Enhancement of Aeribacillus pallidus lipase production through optimization of medium composition using Box behnken design and its application in detergents formulations

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Abstract

Background : Alkaline, thermostable bacterial lipases are very interested at detergent applications, seen that they replace the use of synthetic detergents which cause substantial environmental problems. These enzymes based detergent are eco friendly and produce a waste water with low level of COD (Chemical Oxygen Demand). The present study, investigates a newly isolated Aeribacillus pallidus strain produces, without induction, a novel halophilous, thermo-alkaline and detergent- tolerant lipase. Results: Considerable interest has been given to this lipase by the improvement of its production by the optimization of the pH, the (C/N) ratio and the inoculums size, using the response surface methodology based on the Box-Behnken Design of experiments. A total of 16 experiments were conducted, and the optimized pH, (C/N) ratio and inoculums size were 10, 1 and 0.3 respectively. The results of the analysis of variance (ANOVA) test indicated that the established model was significant (p value < 0.05). Conclusions: A 6.68-fold of increase in enzyme activity was revealed under the optimized conditions with the maximum activity of 68 U/mL. Additionally, lipase of Aeribacillus pallidus is considered as a potential candidate for applications in detergent formulations since it displayed a good stability towards detergents and wash performance.

Background

Lipases are hydrolytic enzymes which are acquiring more importance for industrial applications. Hence, lipases can be incorporated in food industry, in fact, they are used as emulsifiers in the improvement of baked products and pasta (Houde et al., 2004), and also they are used to modify flavours and produce fragrance compounds (Ferreira-Dias et al., 2015). Evenly, they are used in detergence as additives, providing that they are active and stable at high temperatures and alkaline pH, by the removal of oils and fats from cotton fabrics (Pandey et al., 1999; Veerapagu et al., 2013). These hydrolytic enzymes are equally used in wastewater treatment by the biodegradation of oils (Boran et al., 2019), in leather industry by the elimination of fat from animal skin (Das et al., 2016).Further applications of lipases have been described such as medical applications (as new drugs for treatment of digestive aids and high cholesterol levels) (Hasan et al., 2006), textile industry (Hasan et al., 2006)…

These enzymes are found in various microorganisms including yeast, fungi and bacteria (Nagarajan, 2012).

While, despite the high biotechnological potential of lipases in various fields, the yields of their production is one of the most crucial factors to be developed. Thus, the optimization of culture media is substantial, because of lipase production is influenced mainly by the type and concentration of the carbon source, nitrogen source and inducers (Muralidhar et al., 2001) by varying one factor at a time which does guarantee to reach the optimal point (Strobel et Sullivan 1999). As such, recent studies of optimizing the medium components for lipase production have proved that using Plackett-Burman Design (PBD) and Response Surface Methodology (RSM) approaches, whose Box-Benkhen design is one among them (Box and Behnken 1960), were able to be the most effective methods (Gupta et al. 2007; He and Tan, 2006),

which are able to overcome the drawabcks of the one factor at time the fact that, they creates empirical model equations that correlate the relationship between variables and responses. Consequently, RSM has so many advantages, and has successfully been applied to study and optimize the enzymatic processes (Soo et al.2004; Basri et al.2007) and enzyme production from microorganisms (Gaur et al.2008; Teng et Xu, 2008)

In the present investigation, we aimed to improve the yield of lipase production through optimization of the fermentation medium composition for a newly thermostable lipase of *Aeribacillus pallidus.* Additionally, the enzyme showed excellent stability and compatibility with various commercial detergents suggesting its potential as an additive in detergent formulations.

Results

3.1 Preliminary optimization using “one-factor -at-a-time”

Aeribacillus pallidus produced about 24 U/mL of lipase in the production medium, at pH 10 and 65°C in the presence of 1 mM sodium taurodeoxycholate (NaTDC) and 1 mM CaCl2 after 22h of incubation. After this period, lipase activity began to decrease.

The improvement of microbial lipase production is the purpose of several investigations. According to maximizing enzyme production, each strain has its specific requirement in culture conditions. Various components in the media such as carbon and nitrogen substrates which affect the carbon to nitrogen ratio (C/N) which consequently influence the production of extracellular lipase enzyme (Jia et al. 2015) as shown in table 1. We discussed about the effect of C/N ratio thereinafter.

3.2 Effect of carbon source on lipase activity

The major factor affecting expression of lipase is the carbon source. Most of reports available, state that lipases are generally induced by natural oils (He and Tan 2006; Kaushik et al.2006; Abdel-Fattah 2002), and high levels of lipase production were reported from various thermophilic *Bacillus sp* (Eltaweel et al.2005; Bora and Kalita 2007; Lee et al.1999). While the main observation of Box-Behnkhen study was the poor induction of lipase of *Aeribacillus pallidus* by oils. In fact, various carbon sources were tested, glycerol, Tween 80, Olive oil, Glucose, Fructose and Sucrose at final concentration 1% were analyzed independently (Fig. 1). Culture supernatants were sampled at different times during 24 h of growth and assayed for their lipase activity. Statistical analyses of the results showed that the highest lipase activity determined in the culture broth was obtained after 22 h of incubation. Therefore, only the results determined after this time of incubation were taken into account (fig 2). Statistical analysis of the results showed that lipase was significantly improved when glucose was used as the sole carbon source with 1.5 % (Fig. 2), to reach a better GPL activity with 32 U/mL. This result is in contradiction with those having shown that carbon sources are easily broken down and used by bacteria to play an inhibitory role (Lee et al.1999; Gowland et al 1987, Mates and Sudakevitz 1973).

For further comparisons, the glucose effect on *Staphylococcus xylosus* lipase showed the same behaviour, with an optimal activity measured using 2.5% glucose (Ghribi et al.2009).

3.3 Effect of organic and inorganic nitrogen source on lipase activity

In addition to carbon source, the source of nitrogen is an important parameter for lipase production, and to obtain an insight for the effect of different nitrogen sources, various organic and inorganic sources were investigated in lipase production. Generally, when organic nitrogen sources were used such as peptone and yeast extract, high level of lipase production by various thermophilic *Bacillus sp* (Ghanem et al.2000; Sharma et al.2002; Sugihara et al.1991) was observed. So that, yeast extract is one of the most important organic nitrogen sources for high level lipase production by different microorganisms (Bora and Kalita 2007), and this is in accordance with our results. As shown in (Fig.3a), higher lipase activity was obtained when yeast extract was used as organic nitrogen source rather than using Soya Peptone, Tryptone, Pancreatic Digest Peptone and Casein Peptone. Since, 0.05% yeast extract is enough to improve significantly lipase activity reaching 34 U/mL. However, the lipase activity decrease significantly when using Soya Peptone, Tryptone, Pancreatic Digest Peptone and Casein Peptone.

The requirement of the source of nitrogen depend on microorganisms, some of them show preference to organic forms, while others show preference to the inorganic forms. Both organic and inorganic forms are required for some microorganisms. When, we tested the inorganic sources (Ammonium chloride (NH_4Cl), ammonium nitrate (NH_4NO_3), ammonium sulphate ($(NH_4)_2SO_4$), ammonium molybdate ($(NH_4)_6Mo_7O_{24}$ and HgN_2O_4T), it was found that the combination of inorganic and organic nitrogen in the form of NH_4Cl among all the other forms tested with 0.05 % yeast extract improve the lipase production to 36 U/mL (Fig. 3.b).While the omitting of one of them lead to a sharp decrease of the lipase activity.

Several investigation have shown that a combination of organic and inorganic nitrogen sources has been used for lipase production from Bacillus strain A30-1(Wang et al.1995), *Pseudomonas sp.*, (Dong et al.1999)and *P. aeruginosa* LP602 (Dharmsthiti and Kuhasuntisuk 1998) and it was also found that with higher concentration of ammonium chloride (2.5%), lipase production was drastically increased by almost fourfold.

A 6.68-fold increase for lipase production was obtained by one variable at a time. The variation of the concentration of inorganic nitrogen source (Ammonium chloride) in our study affects the C/N ratio. The results shown in table 2, statistically analyzed according to Duncan's test realized after ANOVA analysis, were obtained by using 1.5% glucose, 0.05 %yeast extract and various C/N ratios corresponding to various ammonium chloride concentration. With the values of (C/N=1), which is corresponding to 1.5% glucose and 2.3% NH_4Cl, the optimal lipase activity was detected and reaches 36 U/mL. Up over this C/N ration, a decrease of lipase activity was significantly observed, reaching 18 U/mL for C/N of 6.

In conclusion, using 0.5 g/l yeast extract and applying a C/N ration equal to 1, the optimal activity of GPL reaches 36 U/mL.

These results show that to provide amino acids and vitamins necessary for metabolite production, a balance between organic and inorganic nitrogen sources and suitable values of C/N ratio must be taken into account.

3.4 Optimization of medium components using response surface methodology

In order to optimize medium culture and to improve lipase production by *Aeribacillus pallidus*, a statistical analysis based on experimental design was adopted. This experimental planification methodology is a valuable tool for optimizing the medium of lipase production which provide advantages: the use of multifactor effects, the obtaining of the optimum values and the developing of a system model with substantially less experimental requirements (Wu et al.2007).

Several studies have been shown to increase lipase production for several folds by a large wide of organisms (He and Tan 2006) such as *Aspergillus niger* (Soo et al.2004), *Staphylococcus xylosus* (Sharon et al 1998), *Candida cylindracea* (Jia et al.2015) and *Geobacillus sp* (Abdel-Fattah, 2002).

3.5 Data Analysis and Modeling

Based on the OFAT experiments, which indicated that Glucose, ammonium chloride, inoculums size and pH are the significant factors, affecting the GPL activity, Box-Behnken Design was used to determine the optimum conditions for these significant factors (carbon to nitrogen (C/N) ratio (X1), pH (X2), inoculums size (X3)). The experimental design, experimental and predicted values for lipase production obtained from the regression equation for 16 combinations were shown in (Table 1). The results demonstrated that there is a considerable variation in lipase activity produced by *Aeribacillus pallidus*. Indeed, the highest level of lipase activity was 68 U/mL (run3) and the lowest amount was 30; 34 U/mL (run 6, 8 and 9).

Statistical analysis of variance (Table 3) was used to investigate the effectiveness of the model. The F-value (Fisher's statistical analysis) was used as tool for evaluating the significance of the model. It was estimated to be 87.18, that's mean that there is only a 12.82 % chance of "F-value Model" taking place due to noise.

The coefficient of determination (R^2) is a measure of the quality of the prediction of a linear regression. Generally, its determination and its prediction of the response is significant when it is close to 1 (Haaland, 1989; Kaushik et al.2006). In this model, it is calculated to be 0.992. This is shows a good correlation between experimental and predicted values. Hence, only 21.53% of the total variations are not interpreted by the model. This indicates a satisfactory representation of the process by the model. In addition, a high degree of similarity was obtained between the predicted and experimental values suggesting the significance of the regression model describing the response which was proved the suitability of the model even (fig. 4).

3.6 3D Response Surface and contour Plots Analysis

Response surface and contour plots have been used in order to understand and define the effect of the studied variables within the experimental space. This technique was achieved by depicting the interactions between two variables while keeping the third at a constant level (Ghribi et al.2011). Thus, 3D responses facilated the visual determination of optimum levels of each parameter. Therefore, in the first time (Fig. 5.a) we fixed the inoculum size at its zero-coded level OD600 = 0.3. So, the response was represented as function of the interaction between liquid substrate ratio and pH level. It can be clear that lower than their central level, the carbon to nitrogen (C/N) ratio and pH significantly influenced the enzyme production.

As shown in Fig.5.b and Fig. 5.c, the iso-responses are near parallel to the inoculum size axis. This suggests that neither an increase nor a decrease in inoculum size can affect significantly the lipase activity.

As analyzed in the present study, a successful and significant improvement from 30 U/mL to 68 U/mL in the production of lipase was accomplished, when the respective values of carbon to nitrogen (C/N) ratio (X1), pH (X2), and inoculums size (X3) are 1, 10 and 0.3 respectively. Indeed, a 3-fold increase in lipase yield after optimisation was observed, with 300% increase in T1 lipase production by *Pichia guilliermondii* (Ladidi et al.2017).

3.7 Stability and compatibility on GPL with laundry detergents

A detergent is a mixture of surfactants and oxidizing agents that show their cleaning efficiency at alkaline pH (9–11). Fig.6 proves that lipase of *Aeribacillus pallidus* was highly stable and compatible with commercial solid detergents used; in fact, it retained 98%, 96%, 95 % and 90% of its initial activity with Dixan,OMO, Nadhif and Ariel, respectively, after incubation of 30 min. In addition, in the present of liquid laundry detergents, GPL was highly stable; it retained 100% of its original activity in Dipex when incubated 1h at 50°C, while only 80% were retained when incubated in Ariel.

Enzyme activity of the control sample, which contained no additive and incubated under similar conditions, was taken as 100%. Each point represents the mean of three independent experiments.

These results are consistent with those reported for alkaline lipases from *Bacillus stearothermophilus* (Ben bacha et al.2015), *Bacillus stratosphericus* (Zin et al.2017). Results show clearly that lipase of *Aeribacillus pallidus* can provide further support for its usefulness for future industrial application as a cleaning bioadditive in detergent compositions.

3.8 Removal of oil spot from cotton fabrics

To evaluate the performance of lipase of *Aeribacillus pallidus* in terms of its ability to remove oil spot, several pieces of stained white cotton were incubated at different conditions. As shown in Fig. 7, the limited washing performance was observed with detergent (Dixan) only, and its supplementation seems to improve the cleaning process as evidenced by oil spot removal when compared to detergent alone.

Furthermore, the combination of this enzyme with the Dixan detergent resulted a complete oil spot removal.

Discussion

In the present investigation, we aimed to produce a high level of lipase production of *Aeribacillus pallidus,* using the optimization of response surface methodology, due to its relevance. Results presented in this paper, showed that an overproduction of GPL was reached under aerobic conditions by glucose at high concentrations , a combination between organic and inorganic nitrogen sources, pH 10 and inoculums size equal to 0.3.

As long as, the major factor affecting the lipase activity has always been carbon source, as lipases are generally inducible enzymes (Lotti et al. 1998). In fact, the use of glucose as carbon source to the culture medium improve well the aerobic growth, the biomass formation and the productivity of the lipase (Fig.2), while its decrease in the presence of lipid source such as tween, glycerol and olive oil, this can be explained by he fact that glucose is the most readily metabolized carbohydrates and a source of fast energy (Madigan et al. 2003).

After 22h of incubation, lipase activity began to decrease, might be due to the exhaustion of nutrients, accumulation of toxic, and the change in pH of the medium, or proteolysis of lipase by proteases which were produced simultaneously (Nouroozi et al. 2015). An excessive increase of the glucose concentration decrease both the lipase activity and cells formation (Fig. 2), it could be explained by the fact that the cells produced at high glucose concentrations are not physiologically able to synthesize lipases, this phenomenon can be observed in facultative anaerobic bacteria such as *Bacillus thuringiensis* (Al-mhanna, 2010)..

Yeast extract increase the productivity for the most of microorganisms (Bora and Kalita 2007), thanks to its wealth of vitamins and trace elements for the growth of bacteria and increases their lipase production (Gupta et al. 2007). Whereas, the decrease of lipase activity when using peptone as organic nitrogen source, could due to its complex composition which can cause toxic effects by one of its components (Sooch and Kauldhar 2013).

An other substantial factor for the productivity of lipase is the pH. The pH not only acts on enzymatic activity but also on the properties of the interface in a multiphasic system, on the solubility of the reagents in the medium, as well as the sharing of the enzyme between the aqueous phase and the interface. The optimum pH of lipase activity is usually around 7 while bacterial lipases generally have a slightly basic optimum pH (8- 8.5) (Sharma et al. 2001); (Gargouri et al. 2008).

Most halophilic lipases showed maximal production at pH alkalin and temperature up to 40° C (Esakkiraj et al. 2014); (Li et al. 2014). Seen that *Aeribacillus pallidus* grew at 30 g/l NaCl in the culture medium, at 55 °C and lipase was active under these conditions (Ktata et al. 2018) , GPL was considered as a thermoactive and haloalkaliphilic lipases (Gupta et al. 2007 ; Marques et al. 2014; Yoo et al. 2011).

Lipases used in detergents must have a broad spectrum of substrates and be able to withstand washing conditions such as pH values between 10 and 11, and a temperature of between 30 ° C and 60 ° C, thus, this novel lipase are especially sought-after. Once the lipids are partially or fully hydrolyzed by the enzyme, becomes easier to extract from the washed fabric. In fact, an alkaline lipase was reported, produced by *Pseudomonas alcaligenes* M-1, which was well adapted to remove fatty stains under wash conditions (Gerritse et al. 1998).

Thus, the performance of a good detergent lipase is defined by multiple parameters such as: the thermostability feature of lipase of *Aeribacillus pallidus* (Bora and Bora 2012), the ability to hydrolyze several types of triglycerides (short, medium and long chain) and its compatibility with other detergent components, support the usefulness of lipase of *Aeribacillus pallidus* in future industrial applications as a cleaning bioadditive in detergent formulations. In summary, GPL could have a good capacity for removing fatty stains in alkalin environment, suggesting its potential use as an additive in detergent formulations.

Conclusion

Experimental planning methodology was used in our study as a tool to significantly increase the production yields of *Aeribacillus pallidus* lipase GPL and predict the optimal values of the important influent factors. A Box-Behnken design with three optimal factors was applied (C/N ratio, pH value and inoculums size). A high degree of similarity was obtained between the predicted and experimental values suggesting the significance of the regression model describing the response. This novel lipase showed excellent stability and compatibility with various commercial detergents, suggesting its potential use as an additive in detergent formulations.

Materials And Methods

2.1 Source of strains

Strains were previously isolated from the production water (an oil/water mixture) of the oil-field managed by Thyna Petroleum Services (TPS), located at 11 km northwest of Sfax city, Tunisia (Mnif et al.2014). The formation water deposit from depths of 1300m at a temperature of 78 °C, a salinity of 100 g /L and a pH of 7.6. Samples were directly collected in sterile bottles and stored in dark at 4 °C until use.

2.2 Lipase production at shake flask scale

The thermoalkaline lipase from *Aeribacillus pallidus* (GPL) was produced using the optimized medium which is composed of (g/1): 30 g NaCl, 1 g KH2PO4, 0.4 g NH4Cl, 1 g MgSO4.6H2O, 1g CaCl2, 0.5g yeast extract and 1mL trace-element solution (Jaouadi et al.2009) in 1 L of distilled water. The pH was adjusted with 4M KOH solution to 7.4. Aliquots of 50 mL were dispensed into flasks and sterilized by autoclaving at 121 °C for 20 min. These flasks were incubated at 55 °C under aerobic conditions for 22 h

with shaking at 200 rpm. Before each assay, the microbial cell debris was removed by centrifugation at 13000 rpm for 30 min. Next, the obtained clear supernatant was used as a crude enzyme preparation.

2.3 Lipase activity and protein estimation

The lipase activity was measured titrimetrically at pH 10 and 65°C with a pH-stat under standard conditions, using TC4 (250µl) in 30 mL of buffer containing 2.5 mM Tris-HCL,150 mM NaCl pH 10, 1mM $CaCl_2$ and 1 mM sodium taurodeoxycholate (NaTDC) or olive oil emulsion whose obtained by mixing 10 mL of olive oil in 90 mL of 10 % (w/w) gum Arabic (for 3x 30s) in a Waring blender. One unit of lipase activity corresponds to 1 µmol of fatty acid released per minute under the assay conditions used.

Protein content was measured by Bradford method (Bradford, 1976), using bovine serum albumin as standard.

2.4 Medium optimization for maximum lipase production by *Aeribacillus pallidus* using Statistical Procedure

Lipase production optimization was carried out using experimental planification methodology through the response surface methodology. Preliminary studies through one factor at a time have proved that ratio C/N, pH and inoculum size affect significantly lipase production . Therefore, in order to determine the optimum levels of these three significant variables, to predict the possible interaction between them and to enhance lipase activity, a Box-Benkhen design for three independent variables was adopted. It was generated using NemrodW version 2007 software (LPRAI,Marseille, France).

2.5 One-factor-at-a-time (OFAT)

Firstly, the classical one-factor-at-a-time (OFAT) approach was employed to evaluate the influential parameters on the lipase production. In the first step of culture conditions optimization, we have started with optimizing the carbon source, the organic and inorganic nitrogen source, and their concentrations, the inoculums size and pH.

These parameters were optimized by keeping all factors at a constant level in the basal medium, except the one under study and each subsequent factor was examined after taking into account the previously optimized factor(s).

2.6 Optimization of nutritional parameters

In one's element, the name of *Aeribacillus pallidus* has been reassigned to *Geobacillus pallidus* (Minana Galbis et al.2010), owing to DNA composition level , fatty acid composition and the polar lipid profile (Chamkha et al.2008). This strain is a Gram-positive bacterium, aerobic, thermophilic, halotolerant (Minana Galbis et al.2010).

To select the best carbon source that maximizes lipase production for this strain, various carbon sources were tested, as, glycerol, Tween 80, Olive oil, Glucose, Fructose and Sucrose at final concentration 1%. The concentration of the selected carbon source was then varied in the range of 0–2% to work out the optimum concentrations.

The effects of nitrogen sources were evenly evaluated, with various organic and inorganic nitrogen sources at the same final nitrogen concentration. For organic sources, we have already used (yeast extract, peptone, soya peptone, casein peptone and tryptone) when the Ammonium chloride (NH_4Cl), ammonium nitrate (NH_4NO_3), ammonium sulphate ($(NH_4)_2SO_4$), ammonium molybdate ($(NH_4)_6Mo_7O_{24}$ and HgN_2O_4T) were applied in addition to 0.5 % w/v as inorganic nitrogen sources. After selecting yeast extract as the best organic nitrogen source, it was considered as a constant level in the basal medium.

2.7 Optimization of physicochemical parameters

Similarly, the effects of pH (6-10) and the inoculums size (OD 0.1-0.6) were already evaluated on strain growth and lipase production, as the physical parameters before being subjected to statistical optimization. Then, fermentation was performed in 250 mL shake flasks with 50mL medium and incubated at 55°C in a shaker (200 rpm) for about. All components were analyzed independently, and every test was performed in triplicate. The influential factors and levels for the enzyme activity were evaluated.

2.8 Optimization of medium components by response surface methodology

To analyze the experimental design data and to determine the optimum conditions for lipase production by *Aeribacillus pallidus*, response surface methodology (RSM) was applied. Box-Behnken Design (BBD) is one of the response surface methodology, with a three-level factorial design was used as the experimental design, to optimize the concentrations of three significant factors namely ratio C/N, pH and inoculums size for enhancing lipase production. The remaining factors were maintained at fixed concentration. The independent variables were studied at three different levels, low (−1), medium (0) and high (+1). All the experiments were carried out in triplicate and the average of lipase production obtained was taken as the response (Y).

The relationship between dependent and independent variables is explained by the following second-order polynomial equation and optimum levels were represented in response surface plots.

$$\hat{Y} = b0 + b1\,X1 + b2\,X2 + b3\,X3 + b11\,X12 + b22\,X22 + b33\,X32 + b12\,X1\,X2 + b13\,X1\,X3 + b23\,X2\,X3$$

Where X1, X2 and X3 are the coded factors studied, b0 intercept, b1, b2, b3 linear coefficients, b11, b22, b33 squared coefficients, b12 , b13, b23 interaction coefficients. The model coefficients were estimated using multi linear regression. The significance of the coefficients is evaluated by multiple regression analysis based upon the F-test with unequal variance ($P<0.05$). To check the compatibility of the proposed model with the obtained experimental data, we performed an analysis of variance.

2.9 Effects of commercial detergents on lipase activity

The suitability of the lipase of *Aeribacillus pallidus* as a detergent additive, was determined by testing its stabilities and compatibilities towards a wide range of commercialized solid and liquid detergents.The list of liquid detergents included Dipex, (SOTUP, Sfax, Tunisia), Ariel (Procter & Gamble, Switzerland), Skip (Unilever, France). and Judy (Ennadhafa, Sfax, Tunisia).. The solid detergents used were Nadhif(Henkel-Alki, Tunisia), Dixan (Henkel-Alki, Tunisia)„ Ariel(Procter & Gamble, Switzerland), and Omo (Unilever, France).

In order to check lipase of *Aeribacillus pallidus* stability and compatibility with detergents, the mentioned commercial detergents were diluted in tap water to obtain a final concentration of 7 mg/mL (to simulate washing conditions). The endogenous lipolytic enzymes present in these laundry detergents were inactivated by heating the diluted detergents for 1 h at 90 °C, prior to the addition of the purified enzymes. 15 U/mL of lipase of *Aeribacillus pallidus* was shake-incubated with each laundry detergent for 30 min at different temperature 30, 40, 50 and 60°C, and residual activity was determined at pH 10 and 65 °C using TC4 as a substrate.

The enzyme activity of a control (without any detergent), incubated under similar conditions, was taken as 100%.

2.10 Removal of oil spot from cotton fabrics

New white cotton cloth pieces (5 × 5 cm) were speckled with lubricating oil and used to stimulate the washing conditions and determine the efficiency of lipase of *Aeribacillus pallidus* as a biodetergent additive compared. The endogenous lipases found in Dixan liquid laundry detergent were inactivated by heating the diluted detergents for 1 h at 90 °C prior to the addition of the purified tested enzyme. The stained cloth pieces were shake-incubated (220 rpm) in different wash treatments at 50 °C for 30 min in Erlenmeyer 250 mL containing a total volume of 100 mL of: tap water, Dixan detergent (7 mg/mL in tap water), and detergent added with GPL (15 U/mL). After treatment, the cloth pieces were taken out, rinsed with water, dried and submitted to visual observation to examine the stain removal effects of the enzymes. The untreated blood-stained piece of cloth was taken as a control.

Abbreviations

one-factor-at-a-time (OFAT)

Aeribacillus pallidus lipase (GPL)

Thyna Petroleum Services (TPS)

Carbone/azote ratio (C/N ratio)

Response Surface Methodology (RSM)

Declarations

Conflict of interest The authors declare that they have no confict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Availability of data and material : The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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Tables

Table 1: Experimental design using Box-Behnken of three independent variables with their actual values showing the experimental and predicted responses.
The experiments were conducted three times.

Run order	X1 C/N	X2 pH	X3 Inoculum Size (DO600)	Lipase Activity U/ml	
				Experimental	Predicted
1	1	6	0.3	50 ± 0.9	51
2	4	6	0.3	40 ± 0.12	41
3	1	10	0.3	68 ± 0.92	67
4	4	10	0.3	52 ± 0.13	51
5	1	8	0.1	48 ± 0.24	47.5
6	4	8	0.1	30 ± 0.22	29.5
7	1	8	0.5	42 ± 0.84	42.5
8	4	8	0.5	34 ± 0.71	34.5
9	2.5	6	0.1	30 ± 0.31	29.5
10	2.5	10	0.1	48 ± 0.42	49.5
11	2.5	6	0.5	38 ± 0.1	36.5
12	2.5	10	0.5	42 ± 0.2	42.5
13	2.5	8	0.3	48 ± 0.12	48
14	2.5	8	0.3	48 ± 0.12	48
15	2.5	8	0.3	48 ± 0.12	48
16	2.5	8	0.3	48 ± 0.12	48

Table 2: ANOVA analysis for lipase activity in Box-Behnken experiments obtained by *Aeribacillus pallidus*.

Source of variation	Sum of squares	Degree of freedom	Mean square	F-value	Significance
Regression	1.3078E+0003	9	1.4531E+0003	87.183	***
Residual	1.000E+0001	6	1.6667E+0000		
Total	1.6667E+0003	15			

Table 3: Estimated effect, regression coefficient and corresponding t and P values for lipase activity in Box-Benkhen design experiments

Noun	Estimate Coefficient	Inflation Factor	Standard deviation	t.exp	Confidence level (%)	Signification
b_0	48	1.00	0.645	74.36	< 0.01	***
b_1	- 6.500	1.00	0. 456	- 14.24	< 0.01	***
b_2	6.500	1.00	0. 456	14.24	< 0.01	***
b_3	0.000	1.00	0. 456	0.000	100	
b_{1-1}	1.750	1.00	0.645	2.71	3.512	*
b_{2-2}	2.750	1.00	0.645	4.26	0.532	**
b_{3-3}	- 11.250	1.00	0.645	-17.43	< 0.01	***
b_{1-2}	- 1.500	1.00	0.645	-2.32	5.9	
b_{1-3}	2.500	1.00	0.645	3.87	0.824	***
b_{2-3}	- 3.500	1.00	0.645	-5.42	0.163	**

t.exp is the value of variables determined by student’s test.

***: Significant for 0.0001< p-value < 0.001

**: Significant for 0.001< p-value < 0.01

* : Non significant for p-value > 0.05

Figures

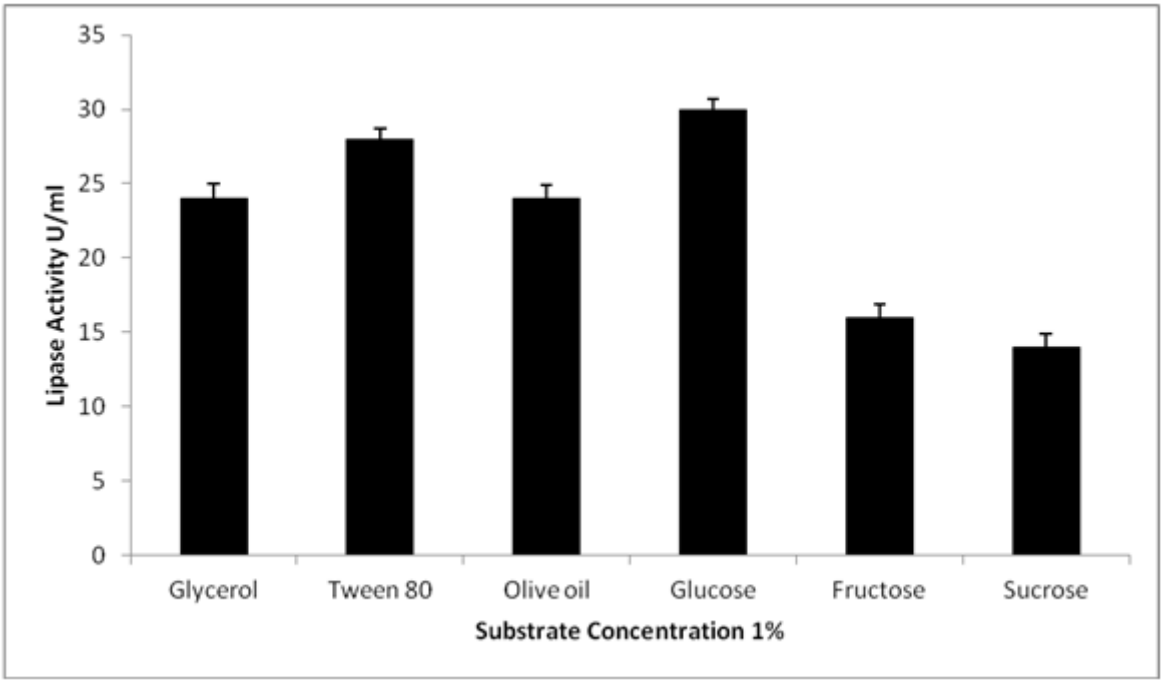

Figure 1

The effect of different carbon sources on lipase activity obtained at 65 °C, pH 10, for 22 h by Aeribacillus pallidus. The values are average of three independent experiments and the error bars represent standanrd deviation.

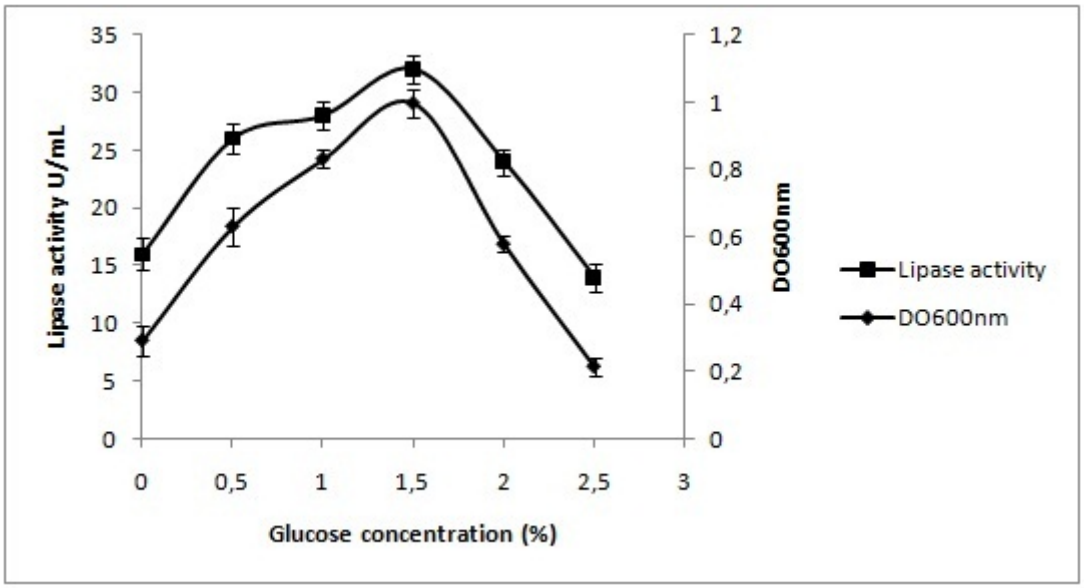

Figure 2

Aerobic growth of Aeribacillus pallidus on the optimized culture medium with different glucose concentration (filled triangle). The effect of different concentrations of glucose on lipase production at 65 °C, pH 10, for 22 h (filled square). The values are average of three independent experiments and the error bars represent standard deviation.

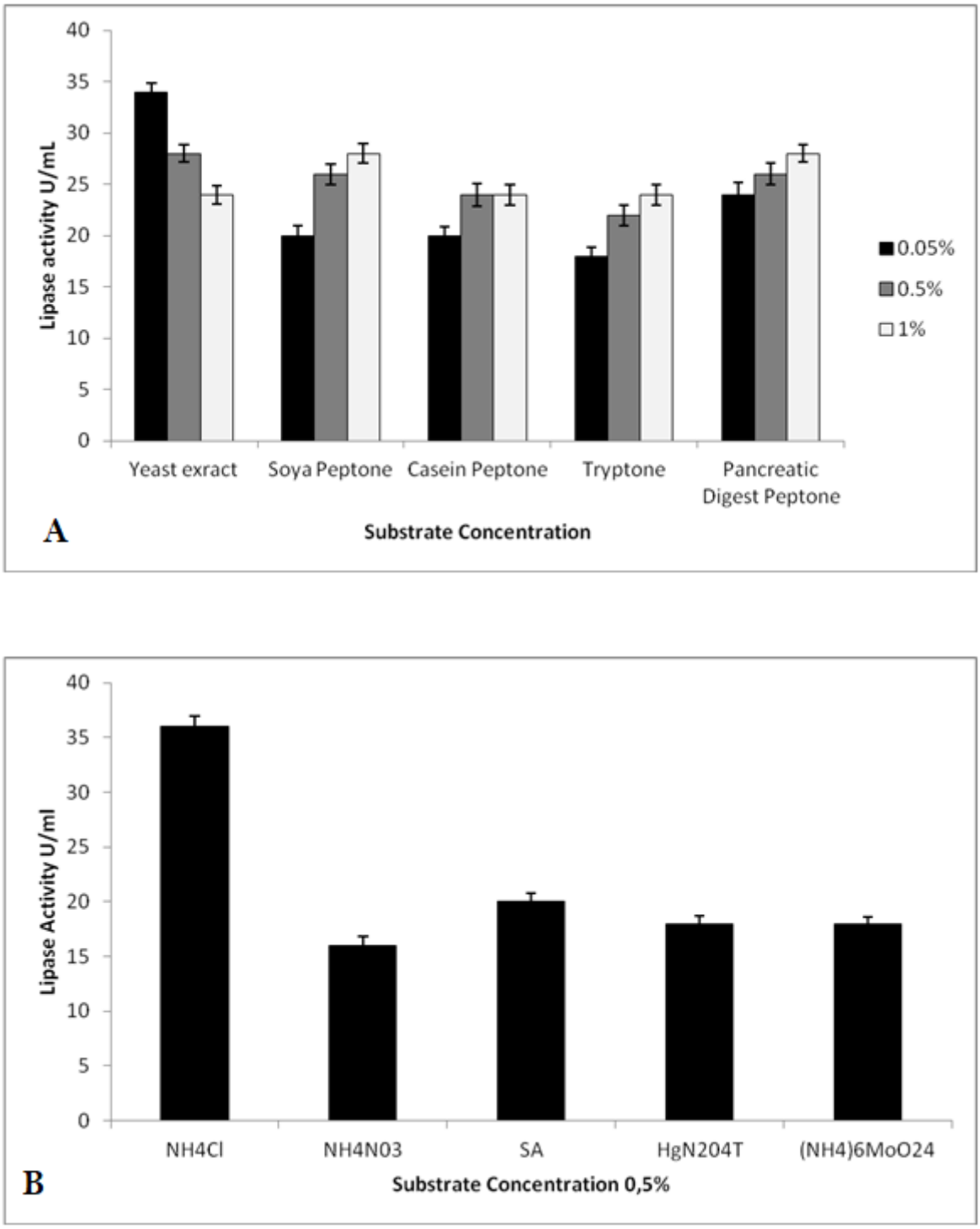

Figure 3

(a) Effects of different organic nitrogen sources on lipase production at 65 °C, pH 10, for 22 h of incubation by Aeribacillus pallidus using glucose as a carbon source kept at 15 g/l.. (b) Effects of

different inorganic nitrogen sources on lipase production at 65 °C, pH 10, for 22 h of incubation by Aeribacillus pallidus

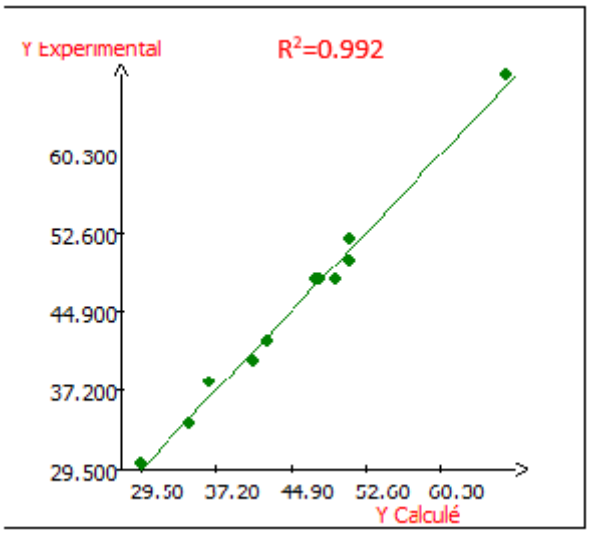

Figure 4

Validation of Predicted versus Actual values. The straight line of regression with data points across indicates the suitability of the model, agreement between predicted and actual values and it conforms to the assumption of data point representation

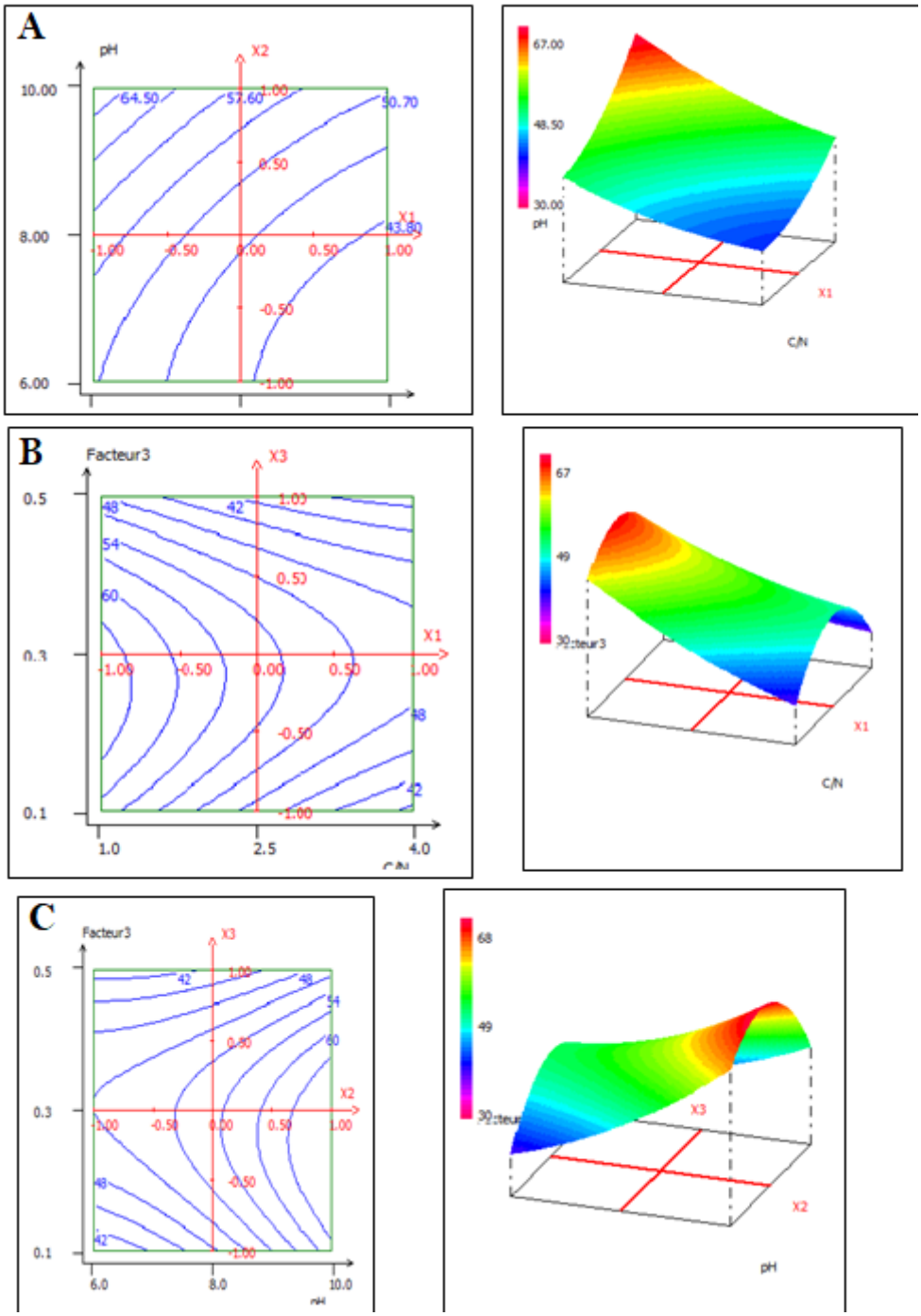

Figure 5

(a) Effect of ration C/N and pH on lipase activity: response surface plot (right) and its contour plot (left) of interaction between the two factors. (b) Effect of ration C/N and inoculums size on lipase activity: response surface plot (right) and its contour plot (left) of interaction between the two factors. (c) Effect of ration pH and inoculums size on lipase activity: response surface plot (right) and its contour plot (left) of interaction between the two factors.

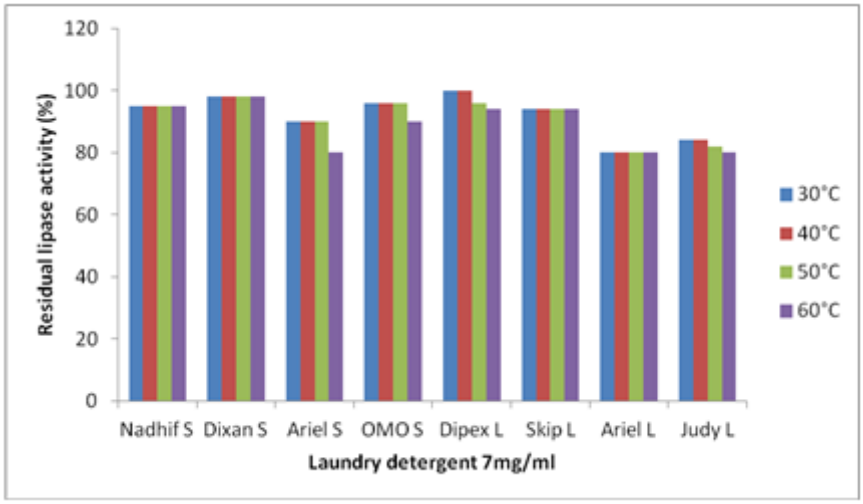

Figure 6

Stability of the purified lipase (GPL) in the presence of liquid and solid laundry detergents. Enzyme activity of the control sample, which contained no additive and incubated under similar conditions, was taken as 100%. Each point represents the mean of three independent experiments.

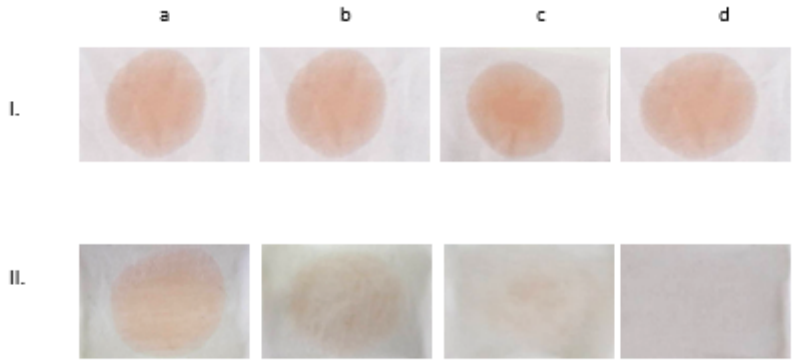

Figure 7

Washing performance analysis test of GPL in the presence of the commercial detergent Dixan (a) Oil-stained cloth washed with tap water; (b) Oil-stained cloth washedwith Dixan detergent (7 mg/ml), (c) Oil-stained cloth washed with GPL only (d) Oil-stained cloth washed with Dixan added with GPL (15 U/ml). I: untreated cloths (control) and II: treated cloths.