Optimization of fermentation conditions for recombinant lipase expression at small scale using response surface methodology for preliminary production in two bioreactor platforms.

Angela Liliana Meza López1, Alejandro Acosta-González1, Ingrid Yamile Pulido Manrique1, Rosa Erlide Prieto1, Carlos Jiménez Junca1*

1Engineering Faculty, Bioprospecting Research Group, Universidad de La Sabana, 140013, Chía, Colombia.

*Correspondence: carlos.jimenez@unisabana.edu.co

Abstract

Background: Pseudomonas lipases are widely used in industrial applications due to its unique biochemical properties, but one of the biggest limitations are the low yields obtained in native strains therefore, organisms as E. coli are used for the recombinant lipase overexpression. However, the recombinant lipase is accumulated as inclusion bodies and it affects biological activity, making that researchers evaluate different fermentation conditions to improve the activity of recombinant enzymes. In this study, a statistical experimental design was implemented to evaluate the effects of temperature, agitation rate and osmolyte concentration on the recombinant lipase activity produced in E. coli BL21 (DE3). Once the significant variables were identified, an optimization by a Response Surface Methodology was applied to maximize the lipase production.

Results: The Box-Behnken designs revealed different optimal fermentation conditions for each osmolyte experiment. The glycerol showed the highest specific lipase activity compared to the other osmolytes and 0.1 M of osmolyte glycerol, 5°C and 110 rpm showed the highest significant increase on the specific lipase activity and the data fitted the model very well. The validation showed that 452.01 U/mg of specific lipase activity was obtained which was significantly higher compared to the group where no glycerol was added (271.38 U/mg). The relative recombinant lipase expression was 2.7-fold lower at 5°C compared to 25 °C, but at 5°C the lipase activity was significantly higher. In addition, when the 3 L shaken Erlenmeyer Bioreactor was used to produce the recombinant lipase based on the
power input parameter, the specific lipase activity was not significantly different from that found in Schott (408,4 U/mg and 452 U/mg, respectively), which means that this Bioreactor platform should be used for future scale-up processes.

**Conclusion:** Low temperatures, low agitation rates and 0.1 M of glycerol in the autoinduction media enhanced the activity of the recombinant lipase produced in *E. coli* BL21(DE3). The optimized conditions and the 3 L shaken Erlenmeyer Bioreactor can be used to produce the recombinant enzyme in a higher volume based on the power input parameter. Further studies using this strategy may lead to the identification of optimal culture conditions for a given recombinant enzyme facilitating the large-scale bioprocess implementation.

**Keywords:** *Pseudomonas*, *E. coli* BL21 (DE3), Recombinant lipase, Enzyme activity, Fermentation conditions, Box-Behnken Design Optimization, Shaken Flask Bioreactor.

**Background.**

Lipases are the most industrially important biocatalysts due to their food, pharmaceutical, leather, cosmetics, detergent, dairy and paper industrial applications [1]. Lipases (triacylglycerol ester hydrolases E.C. 3.1.1.3) are hydrolase enzymes that can catalyze the hydrolysis of long triacylglycerol substrates into fatty acids and glycerol [1, 2]. They can be found in plants, animals and microorganisms, but lipases from microorganisms are of particular importance due to the low cost and easy production [3]. *Pseudomonas aeruginosa* is a gram-negative bacterium widely known because it is an opportunistic pathogen that cause several infections in humans by the secretion of different proteins to the extracellular medium such as phospholipases and lipases [4]. Lipases from *Pseudomonas* species show a wide substrate specificity, high enantioselectivity concerning hydrolysis of racemic esters and activity in organic solvents, making them attractive to harsh industrial applications [5, 6] However, lipase production is limited to match the biotechnological demand because of low stability and low yields in the native strain [7, 8]. For this reason, the use of recombinant DNA technology to increase the lipase production is attractive for many researchers.
Recombinant DNA technology has facilitated heterologous enzyme's production in large quantities in several prokaryotic and eukaryotic expression systems [9]. However, prokaryotic organisms such as *Escherichia coli* is one of the preferred expression system due to its rapid growth, easy genetic manipulation and simplicity of cultivation [10]. Among the different *E. coli*-based expression systems, the T7 RNA polymerase system is the most popular for protein production. In *E. coli* strains like BL21 (DE3) [11]. Generally, Isopropyl β-D-1-Thiogalactopyranoside (IPTG) or lactose induce the protein production, however IPTG induction commonly leads to the formation of inclusion bodies (IBs) [12, 13]. IBs are misfolded and biologically inactive proteins, formed by bacterial folding system's failures when the polypeptide chain is folded, leading to protein accumulation in the cytoplasm [14]. Some studies recover proteins from IBs after several cycles of denaturation and renaturation, resulting in time-consuming procedures, poor recovery yields of bioactive enzyme (15-25%) and affecting the enzyme conformation [14, 15]. On the other hand, the use of fusion tags, co-expression of molecular chaperones and site-directed mutagenesis have been implemented to increase the recombinant enzyme activity. However, the enzyme activity could potentially be affected, despite this varies for each case [13, 16–18].

A novel strategy to improve the recombinant enzyme expression is the auto-induction system. While glucose is consumed, the system is repressed. Then, lactose consumption activates the *lac* promoter and induces protein expression [19, 20]. The auto-induction method is better than IPTG induction because cell density for induction do not have to be monitored, which is more effective and economical than IPTG induction. Also the protein yield is increased up to four times and the enzyme activity of the target protein is improved when using auto-inducing media [12, 21, 22]. Despite these advantages, enzyme aggregation is unavoidable and it seriously affects the enzyme activity [10]. Therefore, the optimization of fermentation conditions could significantly improve the recombinant enzyme's productivity in terms of enzyme activity.

Commonly, methods developed for recombinant protein technology are optimized using one factor at one time (OFAT), which is time-consuming and less efficient than other strategies. A better approach to evaluate the effect of different factors, using a minimum number of experiments and at the same time looking for interactions between the variables is the *design of experiments* methodology (DoE). There are distinct DoE methods, including three-level box behnken designs (BBD) that are slightly more efficient than Central Composite Designs.
, and more effective than full factorial Designs, because BBD do not contain points in which the variables are simultaneously at the highest or lowest levels and use less number of runs [10, 23]. BBD allows identifying significant variables in the process and the optimization of the response using response surface methodology (RSM) [23]. RSM is an efficient tool to optimize the culture parameters and it has been used to improve the production of enzymes such as thermophilic cellulases in *E. coli*, superoxide dismutases in *E. coli* and lipases from *Geotrichum candidum*, [24–26]. However, only a few studies have focused on the optimization of the recombinant lipase. Khurana et al. in 2017 optimized the culture conditions to enhance the lipase activity derived from *Bacillus* sp. and cloned in *E. coli* BL21 (DE3), Abu et al., in 2017 optimized the physical conditions to maximize the lipase activity derived from the yeast *Pichia guilliermondii* and cloned in the same host and Nelofer et al. in 2011 optimized the lipase production derived from *Bacillus* sp. and cloned in eight *E. coli* strains [27–29]. However, until now, there are no studies focused on optimizing fermentation conditions to maximize the activity of the recombinant lipase derived from *P. aeruginosa* and cloned in *E. coli* BL21 (DE3).

Several fermentation conditions have been implemented to enhance the production of recombinant proteins. But, low temperatures have demonstrated to be one of the most influencing parameters to increase the solubility and enzyme activity of different unstable proteins in *E. coli* [30]. Most of the studies have worked with temperatures above 23 °C for recombinant *E. coli* strains grown in auto-inducing media. Nevertheless, this is the first report that enhances the recombinant lipase activity in *E. coli* BL21 (DE3) grown at temperatures below 10 °C using auto-inducing media. The expression changes of the recombinant lipase at low temperatures were also investigated using quantitative real-time PCR (q PCR-RT). This method was chosen to quantify the relative lipase expression versus the 16 S reference gene due to its specificity, accuracy, and sensitivity. The ribosomal RNA 16 S housekeeping gene was used because it is necessary for cell survival and is considered stable in all microorganisms, even under various experimental treatments [31]. Because most of the recombinant lipase studies have mainly focused on cloning and expression of the target protein, which is usually not correctly expressed in the cell, there is still a need for improving the recombinant lipase activity produced in *E. coli* BL21 (DE3) using optimization strategies [32]. The optimal culture conditions identified by RSM that enhanced the specific lipase activity in *E. coli* BL21(DE3) at small scale were used for producing the recombinant enzyme in the two bioreactor platforms at large scale.
Few studies have reported the production on a large scale of the recombinant lipase. Gerritse et al., in 1998 showed the scale-up production of the lipase obtained from *Pseudomonas alcaligenes* and cloned in *E. coli* from 10 to 100 L and the Zhao et al., in 2008 reported the scaling-up process of the recombinant lipase obtained from *Candida rugosa* and expressed in *Pichia pastoris*, from 5 to 800 L [33, 35]. Therefore, it was important to evaluate if the optimized culture conditions could be implemented in two bioreactor platforms containing higher volumes while maintaining the same enzyme productivity and bacterial growth at the end of the cultures.

Because there are not reports performing the production of this recombinant lipase in the bioreactor platforms mentioned above and the optimal fermentation conditions to improve the recombinant lipase productivity derived from *P. aeruginosa* and cloned in *E. coli* BL21 (DE3) still remain unknown, especially at low temperatures [36–38], the goal of this research was to find the optimal fermentation conditions from temperature, agitation rate and osmolyte concentration at a small scale to enhance the recombinant lipase activity using RSM and to produce the recombinant lipase in two different bioreactor platforms a 3 L shaken Flask Bioreactor and a 2 L bioreactor using the optimized conditions found at small scale.

**Results**

**Pre-inoculum preparation and determination of inoculum growth conditions**

The recombinant *E. coli* BL21 (DE3) growth was investigated in LB and ZYM-505 medium to choose the one that exhibited the highest growth and determine the appropriate incubation time of the inoculum. The experiment shows that cells grown in LB (O.D.: 1.076) showed an optical density lower compared to the ZYM-505 (O.D.: 1.255) (Additional file: Figure 1S). According to the previous result, the ZYM-505 medium was chosen for the inoculum preparation because it enhanced bacterial growth. We also found that cells are in the logarithmic phase between 3-4 hours of incubation, which is the best growth phase in the inoculum for the inoculation of the main cultures.
Growth curves and quantification of the recombinant lipase production

To investigate the effect of temperature on the E. coli BL21 (DE3) growth and to ensure that the main cultures were stopped at the stationary phase, three growth curves were performed at 5°C, 15°C and 25 °C at 110 rpm in autoinduction media without added osmolyte for 120, 80 and 48 h, respectively. At 5°C, E. coli BL21 (DE3) exhibited a slow growth achieving an O.D. of 0.6 and CFU/mL of 3.6 x 10^8 at the end of the culture with a specific growth rate of 0.006 h⁻¹. While cells grown at 15 °C achieved an O.D. of 1.6 and CFU/mL 5.6 x 10^8 with a specific growth rate of 0.01 h⁻¹. E. coli BL21 (DE3) grew better at 25°C because it showed an O.D. of 4.0 and CFU/mL 3.6 x 10⁹ with a specific growth rate of 0.16 h⁻¹. As expected, the temperature had a significant effect on the growth kinetics (Additional File 1: Table S2). With lower temperatures, the lag phase and the specific growth rate are reduced. At the end of the E. coli growth at the three temperatures, cells achieve the stationary phase (Additional File 1: Figure 2S).

As expected with mesophilic microorganisms, temperature not only affected growth rates of E. coli BL21 (DE3), but also affected the consumption rates of carbon sources available in the culture medium. Within the optimal mesophilic growth (15-37°C) the induction of the recombinant lipase gene is performed when cells start lactose consumption once the glucose is depleted in the auto-inducing media. Cells grown at 25°C started the lipase induction earlier (21 h) compared with cells grown at 15°C (48h) (Figure 1 A-B) (Additional File: Table S3 – Figure 3S). On the contrary, at 5°C the lipase band density was visible at the beginning of growth (Figure 1 B), indicating an early induction of the lipase production and possibly an early lactose consumption. Even though, at 15°C and 25°C the recombinant lipase induction times were evident, there was a leaky expression because a faint lipase band was present on the SDS-PAGE gels from the beginning of the cultures (Figure 1 B).

The total recombinant lipase production and the lipase activity were also affected by temperature. The total production of the recombinant lipase at 5°C was 1.5 mg. When the temperature was increased from 5°C to 15°C / 25°C, a rise of 0.8 mg (1.5-fold) and 3.5 mg (3.3-fold) was obtained, respectively. The semi-quantification on the SDS-PAGE gel showed that 1.5 mg and 2.3 mg of total recombinant lipase are produced by 220 mg and 350 mg of wet biomass when cells are grown at 5°C and 15 °C, respectively and up to 5 mg of the target enzyme are produced by 430 mg of wet biomass when E. coli is grown at 25°C. Then, the total recombinant lipase yield (total
lipase / wet biomass) was higher at 25°C compared to 5°C (1.16 and 0.68%, respectively). However, these results were obtained from the total extract, it means that the amount of recombinant lipase produced in the soluble and non-soluble fraction was used for the quantification. Our results in the soluble fraction showed that decreasing the temperature from 15°C to 5°C the enzyme activity is increased in 11,10 U/mL (16 %), and decreasing the temperature from 25°C to 5°C the enzyme activity is increased in 25,31 U/mL (47 %) (Figure 2A).

Figure 2B shows the monitoring of enzyme activity during the *E. coli* cultures grown at 5 °C, 15 °C and 25 °C. During the whole culture, the recombinant lipase activity was higher in cells grown at 5°C compared to those at 15°C. The results obtained demonstrate that reducing temperatures enhances the recombinant lipase activity produced in *E. coli* BL21 (DE3).

**Effect of osmolytes glycerol, sorbitol and glycine on recombinant lipase activity**

To determine the effect of osmolytes on the specific lipase activity, different osmolytes were added to the autoinduction media and the results were compared with a control group where none osmolyte was present (Figure 3). The one-way ANOVA test demonstrated that the osmolytes significantly affected lipase specific activity with a p-value < 0.05. To determine which osmolyte had a significant effect a Dunnett test was performed (Additional File 1: Table S4-S5). The results showed that when glycerol and glycine are added to the culture media the specific lipase activity increase by 7 % and 16 %, respectively, but the increase was not statistically significant. On the other hand, sorbitol had a significant negative effect because it reduced the response by 27%.

**Optimization of lipase specific activity using Box Behnken Design**

After testing that reducing temperatures and adding osmolytes to the culture media positively affected the recombinant lipase activity, we decided to include three different temperatures and three different osmolyte concentrations on the BBD design to evaluate its effect on the specific lipase activity. Finally, we also wanted to determine if agitation rates enhanced the recombinant lipase specific activity and we assessed the effect of three different agitation rates.
We developed three models to determine the optimal fermentation conditions from temperature, agitation rate and osmolyte concentration. For each BBD representing each osmolyte glycerol, sorbitol and glycine, the lipase specific activity values obtained were analyzed by RSM, which retrieved the following equations:

1. Glycerol - Specific lipase activity (U/mg) = -35.51 - 80.25 A - 68.43 B - 47.20 C + 36.21 AB + 28.44 AC + 26.12 BC + 18.97 A² + 172.26 B² + 29.75 C²

2. Sorbitol - Specific lipase activity (U/mg) = +1.92 - 77.41 A - 43.63 B - 8.93 C + 44.10 AB - 0.66 AC + 5.07 BC + 77.27 A² + 42.15 B² - 15.29 C²

3. Glycine - Specific lipase activity (U/mg) = +183.81 + 37.90 A + 54.23 B + 41.62 C

Where Specific lipase activity (U/mg) is the response variable, A refers to temperature, B refers to agitation rate and C represents osmolyte concentration.

The lipase specific activity for each design varied significantly, but glycerol experiments showed the highest specific lipase activity. Every design created for each osmolyte was fitted with different models and specific optimal fermentation conditions could be determined. The models obtained for specific lipase activity when glycerol and sorbitol were added separately to the culture media showed a second-order model for both cases (r = 0.89, model p-value=0.04 and r = 0.90, model p-value= 0.04, respectively), where the significant variables were temperature and agitation rate. Finally, the specific lipase activity when glycine was added showed a first-order model (r= 0.55, model p-value= 0.052). Here only agitation rate was the significant variable. The statistical significance of the linear and quadratic models was determined by the ANOVA presented in Table 1 (Additional File: Table S6).

The p-value= 0.04 from the glycerol and sorbitol models indicates that the model explains the data behaviour. While the p-value= 0.052 for glycine implied that the model was not significant. The lack-of-fit values of 0.056, 0.054 and 0.249 for glycerol, sorbitol and glycine models indicate that were not significant (Table 1).
To understand the interactions of fermentation conditions and to find the optimal conditions required for maximum specific lipase activity, 3-D response surface curves were plotted. Response Surface graphs allowed to establish a relationship between the response variable and the experimental levels of the significant variables. Figure 4 A-B shows the interaction effects between the temperature and agitation rate while the third variable (osmolyte concentration) remained constant. As shown in figure 4 A-B, the specific lipase activity is maximized when the temperature and agitation rate are low. Besides, the glycerol model exhibited a higher specific lipase activity compared to the sorbitol model. However, the interaction between these two variables was not significant for no model. On the contrary, the glycine model was not subjected to optimization because only the agitation rate had a significant effect on the specific lipase activity and the model did not explained the data behavior.

According to the models, the optimum fermentation conditions for specific lipase activity in each design were 5°C - 110 rpm - 0.1 M of glycerol and 5°C - 110 rpm - 0.26 M of sorbitol. To validate the models generated by RSM, we performed 3 independent runs at the predicted optimal condition. The experimental specific lipase activity in glycerol model lead a value of 452.01 U/mg that was close (difference of 4.1 %) to the predicted value of 471.06 U/mg. The experimental specific lipase activity in sorbitol model produced a value of 269.64 U/mg, which was close (difference of 11.1%) to the predicted value 301.4 U/mg (Additional file: Figure 4S).

Additionally, the validated experiments showed that when glycerol was added to culture media, the specific enzyme activity increased significantly 1.67 times (452 U/mg) compared to the culture without glycerol (271.3 U/mg). On the other hand, when sorbitol was added, there was no significant increase in the response variable (269 U/mg) compared to the control group (271.3 U/mg). The Dunnet test was performed on each validated experiment to determine significant differences. Three statistical assumptions were tested to ensure that the experimental data obtained in the glycerol and sorbitol designs were appropriate to conduct the response surface optimization (Additional File 1: Table S7 and Figure 5S – 7S).
Quantification of the recombinant lipase for optimal fermentation conditions

Once the recombinant lipase was obtained using the optimal fermentation conditions, a semi-quantification was performed. We found that the amount of lipase produced in the soluble fraction was very low and high amount of the enzyme was produced in the insoluble fraction as inclusion bodies (Additional file: Figure 8S)

Comparing the recombinant lipase produced in the soluble fraction with the control group, it could be concluded that when we added glycerol to the culture media, the recombinant lipase's solubility increased 1.3-fold.

Sorbitol did not increase the solubility of the recombinant protein (Additional File 1: Figure 9S)

Looking for the lipase yield in the soluble fraction, it can be concluded that when cells were grown at the optimal conditions in the presence of glycerol and sorbitol, 0.13 mg and 0.06 mg of the recombinant lipase active were produced by each 140 and 210 mg of wet biomass. Nevertheless, in the insoluble fraction, we found that 1.9 mg and 1.67 mg of inclusion bodies from the recombinant lipase were produced in the same wet biomass for glycerol and sorbitol, respectively. Then, the protein yield (mg of lipase / mg of wet biomass) was higher when cells were grown in the presence of glycerol (0.09%) compared to sorbitol (0.02 %). A similar effect was seen at the insoluble fraction, where the protein yield was higher when cells were grown with glycerol (1.38%) compared to sorbitol (0.79 %) (Additional File 1: Table S8). These results confirm that 0.1 M of glycerol enhanced the specific lipase activity and the amount of recombinant lipase produced in the soluble fraction by E. coli BL21(DE3).

RNA quantification and relative expression of the recombinant lipase at different temperatures

The qPCR-RT performed to investigate the differences in lipase expression when E. coli BL21 (DE3) was grown at 25°C, 15°C and 5°C (Control) showed that levels of lipase mRNA were markedly elevated when the recombinant lipase was produced at 25°C and 15°C compared with 5°C (2.7-fold and 1.3-fold, respectively). These measurements confirm the low production of the recombinant lipase observed in SDS-PAGE gels at low temperatures and suggest that the lipase gene is highly expressed at higher temperatures compared to the lower ones (Figure 5). It is crucial to highlight here that even though the expression level of the recombinant lipase at 5°C is lower compared to 15°C and 25°C E. coli cultures, the specific lipase activity was higher at 5°C.
than 25°C as reported previously (Figure 2), confirming that the enzyme productivity (U/mg) is higher at low temperatures.

**Production of the recombinant lipase from E. coli BL21(DE3) in two bioreactor platforms**

Once the optimal fermentation conditions were validated in Schott, we used the same optimal conditions to produce the recombinant lipase in two different bioreactor platforms to evaluate if the enzyme productivity and bacterial growth at the end of the culture remained the same as in Schott. The first platform, a 3 L shake flask Erlenmeyer and the second one a 2 L Bioreactor.

When we produced the recombinant lipase in the 3 L Shake Flask Bioreactor and 2 L Bioreactor the optical density remained very similar at the end of the cultivations (O.D. 600 nm = 0.600), but the CFU/mL showed significant differences for both Bioreactors (Additional File 1: Table S9-S11). At the end of the culture the 3 L Shake Flask Erlenmeyer exhibited a higher number of viable bacteria compared to Schott (2.73 x 10^8 and 1.76 x 10^6, respectively) and a higher the specific growth rate compared to Schott (0.003 h^-1 and 0.001 h^-1, respectively). On the other hand, the specific enzyme activity was slightly lower in Shake Flask Erlenmeyer compared to Schott (408.4 U/mg and 452.0 U/mg, respectively). However, this difference was not significant, which means that in terms of enzyme activity, the optimal culture conditions could be effectively used in shaken flask bioreactors with higher volumes, up to 3 L (Figure 6) (Additional File 1: Table S12-S12).

Concerning the production of the recombinant lipase in the 2 L Bioreactor, we found a lower number of viable colonies compared to the Schott (1.32 x 10^8 and 1.76 x 10^6, respectively) and consequently a significant reduction in the specific lipase activity from 452 U/mg in Schott to 337.9 U/mg in the 2 L Bioreactor (Figure 7). Even though the specific growth rate was similar in Bioreactor and Schott (0.002 h^-1 and 0.001 h^-1, respectively), the viability was much lower than in Schott.

**Discussion**

Bacterial growth depends on different nutrients constituting a culture medium and each one can stimulate different growth patterns depending on their combinations [39]. Our first results indicated that the culture media ZYM-505 exhibited greater optical density growth compared to LB medium due to a more complex
These results agree with those reported by Mihasan in 2007 where they found that the biomass production in *E. coli* was higher in ZYP (24.44 g/L) medium compared to LB (14.62 g/L). On the other hand, the inoculum’s logarithmic phase was selected because in the main cultures, the lag phase is shortened and exhibits the maximal growth rate.

It is widely known that the yield and specific growth rate of many bacteria are affected by temperature [42]. The optimal growth temperature for *E. coli* BL21 (DE3) is 37 °C with a $\mu_{max} = 1.05$ h$^{-1}$, however, our study performed experiments below the optimal temperature to enhance the activity of the recombinant enzyme [43]. Our results showed that low temperatures exhibited lower specific growth rates compared with higher temperatures, this is explained because the reaction rates are lowered, conducing to decrease growth and product synthesis [42]. Similar results were reported, where at 18 °C the growth rate for *E. coli* was 0.2 h$^{-1}$ and at 30°C was about 0.5 h$^{-1}$ [44].

Temperature also influenced the induction of the lipase gene expression and the total amount of recombinant lipase produced. Low temperatures delayed the consumption of lactose, but once cells start consuming lactose, the *lac* repressor is released from its binding site in the T7 *lac* promoter and the induction of the T7 RNA polymerase is achieved by the allolactose [20]. On the other side, high temperatures produced a higher total amount of the target enzyme than low temperatures. It is possibly explained because the peptide chain elongation rate during transcription is increased when the temperature rises. Consequently, the synthetic protein capacity at high temperatures is overwhelmed, provoking an increase in proteins not correctly folded [45]. A leaky expression was also observed at the beginning of the cultures, explained because even in the absence of lactose there is always a basal expression of the recombinant gene caused by the negative control of the *lac* promoter [46].

Our study demonstrated that the recombinant lipase activity in the soluble fraction was higher at low temperatures. According to previous studies, this is explained because the hydrophobic interactions in the protein are reduced, transcription and translation rates are lowered, giving enough time to cells to correctly fold chaperone-assisted lipase and consequently enhance the biological activity of the target enzyme [32, 47]. In this study, enzyme activity increased when the temperature was decreased from 25°C to 5°C. Similarly, Wang
and collaborators in 2009 increased the enzyme activity of the alcohol oxidase when decreasing temperature from 30 °C to 22 °C (1120 U/mL to 1611 U/ml, respectively)[48].

Bacterial culture temperature is one of the most crucial factors that must be optimized for increasing the recombinant enzyme activities and yields because it influences cell growth and protein synthesis [49]. Our study showed that the optimal temperature for glycerol and sorbitol experiments was the lowest temperature 5°C, probably due to different events occurring at this condition like induction of cold-shock proteins (CspA) and the reduction in protease activity, inclusion bodies formation and synthesis of cellular proteins [30, 32]. Our results agree with those reported by Jham and Sahoo where the enzyme activity of r-Xynb (recombinant xylanase) was increased by 5-fold when decreasing the temperature from 37 °C to 20 °C [32].

Another variable evaluated was the osmolyte concentration, that did not show a significant effect in the glycerol and sorbitol models. However, when we analyzed separately the osmolyte effect on the specific lipase activity we found that glycerol and glycine increased significantly the response variable. Glycerol may have stabilized the lipase activity by interacting electrostatically with N and O atoms in the enzyme and forming a complex glycerol-water around the protein. This complex decreases the hydrogen-bond rupture enhancing the solvation layer and provoking that hydrophobic groups of the lipase migrate inside the enzyme avoiding this way enzymatic aggregation [50, 51] Our results, agree with those reported by Baumer in 2017 where the catalytic activity of a recombinant esterase produced in *E. coli* BL21 (DE3) is increased when the enzyme was exposed to glycerol (0-10%) [52].

Additionally, glycine enhanced the lipase activity because, according to literature, it modifies the peptidoglycan layer structure changing the membrane permeability and increasing its release to the extracellular space [53] Glycine also might be transported in *E. coli* using the glycine transport system (Cyc) and later is used to help the protein synthesis [54]. These results agree with those reported by Hong in 2019 and Aristidou in 1993, where after supplementation at 1% with glycine, the extracellular enzyme activity of the recombinant Sortase A and alfa-amylase produced in *E. coli* BL21 was 6-fold and 16.3-fold higher than the control group, respectively [53]

The agitation rate was another variable optimized in this study. We found that low agitation rates (110  rpm) enhanced the recombinant lipase activity probably because the oxygen availability influences the lipid and
protein composition of the outer membrane allowing the passive leakage of recombinant proteins [37]. The limitation in the respiration rate due to low agitation rates decreases the cell metabolism and consequently, the protein synthesis, giving more time to the chaperone to assist the lipase folding [55]. Our results agree with those reported by Zafar et al., where the enzyme activity of the recombinant α-amylase produced in *E. coli* BL21 (DE3) was increased when the agitation rates were reduced from 300 rpm (1.18 U mL⁻¹ min⁻¹) to 200 rpm (5.43 U/ml⁻¹ min⁻¹) [56].

The optimization results showed that the lipase expression at the lowest temperature and agitation rate, and glycerol added to the medium displayed the best conditions to enhance the specific lipase activity in *E. coli* BL21 (DE3). Despite finding the optimal culture conditions, most of the recombinant lipase was still produced in inclusion bodies and only a small amount was present in the soluble fraction. According to Kano et al., lowering temperatures can increase recombinant protein solubility, but it is specific of each protein [57]. Similar studies have decreased the temperature from 37°C to temperatures below 20°C but without a significant increase in the solubility of different recombinant proteins produced in *E. coli* [32, 58, 59].

When *E. coli* BL21 (DE3) was grown at low temperatures (5°C) we found that the relative lipase expression level was lower compared to higher temperatures (25°C), but the specific lipase activity was higher at low temperatures. This result might occur because at high temperatures the total recombinant lipase not correctly folded is increased, forming inclusion bodies that drastically reduce the enzyme activity [32, 47]. Therefore, low temperatures can reduce the expression level of the recombinant lipase, but this reduced expression is necessary to enhance the specific lipase activity in *E. coli* BL21 (DE3) and consequently enhance the appropriate folding of the enzyme.

When *E. coli* BL21 (DE3) was grown in the Shaken Flask Bioreactor the viable cell counts were higher than Schott. According to previous studies, cotton plug in shaken flask Bioreactor could have improved the gas transport of oxygen and carbon dioxide through the sterile closure by diffusion and convection [60, 61]. However, in Schott’s, screw caps were used for bacterial cultures instead of cotton plugs, this could have limited
gas diffusion inside the vessel and limit the oxygen transfer rate, affecting bacterial growth [62]. Low agitation rates might have enhanced the exposure time of the liquid film on the bioreactor wall to the air, increasing this way of dissolved oxygen concentration in the liquid [63].

On the other hand, Bioreactors are commonly equipped with Rushton turbines for large-scale applications due to the high oxygen transfer rates they can achieve [64]. On the contrary, the anchor type has a low power number, achieves a high-quality mixture and a homogeneous environment in substrates and dissolved oxygen, enhancing mass transfer [65]. Firstly, it is highly probable that the anchor impeller’s stirring rate used in the 2 L Bioreactor could have provoked cell shear stress, morphological cell changes, lysis, death, and recombinant lipase productivity changes. Similar results, have reported a reduction in 80% of E. coli viability, when exposed to 1250 Pa of threshold tolerance to shear stress and fragmentation of mycelia from Aspergillus oryzae when using this impeller [65, 66]. The previous studies highlight the importance of establishing a threshold to avoid shear stress in E. coli. Secondly, the low cell viability and enzyme productivity can also be associated with a low oxygen transfer rate (OTR) due to the absence of an air sprinkler at the bioreactor’s bottom. Aeration in the system enhances the oxygen mass transfer because the impeller breaks the air bubbles and the superficial area is increased [67].

In conclusion, our study results suggest that the optimization of fermentation conditions in E. coli significantly enhanced the enzyme productivity in terms of specific lipase activity. Low temperatures, low agitation rates and 0.1 M of glycerol maximized the specific lipase activity. Even though, the recombinant lipase relative expression was lower at low temperatures compared to high temperatures, the lipase activity was higher when E. coli BL21(DE3) was grown at 5°C compared to 25°C. On the one hand, when the recombinant lipase was produced in the shaken Erlenmeyer Bioreactor using the same optimal culture conditions for Schott experiments, the enzyme productivity was not significantly different, suggesting that using this bioreactor can be a useful alternative for lipase production for industrial purposes. On the other hand, it is essential to consider the type of impeller for Bioreactor and to establish a threshold to avoid shear stress during cultivation to maintain the lipase productivity and cell growth identical to the small scale. This study represents a reliable and low-cost strategy for recombinant lipase production on E. coli BL21(DE3) through the optimization of
fermentation conditions and its production in two bioreactor platforms of higher volumes, which provides additional information for the future scale-up processes.

**Methods**

**Bacterial strain**

The strain *E. coli* BL21 (DE3) strain containing the plasmid PACYC harbouring the LipA (Lipase) and lif (Lipase specific foldase) genes derived from *Pseudomonas aeruginosa* was used in the present study (Sigma- Aldrich - St. Louis, MO, USA). In the recombinant plasmid PACYC was previously designed by Pulido *et al.* 2020, the LipA sequence was removed from its signal peptide and had 861 bp, which encoded a protein with 285 residues and a molecular weight of 30.1 kDa [68]. The lif sequence had a length of 867 bp after removing 156 bp encoding for an inner membrane anchor and with a molecular weight of 32.4 kDa. The strain was conserved at -80 °C in ultra-pasteurized skim milk until pre-inoculum and inoculum preparation[68].

**Pre-inoculum preparation and determination of inoculum growth conditions**

For the pre-inoculum, one single colony was inoculated in 20 mL of LB and ZYM-505 medium and was grown overnight under continuous shaking 150 rpm and 37°C until cells reached an O.D. 0.9 (3.6 x 10^7). To determine the appropriate culture media and the incubation time for the inoculum, an optical density growth curve was performed in LB (5g/L Yeast Extract, 10g/L Na Cl and 50 μg /mL of chloramphenicol) and ZYM-505 medium (0.5 % yeast extract, 25mM Na₂HPO₄, 25 mM KH₂PO₄, 50 mM NH₄Cl, 5 mM Na₂SO₄, 2 mM MgSO₄ and 0.2x Trace metals, 0.05% glucose and 50 μg /mL of chloramphenicol). For the inoculum preparation, 10% of the pre-inoculum from each culture media was used to inoculate 20 mL of LB and ZYM-505 medium respectively and were grown under continuous shaking 150 rpm at 37°C. Absorbance was measured at 600 nm in a spectrophotometer (Genesis10S UV-VIS, Thermo scientific, MA, USA) each 1 hour for 8 hours until cells reached stationary phase. At the end, the culture media with the higher optical density and the incubation time that represented the exponential phase were chosen for the inoculum preparation and for the inoculation of the main cultures containing auto-inducing media at the different temperatures, agitation rates and osmolyte concentration mentioned later.
Growth curves and quantification of the recombinant lipase production

Three different *E. coli* BL21 (DE3) growth curves for optical density and CFU/mL were performed at 5, 15 and 25°C for 120, 80 and 48 h respectively (Incubator shaker - New Brunswick Innova 42). The agitation rate of 110 rpm was kept constant and cells were grown in auto-inducing media. The pre-inoculum and inoculum were prepared as mentioned above for each growth curve and 10% of the inoculum was inoculated in each flask containing 20 mL of auto-inducing media. All growth curves were performed using the destructive method and approximately 3-5 points were taken per day to build the curves.

The growth was monitored by measuring the absorbance at 600 nm in a spectrophotometer (Genesis10S UV-VIS, Thermo scientific, MA, USA). For viable cell count 50 µL of each sample was serially diluted 10-fold with saline water (0.85%), 100 µL of each dilution was separately plated on LB agar supplemented with 50 µg/mL of chloramphenicol and colonies were counted after incubation for 24 h at 37°C. For all the growth-curve measurements, two replicates were carried out. The specific growth rate, \( \mu \), was calculated from the slope of the line obtained by plotting the natural log of cell biomass (CFU/mL) against time (h), according to the equation

\[
y = mx + c, \text{ where } y = \ln \text{(CFU/mL)}, m = \text{the slope, } x = \text{time (h) and } c = \text{intercept.}
\]

The relative band density of the recombinant lipase in the total extract was quantified in the gels using Image J software to determine the total amount of the recombinant lipase at the end of the culture and the induction time of the recombinant lipase at each temperature. The enzyme activity in the soluble fraction was monitored in each sample time for the different growth curves.

The total recombinant lipase production yields were calculated for each temperature from the densitometric analysis in the Image J software (mg of total recombinant lipase / mg wet biomass).

Effect of osmolytes glycerol, sorbitol and glycine on recombinant lipase activity

For this experiment, 10% was picked from the inoculum and grown in 20 mL of autoinduction media at 5°C and 150 rpm for 120 h. Four different treatments were carried out: 1. Control group (Without osmolyte) 2. Glycerol (0.1 M) 3. Sorbitol (0.1 M) 4. Glycine (0.07 M). When the culture finished, cells were harvested by centrifugation...
at 6500 rpm for 10 min at 4 °C. Harvested cells were lysed and the specific lipase activity was determined in the soluble fraction following the protocol mentioned later.

**Main cultures: Determination of optimal fermentation conditions**

Three Box Behnken Design (BBD) were created in Design Expert Software (V. 12.0 MN, USA), each one representing each osmolyte evaluated. Based on the literature, three factors were selected as independent variables: temperature, agitation rate and osmolyte concentration. Each variable was evaluated in three different levels: Temperature 5 °C, 15 °C and 25 °C, Agitation rate 110 rpm, 150 rpm and 250 rpm and osmolyte concentration for glycerol and sorbitol (0.1 M, 0.4 M and 0.7 M) and glycine (0.03 M, 0.07 M and 0.1 M). Each design comprised a total of 17 experiments, including 12 factorial points and 5 center points. All experiments were carried out in triplicate and the average of the lipase specific activity (U/mg) was taken as a response (Additional file 1: Table S1). The predicted lipase specific activity was calculated using first and second-order models equations:

\[ Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 \]

\[ Y = \beta_0 + \sum \beta_{ij} x_i x_j + \sum \beta_{ii} x_i^2 \]

Where \( Y \) is the predicted response, \( \beta_0 \) is the coefficient of interception, \( X_1 \) is the temperature, \( X_2 \) is the agitation rate, \( X_3 \) is the osmolyte concentration, \( \beta_i \) is the coefficient of linear effect, \( \beta_{ii} \) is the coefficient of quadratic effect and \( \beta_{ij} \) is the coefficient of interaction effect.

Once the BBD were created, the experiments were performed at the different fermentation conditions and were auto induced for 120, 80 and 48 h for the temperatures 5 °C, 15 °C, 25 °C, respectively. For this, cells were grown in autoinduction media that contained ZY (10 g/L bacto tryptone, 5 g/L yeast extract), solution 20 x NPS (For 100 mL: 6.6 g 0.5 M (NH₄)₂ SO₄, 13.6 g 1 M KH₂PO₄ and 14.2 g 1 M Na₂HPO₄) (PanReac, Applichem, Germany) solution 50 x 5052 (For 100 mL: 25 g glycerol, 73 ml water, 2.5 g glucose and 10 g α-lactose), trace elements (20 µM CaCl₂, 10 µM of FeCl₃, MnCl₂ and ZnSO₄, 0.1 µM CoCl₂, and 0.05 µM of CuCl₂ and NiCl₂) and 1 M Mg SO₄ supplemented with 50 µg/mL of chloramphenicol (J.T. Barker, USA and Sigma-Aldrich Co., St. Louis, MO, USA) [20].
When the cultivation period ended, cells were harvested by centrifugation at 6500 rpm for 10 min (Benchtop centrifuge, Thermo Scientific, MA, USA). The bacterial pellet was resuspended in lysis buffer (5 mL/g of cell pellet) containing 20 mM Tris HCl pH 8, NaCl 250 mM, glycerol 5%, DNase, 0.3% v/v of triton X-100, lysozyme 60 µg/mL, PMSF 0.5 mM and incubated at 37 °C for 40 min [69, 70]. The suspension was centrifuged at 13000 rpm for 20 min at 5°C to obtain the soluble and insoluble fraction (Inclusion bodies). The supernatant containing the soluble recombinant lipase was used to determine enzyme activity, specific lipase activity, total protein content, recombinant lipase quantification by SDS-electrophoresis and lipase yield production (mg of total recombinant lipase/ mg wet biomass).

Lipase assay curves, osmolyte test and main cultures.

The enzymatic activity was determined in the soluble fractions by p-nitrophenyl palmitate (p-NPP) hydrolysis by a microplate reader spectrophotometer (Microplate Absorbance Reader MpM6, Biorad, USA), following the method previously performed by our group. An aliquot (10 μL) from the soluble fraction was added to 440 μL of reaction media containing p-NPP (1 mM), Tris HCl (pH 8.2; 20 mM), Triton X-100 (0.4%), CaCl₂ (5 mM) and gum arabic (100 mg/100 mL). The reaction mixture was incubated for 20 min at 37 °C. The lipase activity was determined by measuring the release of p-nitrophenol (p-NP) at an absorbance of 410 nm. One lipase unit was defined as the amount of lipase capable of releasing 1 nmol of p-NP per 1 min [16]. The enzyme productivity of E. coli BL21 (DE3) was evaluated by enzyme activity assay in the soluble fraction. The lipase activity and the specific lipase activity were calculated using the following formulas:

\[
\text{Lipase activity (U/mL)} = \frac{\Delta \text{Abs/min} \times \text{total volume (mL)}}{\varepsilon \times \text{path length} \times \text{sample volume (mL)}}
\]

\[
\text{Specific Lipase Activity (U/mg)} = \frac{\text{Enzyme activity (U/mL)}}{\text{Total protein (mg/mL)}}
\]

Stock solution of p-NP 10 mM was dissolved in buffer tris-HCl pH 8.2 with 0.4% triton x-100 (v/v) to reach a final concentration of 1 mM (Sigma-Aldrich Co - St. Louis, MO, USA). The p-NP 1 mM was used to build a calibration curve of p-NP in a concentration range of 0 – 0.05 mM at three different absorbance values 400 nm, 410 nm and 415 nm using a Microplate reader [16, 29]
Total protein content was measured in the soluble fraction following the Zor & Selinger Bradford Method Assay (1996) modified by the Research Group of the University [71]. The assay was carried out in a 96-well plate with a final volume of 250 µL per well and the plate was incubated at room temperature for 5 minutes. The analysis was measured at 595 nm and 450 nm in a Microplate reader. A calibration curve was made with bovine serum albumin (BSA) standard protein.

Polyacrylamide gel electrophoresis (SDS-PAGE)

Total extract of samples obtained in the growth curves and soluble/non-soluble fractions of the samples obtained in the optimal fermentation condition experiments were analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% gel slabs in a Mini-protean Precast Gels (Bio-rad, USA) at 200 V for 1 h. A molecular weight marker was added in one lane and samples were mixed with loading buffer (1M Tris HCL pH 6.8, SDS 10%, 0,1% bromophenol blue, 100% glycerol and 14,3M β-mercaptoethanol) and then boiled for 12 minutes. Subsequently, gels were stained with Coomassie Blue R250. The gel obtained from SDS-PAGE was analyzed using the software Image J to quantify the recombinant lipase band's intensity (Additional file: Table S14).

BSA (3 µg) was used as a standard protein to calculate relative band density in gels. The following equation was used to determine the amount in µg of recombinant lipase produced:

\[
\text{µg of recombinant lipase} = \frac{\text{Peak area protein band}}{\text{Peak area BSA band}} \times 3
\]

The supernatant was directly used for soluble protein content analysis with SDS-PAGE, while the insoluble fraction was first solubilized. The insoluble fraction was resuspended in 500 µL of washing buffer (Tris HCL 20 mM, Na Cl 300 mM, Triton 1% and Urea 1 M) and the pellets were washed three times and centrifuged at 13.000 rpm for 20 min. Later, the pellet was washed two times with PBS 50 mM (137 mM NaCl, 2.7 mM K Cl, 8 mM Na₂HPO₄ and 2 mM KH₂PO₄ · pH 8.0) to remove the contaminating detergent and centrifuged again at 13.000 rpm for 20 min. The pellets were resuspended in PBS + 2 M urea and were frozen at -20 °C for 24 h and later...
thawed at room temperature. Finally, samples were centrifuged again and the supernatants were analyzed by SDS-PAGE [14].

**Total RNA extraction and RNA Purity and Integrity.**

Cultured *E. coli* cells (5 mL) at 5°C (optimal culture condition), 15°C and 25 °C were collected by centrifugation (6000 rpm, 10 min, 4°C) and total RNA was extracted from cells using InnuSPEED Bacteria/Fungi RNA kit (Analytik jena, Berlin, Germany) according to the manufacturer’s protocol. RNA pellets were stored at -80°C in DEPC water until analysis. RNA samples were quantified with a Qubit fluorometer (Invitrogen, United States) measured at 260 nm, using RNA kit (Invitrogen, California, United States). The presence of DNA was verified by PCR amplification of 16 S gene and the residual contaminating DNA was removed with RNase-free DNase I (2000 U; AMB). For the DNase treatment, 10 µg of the total RNA was mixed with 10 µL of DNase treatment (0.02 U/µL of DNase, 1 µL of DNase Buffer, 8.7 µL of DEPC water) and incubated at 37°C for 10 min. Finally, the DNase enzyme was inactivated using 1 µL of 25 mM EDTA and tubes were heated for 10 min at 65°C. The DNA degradation was verified again by PCR amplification of 16S gene using RNA extract containing 1 µg of RNA as the PCR template (Additional File 1: Figure 6S)

RNA purity and integrity were verified using denaturing DEPC agarose gel (2.5%) analysis. 8 µL from each sample was added to each lane of the gel. The agarose gel was run at 90 V for 30 min and three bands were visualized under UV light to confirm RNA integrity 23 S, 16 S and 5 S (Additional File 1: Figure 10S-11S)

**Quantitative Real Time PCR for recombinant lipase analysis.**

An SensiFAST™ SYBR® No-ROX One-Step Kit q RT-PCR KIT (Bioline, Tenesse, United States) was used according to the manufacturer’s instructions. Primers were designed to amplify the lipase gene (Nucleotide accession number reported by Pulido et al., 2020 at Genbank: MK336958) [68]. Primers for LipA, forward (CTCGGCTTGCAACACATCCT), reverse (CTTCGGTGACGTAGACCTGG) and primers for 16 s, forward (ACTCCTACGGGAGGCAGCAG), reverse (ATTACCGCGGCTGCTGG) were used to detect the recombinant lipase and 16 s gene for relative expression. Primers were designed using the primer designing tool from the NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and the best primers were selected according to the criteria commonly used for good primer design [72].
Briefly, 10 µL of PCR reaction mixtures contained 5 µL of Sensifast 2X SYBR Green PCR Master Mix, 2 µL of total RNA (Concentration 1 µg), 0.8 µL of Primer Forward and Reverse for LipA (Concentration 0.4 µM), 0.2 µL of reverse transcriptase, 0.4 µL of RNase inhibitor and 3.8 µL of DEPC water. Separate PCR reaction mixtures were performed for LipA and 16 S amplification. Non-template controls (NTC) were run as a negative control of the reaction for each set of primers. The real-time PCR reactions were carried out in duplicates in low profile white PCR tubes for production of 91 pb of lipase and 180 pb for 16 s gene.

In brief, the real-time PCR was performed in a CFX96 touch system (Bio-rad, California, United States) using the following cycles: 45 °C for 10 min, 1 cycle (Reverse Transcription), 95°C for 10 min (Polymerase activation), followed by the following cycling parameters: 95 °C for 5 s (Denaturation) and 55.7°C for 20 s (Annealing/extension). The CFX Maestro Software was used to determine the cycle threshold (Ct) point of each reaction. The Ct point of the three samples 5°C, 15°C and 25°C was defined as the duplicate’s average of the Ct data. Values were normalized to the reference gene 16 S rRNA (E. coli) used as the internal control. The relative quantity of LipA gene expression was calculated using the comparative cycle threshold (ΔΔCt) method. The specificity of PCR amplification was confirmed by melting curve analysis between 50 °C and 95°C.

The optimal annealing temperatures of the LipA and 16S primers were evaluated and the appropriate target sample concentration to achieve a sigmoidal curve during PCR-RT amplification was determined:

- **Optimal annealing temperature**: Five different temperatures were tested for optimal amplification of LipA and 16 s gene (55.0 °C, 55.7 °C, 57.0 °C, 59.0°C, 61.4 °C). NTC were run as a negative control of the reaction for each set of primers.

- **Optimal Sample concentration**: Serial dilutions 1:10 of total RNA were tested (10 ng, 1 ng and 0.01ng). The test was used to choose the appropriate RNA concentration in the original samples for 16 S and LipA genes. NTC were run as a negative control of the reaction for each set of primers.

**Recombinant lipase production and growth curves in two Bioreactor platforms**

The optimal fermentation conditions obtained from the BBD that showed the highest specific lipase activity were used in the 2-L bioreactor (IKA EUROSTAR 200 P4 Control) and the 3 L Shake Flask Bioreactor to produce
the recombinant lipase in higher volumes. Both bioreactors were inoculated with 10% of the total effective volume. For the 2 L Bioreactor containing 1,6 L of autoinduction medium, 10% of the pre-inoculum was inoculated in 160 mL of ZYM medium to prepare the inoculum. This inoculum was used to inoculate the 2 L bioreactor that was maintained at 5°C, 131 rpm for 120 h.

For the Shake Flask Bioreactor containing 600 mL of autoinduction medium, 10% of the pre-inoculum was inoculated in 60 mL of ZYM medium to prepare the inoculum. This inoculum was used to inoculate the Shake Flask Bioreactor that was maintained at 5°C, 162 rpm, for 120 h. The specific lipase activity was calculated at the end of both cultures and cell growth was monitored during the whole cultivation.

In this work, the power input (P/V) in shake flasks was assumed to be the same as reported by Gamboa and collaborators in 2019, where they calculated a P/V of 0.20 kW/m3 in conventional flasks using a C25 Incubator Shaker, New Brunswick Scientific, the same instrument used in our study (Gamboa et al. 2019). The P/V was used as the criterion to change the volume of production of the recombinant lipase from Schott 100 mL to the 2 L stirred-tank bioreactor and from Schott 100 mL to 3 L Shake Flask Bioreactor. The bioprocess was run in duplicate in both bioreactors. The 2 L bioreactor was equipped with an anchor impeller (Geometry dimension 0.9 – 0.98 d2/d1 where d1: container diameter, d2: stirrer diameter).

The unaerated power number (Np), defined by dimensional analysis is given in the following equation and was used to set the stirrer speed of the impeller in the case of the 2 L Bioreactor and the agitation rate in the 3 L Shake Flask Erlenmeyer [73]:

\[ N_p = \frac{P}{\rho N^3 D^2} \]

Where: P = Power (W), \( \rho \) = liquid density (kg/m³), N = shaking frequency, number of revolutions (s⁻¹), D = stirrer diameter or maximum inner diameter of flask (m).

Once the Np number was calculated, the following equation was used to obtain the P/V for each case [74]:

\[ \frac{P}{V} = (N_p \times \rho \times N^3 \times d^5)/V \]

Where Np= power number, \( \rho \)= liquid density (kg/m³), N= shaking frequency, number of revolutions (s⁻¹), d = stirrer diameter or maximum inner diameter of flask (m), V= vessel working volume (m³)
Statistical analysis

The validity of the model and the effect of independent variables on the response were assessed using analysis of variance (ANOVA, p-value < 0.05 = Significance level) [75, 76]. Multiple correlation coefficient ($R^2$) and adjusted $R^2$ were used to assess the first and second-order equation fitness. The ANOVA was also used to determine the significance of the equation model using a lack-of-fit test. Three statistical assumptions were checked in the data: Normality of residuals by Smirnov-Kolmogorov test, homoscedasticity of the residuals by Levene's test with a 95% significance level and finally for independence. Dunnet test was performed to evaluate the effect of osmolytes on the recombinant lipase activity and the significant enzyme activity and growth differences between schott scale, Erlenmeyer and Bioreactor.

The selected significant variables for each BBD were subjected to optimization. Response surface methodology (RSM) was used to optimize the fermentation parameters that maximized the specific recombinant lipase activity. The relationship between the variables and the response was exhibited through three-dimensional surface plots.

Figures

Figure 1. Relative Band Density of recombinant lipase in total extract of cultures grown at different temperatures. A. The band density of the recombinant lipase was determined in the total extract by densitometric analysis of SDS-PAGE. The total extract for each sampling time was analyzed. B. Total extract was separated by SDS-PAGE and stained by Coomassie blue. Error bars are presented in percentage.
Figure 2. Recombinant lipase production and enzyme activity at different temperatures. A. Recombinant lipase production in total extract and the enzyme activity in the soluble fraction were calculated at the end of each culture. B. Enzyme activity for each growth curve in different sampling times. Error bars are presented in percentage.

Figure 3. Effect of osmolytes added to the culture media on the lipase specific activity. *E. coli* BL21 (DE3) was grown in autoinduction media in presence of glycerol, sorbitol and glycine. A control group (without osmolyte added) was used for comparison. Error bars are represented in percentage. (*) Asterisks indicate significant differences against the control group (p-value <0.05).
Figure 4. Surface response plots of specific lipase activity produced by *E. coli* BL21 (DE3). Interaction between temperature and agitation rate and their effect on specific lipase activity. **A.** Glycerol model. **B.** Sorbitol model.
Figure 5. Relative expression ratio of LipA normalized to 16 s gene internal control in *E. coli* BL21(DE3) using q PCR-RT. The bar graph represents the relative mRNA levels for LipA at three different temperatures. Fold change was measured using the comparative (ΔΔCT) method. Error bars are presented in percentage.

Figure 6. Growth *E. coli* BL21(DE3) curves for optical density and CFU/mL in Schott, Erlenmeyer and Bioreactor. Growth curves for Schott (X), Erlenmeyer 3 L (Triangle), Bioreactor 2 L (Circles).
Figure 7. Lipase Specific Activity obtained in *E. coli* BL21(DE3) in Schott, 3 L Erlenmeyer and 2 L Bioreactor. Specific Activity for Schott (Black bar), Erlenmeyer 3 L (Gray Bar) and Bioreactor 2 L (White Bar). Error bars are presented in percentage.

Table 1. ANOVA of independent variables on specific lipase activity for glycerol, sorbitol and glycine model. (*) Asterisk indicates a significant effect (p<0.05)

| Factors                          | Glycerol | Sorbitol | Glycine |
|----------------------------------|----------|----------|---------|
| Model                            | 0.04     | 0.04     | 0.05    |
| A-Temperature (°C)               | 0.01     | 0.01     | 0.09    |
| B-Agitation Rate (rpm)           | 0.02     | 0.05     | 0.02    |
| C-Glycerol Concentration (M)     | 0.1      | 0.63     | 0.82    |
| AB                               | 0.29     | 0.11     | 0.82    |
| AC                               | 0.41     | 0.98     | -       |
| BC                               | 0.43     | 0.83     | -       |
| A²                               | 0.59     | 0.03     | -       |
| B²                               | 0.01     | 0.24     | -       |
| C²                               | 0.41     | 0.57     | -       |
| R²                               | 0.89     | 0.90     | 0.55    |
| Adjusted R²                      | 0.71     | 0.73     | 0.43    |
Supplementary information

Additional file 1: Table S1. Independent variables and levels of the Box Behnken experimental design. Table S2. ANOVA Test to evaluate the effect of temperature on the specific growth rates of E. coli. Table S3. Dunnet Test for optical density (O.D. 600 nm) at the three different flasks Schott, 3 L shaken Erlenmeyer and 2 L Bioreactor. Table S4 ANOVA Test to evaluate the effect of osmolytes on recombinant lipase activity. Table S5. Dunnet Test to determine the effect of osmolytes on recombinant lipase activity. Table S6. ANOVA of independent variables on specific lipase activity for glycerol, sorbitol and glycine model. Table S7. Dunnet test for validation of glycerol and sorbitol models Table S8. Recombinant lipase yield in soluble and non-soluble fraction obtained from wet biomass in E. coli BL21(DE3) at the optimal culture conditions.. Table S9. ANOVA test for optical density (O.D. 600 nm) at the three different flasks Schott, 3 L shaken Erlenmeyer and 2 L Bioreactor. Table S10. ANOVA Test for CFU/mL bacterial growth at the three different flasks schott, 3 L shaken Erlenmeyer and 2 L Bioreactor. Table S11. Dunnet Test for CFU/mL bacterial growth at the three different flasks schott, 3 L shaken Erlenmeyer and 2 L Bioreactor. Table S12. ANOVA Test to evaluate the effect of flasks on specific lipase activity at the three different flasks schott, 3 L shaken Erlenmeyer and 2 L Bioreactor. Table S13. Dunnet Test to evaluate which flask did show significative differences in the specific lipase activity at the three different flasks schott, 3 L shaken Erlenmeyer and 2 L Bioreactor. Table S14. Table that represents the sampling time. Figure 1s: Growth curves for E. coli BL21(DE3) inoculum. Figure 2S. Growth curves at different temperatures 5°C, 15°C and 25°C for E. coli BL21(DE3). Figure 3S. SDS-PAGE analysis of total extract of recombinant lipase for each sampling time in growth curves. Figure 4S. Validation of optimal fermentation conditions for glycerol and sorbitol models. Figure 5S. Normal (%) probability plot of the Standardized residuals. Figure 6S. Test of Homoscedasticity of Residuals (Scatter Plot for Homoscedasticity). Figure 7S. Test of Independence (Residuals versus order) Figure 8S. SDS-PAGE protein gel of soluble and non-soluble fractions for glycerol, sorbitol and glycine models Figure 9S. Densitometric analysis for optimized glycerol, sorbitol and glycine models. Figure 10S. 16 s PCR of total RNA samples before and after DNAse Treatment. Figure 11S. RNA integrity of target samples on DEPC agarose gel.

Abbreviations

ANOVA: Analysis of variance; BBD: Box Behnken Design; BSA: Bovine serum albumin; CFU/mL: Colony forming units per milliliter; DoE: Design of Experiments Methodology; IBs: Inclusion bodies; IPTG: Isopropyl-B-D-1-thiogalactopyranoside; KLa: Oxygen mass transfer coefficient, NF: Conventional flasks; OFAT: One factor at one time; p – NPP: p-nitrophenyl palmitate; p – NP: p-nitrophenol; P/V: Power Input; RSM: Response surface methodology; SDS-PAGE: Sodium dodecyl sulphate-polyacrylamide gel electrophoresis, SDS: Sodium dodecyl sulfate. NTC: Non-Template Control. q PCR-RT: Quantitative Polymerase Chain Reaction – Real Time

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Author’s contributions

ALML performed the experiments, analyzed and interpreted the data. IYPM constructed the strain for the research. AAG and CAJJ-REP directed the experiments, contributed to the interpretation of the results and revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

Data generated is available in this article and in additional file. Data and materials can be requested to the corresponding author.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests

Author details

1Engineering Faculty, Universidad de La Sabana, Campus del Puente del Común, Km. 7, Autopista Norte de Bogotá, Chía, Colombia.
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