 1 mM IPTG was the concentration of choice for maximal HPL production. According to Noordermeer et al. [37], for the optimal expression of alfalfa HPL in the M15 *E. coli* strain, 1 mM IPTG had to be added at A600 less than 0.8. Delcarte et al., 2003 reported that the best expression of HPL from immature bell peppers in M15 *E. coli* was obtained with 0.5 mM IPTG.

Globally, this study shows that fairly high HPL activity (up to 2285 U L⁻¹ of culture in microplates with a B19 expression system) can be recovered in the soluble fraction of the bacterial lysate produced when key parameters are tightly controlled. Optimization of HPL expression in the B19 system represents a 5.7-fold increase of the soluble enzyme production compared to the value obtained with the protocol published previously. To this end, the RSM approach is quite efficient. In the previous expression protocol, the cell growth phase was carried out at 37 °C, and the induction of protein expression with IPTG was carried out at 15 °C. This was likely to result in the production of large quantities of incorrectly folded proteins that aggregated in an inactive state. By lowering the growth temperature and keeping it identical for the induction, the correct folding of the protein

would therefore be favored. Moreover, at the temperature of 15 °C used in the previous protocol, *E. coli* chaperonins, which facilitate proper protein folding by binding to and stabilizing unfolded or partially folded proteins, could lose activity at this reduced temperature. Specifically, it has been shown that the activity of the *E. coli* chaperonin complex GroEL/ES retains only about 30% refolding activity at 12 °C [63].

During the scale-up in Erlenmeyer, the highest soluble HPL activities were obtained (up to $21,920 \pm 356 \text{ U L}^{-1}$ of culture using B19 expression system, which represents a 79-fold increase of the soluble enzymatic activity recovered compared to the value obtained in the previous unoptimized work). As it was previously described, notably by Kimata et al. [64] and Vuillemin et al. [61], levels of protein expression are often reduced in small-scale cultivation format compared to production in Erlenmeyer flasks probably due to aeration and agitation parameters that are more difficult to control. Therefore, the production of the soluble and active HPL would appear to be fairly equivalent in T5 as well as in T7 expression systems. Arctic Express cells (A19 expression system) have been specially engineered for improved protein processing at low temperatures. These cells co-express the Cpn10 and Cpn60 chaperonins, which show high protein refolding activities at temperatures of 4–12 °C. When expressed in Arctic Express cells, these cold-adapted chaperonins can provide improved protein processing at low temperatures. Analysis by SDS-PAGE of protein expression with A19 expression system showed very high amounts of produced Cpn60 and Cpn10 chaperones whose molecular weights are around 55 kDa. This value is close to the molecular weight of the olive recombinant HPL, which is 54.57 kDa. On the gel stained with Coomassie blue, chaperones and HPL appeared as a single very thick band, and it is likely that the amount of produced chaperones was higher than that of the protein of interest. Moreover, according to Vuillemin et al. [61], purification assays rapidly showed that highly overexpressed chaperones in Arctic Express DE3 *E. coli* extract troubled the automated purification (FPLC) process. The A19 expression system results in the production of a high level of the soluble and active HPL but also has drawbacks that limit its use and make it undesirable if purification of the enzyme of interest is envisaged subsequently. The B19 expression system expressing a high amount of the soluble and active enzyme ($21,920 \pm 356 \text{ U L}^{-1}$ of culture) seems suitable for the production of recombinant olive HPL. The cell growth phase to reach an A600 of 0.6 is shortened with this system compared to the A19 system (because it is done at 23 °C instead of 16 °C), making it easier and faster to use in the laboratory. The soluble HPL activity recovered in this study is much higher than that obtained by Delcarte et al. [38] who found 7900 U L^{-1} of culture of HPL activity from extracts of *E. coli* cells culture, and that obtained by Noordermeer et al. [37] who found up to 800 U L^{-1} of culture for expression of recombinant HPL alfalfa in a 10 L of *E. coli* M15 culture under optimal conditions. Bourel et al. [39] and Santiago-Gomez et al. [40] obtained 1200 to 1800 units of HPL of green pepper per liter of culture medium during expression in the yeast *Yarrowia lipolytica*. In these protocols, the biomass is obtained in batch culture, and therefore the bacterial growth will influence the conditions of the environment. The concentration of nutrients, the accumulation of inhibitor products, the concentration of dissolved oxygen, or even the change in pH are all factors that are not controlled and vary in batches. Culture in a fermenter makes it possible to control all these parameters and to eliminate those which may contribute to the appearance of inclusion bodies.

Otherwise, for each system, HPL stability during long-term storage was checked. For each system, the HPL activity was measured in the soluble fraction after a period of one month of storage at 4 °C and then compared to the values mentioned in paragraph 2.2. The results showed that 98, 96, 99 and 99% of HPL activity was recovered respectively for M30, I30, B19 and A19 expression systems. These results were in accordance with those of Noordermeer et al. [37] that showed that alfalfa HPL preparation could be stored at 4 °C for at least 1 month without significant loss of activity.

4. Material and Methods

4.1. Bacterial Strains and Expression Vectors

Four strains of *E. coli* were selected for testing. Two of these strains, M15 (QIAGEN, Hilden, Germany) and IqExpress (New England BioLabs, Ipswich, MA, USA), were used with the pQE30 (QIAGEN, Hilden, Germany) expression vector and are based on the T5 expression system. The other two strains, BL21 (DE3) (New England BioLabs, Ipswich, MA, USA) and Arctic Express (DE3) (Agilent Technologies, Santa Clara, CA, USA), were used with the pET19b (Novagen, Merck, Darmstadt, Germany) expression vector and are based on the T7 system. Genotypes of these different strains are presented in Supplementary Materials, Table S3. For all hosts/vector systems, the expression of recombinant proteins can be rapidly induced by the addition of isopropyl- β -D-thiogalactoside (IPTG).

M15 strain contains the plasmid pREP4, which confers kanamycin resistance and constitutively expresses the lac repressor protein encoded by the lac I gene. Unfortunately, the M15 strain is no longer commercially available. IqExpress strain is deficient in *lon* and *ompT* proteases and contains the lac I gene on miniF, therefore, requiring no antibiotic selection pressure to propagate. The pQE30 vector allows the cloning of the desired gene in fusion with an N-terminal 6xHis tag coding sequence and under control of the T5 promoter. Expression of the recombinant protein is induced by the addition of IPTG, which binds to the lac repressor protein and inactivates it. Once the lac repressor is inactivated, the host cell's RNA polymerase can transcribe the gene of interest cloned downstream from the T5 promoter.

BL21 DE3 and Arctic Express DE3 strains express the lac repressor protein encoded by the lac I gene and contain the λ DE3 prophage present within the chromosome. Both of these DE3 strains express T7 RNA polymerase from the lacUV5 promoter. They are used with the expression vector pET19b allowing the cloning of the desired gene under the control of the T7 promoter. The addition of IPTG induces the expression of T7 RNA polymerase, which can then transcribe the gene of interest cloned downstream of the T7 promoter. BL21 DE3 and Arctic Express DE3 strains are deficient in *lon* and *ompT* proteases. Arctic Express DE3 cells are derived from BL21 cells and are specially designed to overcome the obstacle of protein insolubility during heterologous expression. They are particularly suitable for the heterologous expression of proteins at low temperatures, which represents one interesting strategy for increasing the recovery of the soluble protein. Indeed, in Arctic Express DE3, the chaperonins Cpn10 and Cpn60 from the psychrophilic bacterium *Oleispira antarctica* are constitutively expressed from a pACYC-based plasmid that contains a gentamycin resistance gene. These chaperonins show high protein refolding activities at temperatures of 4–12 °C, which results in improved protein processing at lower temperatures, potentially increasing the yield of active and soluble recombinant protein.

4.2. Molecular Cloning and Construction of Expression Clones

In a previous work [42], the cloned olive HPL cDNA was introduced in the pQE30 expression vector at the SphI and XmaI restriction sites. The recombinant construct (pQE30-HPL) was used to transform M15 *E. coli* cells, which then allowed the expression of the olive recombinant HPL.

In this study, in order to test another strain for the T5 expression system, the recombinant construct pQE30-HPL was used to transform IqExpress *E. coli* cells. Expression clones were selected on Luria–Bertani (LB) agar plates with 50 $\mu\text{g}\cdot\text{mL}^{-1}$ ampicillin. Plasmids were extracted with Promega Wizard[®] Plus SV Minipreps DNA purification system kit and verified by restriction analyses and sequencing (GATC Biotech, Ebersberg, Germany).

Furthermore, in order to test the T7 system for the expression of the olive recombinant HPL, olive HPL cDNA was inserted into the pET19b vector using a PCR strategy with pQE30-HPL as a template. The specific primers NcoI6xHisHPLfor: 5'-atcatgccatggccatcatcatcatcatcatcagggttcgacctccgtgact-3' and XhoIHPLrev: 5'-gtgccgtctcgagtcacttggaacttcagtgattga-3', were used to introduce, respectively a NcoI restriction site at the 5' end upstream of the ATG start codon itself upstream the 6xHis tag coding sequence, and an

XhoI restriction site at the 3' end downstream of the stop codon. The PCR products were cloned in the NcoI-XhoI-oriented direction into the pET19b vector. The recombinant construct (pET19b-HPL) was then used to transform BL21 DE3 and Arctic Express DE3 *E. coli* cells. Expression clones were selected on LB agar plates with 50 µg·mL⁻¹ ampicillin (supplemented with 20 µg·mL⁻¹ gentamycin for Arctic Express). Plasmids were extracted with Promega Wizard® Plus SV Minipreps DNA purification system kit and verified by restriction analyses and sequencing (GATC Biotech, Ebersberg, Germany).

4.3. Statistical Analyses for Optimization of Culture Conditions Using Response Surface Methodology (RSM)

For each of the four *E. coli* strain/recombinant vector couples, a three-factor Box–Behnken design was used to study the combined effect of the temperature, inducer concentration and induction duration on the production of the soluble and active olive recombinant HPL. Table 4 provides details on the factor levels coded −1, 0 and 1 for low, middle and high values, respectively.

Table 4. Experimental level of factors affecting the olive recombinant HPL production for each host-vector combination.

Factor	<i>E. coli</i> Strain/Vector Construction					
	M30 I30 B19			A19		
	Coded Factor Levels			Coded Factor Levels		
	−1 (Low)	0 (Middle)	+1 (High)	−1 (Low)	0 (Middle)	+1 (High)
Temperature (T), °C	20	25	30	10	15	20
Inducer conc. (I), mM	0.1	0.55	1	0.1	0.55	1
Duration (D), h	18	42	66	18	42	66

M30 for M15 transformed with pQE30. I30 for IqExpress transformed with pQE30. B19 for BL21 DE3 transformed with pET19b. A19 for Arctic Express DE3 transformed with pET19b.

The response surface methodology (RSM) was applied to correlate the relationship between factors and to identify optimum levels of the three factors to obtain the combination of values that optimize the response. For each *E. coli* strain/recombinant vector couple, the response surface design was generated using Minitab Software (Release 18) and consisted of 45 experiments, including three repetitions. The experimental design with response values are shown in Supplementary Materials, Table S1. All experiments were conducted in randomized order.

The analyses were performed using the response surface analysis module of the Minitab software. Regression analyses were carried out to fit the mathematical model to the experimental data obtained. The following generalized second-order polynomial equation describes the contribution of the various factors on the response and was used for predicting the optimal values:

$$\hat{Y} = \beta_0 + \sum_{i=1}^I \beta_i X_i + \sum_{i=1}^I \beta_{ii} X_i^2 + \sum_i \sum_j \beta_{ij} X_i X_j \quad (1)$$

where $\hat{Y}$ is the predicted response, I is the number of factors (three in this study), β_0 is the model constant, β_i is the linear coefficient associated with factor X_i , β_{ii} is the quadratic coefficient associated with factor X_i , and β_{ij} is the interaction coefficient between factors X_i and X_j . To achieve clarity, factors X_i will be coded as follows: temperature (T), inducer (IPTG) concentration (I) and induction duration (D).

An analysis of variance (ANOVA) was carried out, and the estimates of each coefficient as well as their significance level were determined. The adequacy of models is checked by determination coefficient (R^2) analysis. p -value is checked to find out the significance of all the fitted equation terms at a 5% level of significance. The response surface plots of the predicted model were obtained by depicting two significant factors within the

experimental range and keeping the other factors at their central level. Finally, the model allowed determining each factor value leading to the optimal production of the soluble active olive recombinant HPL.

4.4. Culture in Microplates

Five milliliters of LB Broth with $50 \mu\text{g}\cdot\text{mL}^{-1}$ of ampicillin were inoculated with a single bacterial colony of freshly transformed *E. coli* BL21 DE3, Arctic Express DE3 (supplemented with gentamycin $20 \mu\text{g}\cdot\text{mL}^{-1}$), M15 (supplemented with $25 \mu\text{g}\cdot\text{mL}^{-1}$ kanamycin) or IqExpress. The cultures were incubated overnight (18 h) at 37°C with shaking (250 rpm).

Six hundred microliters of each overnight culture was used to inoculate 10 mL of culture TB medium ($24 \text{ g}\cdot\text{L}^{-1}$ yeast extract; $12 \text{ g}\cdot\text{L}^{-1}$ tryptone, 0.4% (*v/v*) glycerol; $0.17 \text{ M KH}_2\text{PO}_4$; $0.72 \text{ M K}_2\text{HPO}_4$) supplemented with appropriated antibiotics. Each culture was then incubated at different temperatures defined by the experimental design (10 – 30°C) until the OD (600 nm) reached 0.6. The expression of the recombinant enzyme is then induced by adding isopropyl β -D-1-thiogalactopyranoside (IPTG) at different concentrations defined by the experimental design (0.1 – 1 mM). δ -aminolevulinic acid (2.5 mM), which serves as the precursor for heme synthesis, is also added. The experimental design assays were performed in 24-well format plates using 2 mL of induced culture volume. The plates were incubated at desired temperatures under horizontal agitation (200 rpm) and centrifuged (30 min, $2000\times g$, 4°C) after different induction duration defined by the experimental design (18–66 h). The cell pellets were washed with 1 mL of phosphate-buffered saline (PBS). After centrifugation (30 min, $2000\times g$, 4°C), the cells pellets were resuspended in $500 \mu\text{L}$ of lysis buffer (50 mM sodium phosphate buffer pH 7 containing $0.25 \text{ mg}\cdot\text{mL}^{-1}$ lysozyme, 500 mM NaCl and 0.1% (*v/v*) Triton X-100), followed by freezing overnight at -80°C . After thawing, $0.05 \text{ mg}\cdot\text{mL}^{-1}$ of DNase I, 50 mM MgSO_4 and 1 mM of PMSF are added to each well, and then the plates were incubated for 1 h at 4°C . The HPL activity recovered in the soluble fraction (supernatant) after centrifugation (30 min, $2000\times g$, 4°C) of the crude cell extract was determined by spectrophotometry and expressed in units per liter of culture (U L^{-1} of culture).

4.5. Culture in 1 L Erlenmeyer Flask

Twenty-five milliliters of LB Broth with ampicillin $50 \mu\text{g}\cdot\text{mL}^{-1}$ were inoculated with a single bacterial colony of freshly transformed *E. coli* BL21 DE3, Arctic Express DE3 (supplemented with gentamycin $20 \mu\text{g}\cdot\text{mL}^{-1}$), M15 (supplemented with kanamycin $25 \mu\text{g}\cdot\text{mL}^{-1}$) or IqExpress. The cultures were incubated overnight (18 h) at 37°C with shaking (250 rpm).

Twenty-four milliliters of each overnight culture was used to inoculate 400 mL of culture TB medium supplemented with appropriate antibiotics. Each culture was then incubated at a temperature defined by the experimental design analyses until the OD (600 nm) reached 0.6. After the addition of δ -aminolevulinic acid (2.5 mM) and IPTG at the predicted optimal concentration, each culture was incubated for an induction duration defined by the experimental design analyses. Cells were harvested by centrifugation (30 min, $7000\times g$, 4°C) and resuspended in 50 mM sodium phosphate buffer pH 7 containing $0.25 \text{ mg}\cdot\text{mL}^{-1}$ lysozyme, 300 mM NaCl , $0.01 \text{ mg}\cdot\text{mL}^{-1}$ of DNase I and 1% emulfozene, in the proportions of 6 mL of buffer for 40 mL of culture. Then cells are disrupted by sonication before adding 1 mM of PMSF. After centrifugation (30 min, $2000\times g$, 4°C), the supernatant was collected. The HPL activity was spectrophotometrically measured and expressed in U L^{-1} of culture.

4.6. Enzymatic Assays

Hydroperoxides substrates of HPL were prepared as described by Vick [65] with adaptations. The reaction mixture (30 mL final volume) containing 70 mM sodium borate buffer pH 9 with 6.35 mM linoleic or linolenic acids, 11 mM NaOH , and 30 units of commercial soybean LOX (Sigma-Aldrich, St. Louis, MI, USA) was stirred for 30 min at 4°C under oxygen bubbling. The reaction was stopped by acidification (pH 3) with 1 M HCl .

Hydroperoxides were extracted with 3×30 mL of diethyl ether and washed with 2×30 mL of water. The synthesis of hydroperoxides was checked by TLC in solvent heptane/diethyl ether/acetic acid (55/45/1) and revealed by copper acetate/phosphoric acid (50/50). The identification of products was performed by ^{13}C -NMR, and the concentration of hydroperoxide solutions was determined by measuring absorbance at 234 nm using the extinction coefficient of $\epsilon = 25,000 \text{ M}^{-1} \cdot \text{cm}^{-1}$.

HPL activity was determined at 25 °C by continuously monitoring the decrease of absorbance at 234 nm corresponding to the cleavage of the conjugated diene system of hydroperoxide by the enzyme. The standard 1 mL reaction mixture consisted of 50 mM sodium phosphate buffer pH 7.5 and 10 μL of enzyme extract. The reaction was started by adding 43 μM hydroperoxide. One unit of HPL activity was defined as the amount of enzyme that converts 1 μmol of substrate per minute at 25 °C.

4.7. Protein Expression Analysis by SDS–PAGE and Western Blot

SDS–PAGE was performed using 12% polyacrylamide gels in the presence of 2% SDS. Proteins were stained with EZBlue gel staining reagent (Sigma-Aldrich, St. Louis, MI, USA) or transferred onto nitrocellulose (Bio-Rad, Hercules, CA, USA) and immune-revealed using the monoclonal peroxidase-conjugated anti-His antibody (Sigma-Aldrich, St. Louis, MI, USA) at 1:4000. Peroxidase activity was revealed using 1.3 mM 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Merck, Darmstadt, Germany) in PBS containing 0.01% H_2O_2 .

5. Conclusions

In this study, we were interested in optimizing the expression of olive recombinant 13-HPL, an enzyme responsible for the synthesis of flavoring compounds of industrial interest. We tested different suitable systems of *E. coli* strains/expression vectors and many expression conditions (by varying factors including temperature, inducer concentration and induction duration). Experimental design and RSM were used to find the expression conditions that result in higher soluble enzyme production.

Best levels of the soluble HPL activity were obtained with *E. coli* BL21 DE3 cells. Using the optimal expression conditions, 2277 U L^{-1} of culture of the soluble enzyme was produced in microplates. Obtaining such activity during the expression of recombinant HPL in microplates allows the development of new enzymatic engineering strategies to extend applications. Moreover, scaling-up the culture conditions in a 1 L Erlenmeyer flask allowed to produce of higher soluble enzyme activities (up to $21,920 \text{ U L}^{-1}$ of culture) that were never reported before for any HPL, which are membrane-bound enzymes and having a tendency to form inclusion bodies in *E. coli*. This result represents a 79-fold increase compared to the production levels reported with the previous protocol [42,43]. To this end, the RSM approach is quite efficient and allows to show that high yields of recombinant protein can be reached when key parameters are tightly controlled. The optimized production achieved in this study can now allow using of the olive recombinant HPL as a biocatalyst in higher-scale reactions to produce natural flavoring aldehydes with high added value.

Supplementary Materials: The following are available online at <https://www.mdpi.com/2073-4344/11/2/176/s1>, Table S1: Experimental design (Box-Behnken design) for each expression system (*E. coli* strain/expression vector). Table S2: Response surface quadratic model analysis of soluble enzyme production with M15 *E. coli* strain/pQE30 expression vector (M30), with IqExpress *E. coli* strain/pQE30 expression vector (I30), BL21 DE3 *E. coli* strain/pET19b expression vector (B19) and ArcticExpress DE3 *E. coli* strain/pET19b expression vector (A19). Figure S1: Contour plots showing the effects of cross term product factors on the soluble HPL activity level. (Only significant interactions were kept). Table S3: *E. coli* strain genotypes used in this study for recombinant protein expression and for cloning experiments.

Author Contributions: Conceptualization: S.V., V.B.-B.d.C., L.B. and J.M.; methodology: S.V., J.C., E.F. and M.M.; validation: L.B. and J.M.; formal analysis: S.V., V.B.-B.d.C., L.B. and J.M.; investigation: S.V., J.C., E.F. and M.M.; resources: S.V., V.B.-B.d.C., L.B. and J.M.; writing—original draft preparation, S.V. and J.M.; writing—review and editing, S.V., V.B.-B.d.C., L.B. and J.M.; supervision: L.B. and J.M.;

project administration: L.B. and J.M.; funding acquisition: L.B. and J.M. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Acknowledgments: This work was supported by the Collectivité de Corse and the FEDER project 2014–2020, AGRIEX No. CO 0009204.

Conflicts of Interest: The authors declare no conflict of interest.

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