Protease production from *Lysinibacillus fusiformis* strain C250R: Statistical optimization and compatibility study for use in detergent formulations

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Graphical Abstract

Abstract.

In this study, we aimed to optimize the cultural and nutritional conditions for protease production by *Lysinibacillus fusiformis* strain C250R in submerged fermentation process using statistical methodology. The most significant factors (gruel, wheat bran, yeast extract, and $FeSO_4$) were identified by Plackett-Burman design. Response surface methodology (RSM) was used to determine the optimum levels of the screened factors and their interaction. Under the optimized conditions, protease yield 3045 U/mL was 4.5 folds higher than those obtained by the use of the initial conditions (680 U/mL). Additionally, this research demonstrated that the protease activity of strain C250R has a high detergent compatibility and an excellent stain removal compared to Alcalase Ultra 2.5 L; which offers an interesting potential for its application in the laundry detergent industry.

Keywords: Protease; *Lysinibacillus fusiformis*; Statistical methodology; Detergent formulations.

1. Introduction

The biocatalysts are beautiful creatures and the best solution given by nature. They can be used to improve some biotechnological applications. Catalysis is a process that increases the speed with which a reaction reaches equilibrium. Since the reaction rate depends on the free energy of activation, a catalyst causes the decrease of the energy barrier and accelerates the catalytic stage. Enzymes are molecules which can reduce the activation energy of reaction and thus accelerate the biochemical reactions that take place in the cell (Bouhlel et al. 2021). Therefore, the functionality of a cell, its reproduction requires that all reactions occur in coordinated and controlled synergy. The enzymes are thus key players in life. Proteases, as multifunctional biocatalysts, account for about 60% of global markets enzymes, which have a wide range of applications. They are used in several industrial processes including detergents, baking, food, photography, and pharmaceuticals (Mechri et al. 2017).

Many bacterial species, such as *Caldicoprobacter guelmensis* strain D2C22[T] (Bouacem et al. 2015), *Gracilibacillus boraciitolerans* strain LO15 (Ouelhadj et al. 2020), *Melghiribacillus thermohalophilus* Nari2A[T] (Mechri et al. 2021), and *Lysinibacillus fusiformis* Strain SK (Khadka et al. 2020), are known to produce thermostable proteases.

The genus *Lysinibacillus* was found to be metabolically versatile with the ability to produce wide range of enzymes. *Lysinibacillus fusiformis*, a Gram-positive bacterium closely related to *Bacillus sphaericus*, was formerly known as *Bacillus fusiformis* before 2007 and later on reclassified as genus *Lysinibacillus* because of the presence of the specific amino acids like lysine, alanine, glutamic acid and aspartic acid in its cell wall. This bacterium can produce inactive spherical endospores resistant to high temperatures, detrimental chemicals and UV light under the active situations (Ahmed et al. 2007). Some study has revealed that *Lysinibacillus fusiformis* is capable of producing thermostable protease at a higher temperature (Khadka et al. 2020).

The main purpose of the present study is the statistical optimization of protease production from *Lysinibacillus fusiformis* strain C250R. The produced enzyme was characterized and evaluated for possible application in the laundry detergent industry.

2. Material and methods

2.1. Screening for proteolytic microorganism isolate

Lysinibacillus fusiformis strain C250R was isolated from the production water of the Ramoura oil field (onshore) at Tunisia, with a temperature of 95°C, and salinity of 106 g/L (Mnif et al. 2011). C250R strain was maintained on Luria-Bertani broth-agar (LB-agar) at 4 °C until further uses. The ability of the isolate for proteolytic activity was tested on skimmed milk agar plates (SMAP) as detailed beforehand by the authors (Ouelhadj et al. 2020) and then incubated at 37°C for 24 h. A clear zone of hydrolysis indicating protease activity was measured in millimeters.

2.2. Protease production

A preliminary study for the production of protease production was carried out using a production medium containing per Liter 5 g gelatin, 1 g yeast extract, 50 g NaCl, 2 g $CaCl_2$, 1 g $MgSO_4.7H_2O$, 1 g K_2HPO_4, and 1 g KH_2PO_4 at pH 7.4.

2.3. Optimization of enzyme production

The optimization of protease production from *Lysinibacillus fusiformis* strain C250R was started with the selection for the best carbon and nitrogen sources using the traditional method 'one variable at a time. The experiments were carried out in 250 mL Erlermeyer flasks containing 25 mL of production liquid medium.

The list of various used carbon sources was casein, gelatin, gruel, glucose, starch, and maltose. While, the list of used nitrogen sources was yeast extract, soya flour, meat extract, beef extract, (NH4)2SO4, and (NH$_4$)$_3$FeSO$_3$).

2.3.1. Screening of factors with the Plackett–Burman design

Seventeen variables (incubation temperature, initial pH, speed of agitation, gruel, sucrose, lentil flour, wheat bran, yeast extract, urea, NaNO$_3$, crude oil, NaCl, CaCl$_2$, FeSO$_4$, MnSO$_4$, trace elements, and KH$_2$PO$_4$) were studied at low (-1) and high (+1) levels of each factor (**Table 1**). The effect of each variable was carried out by the determination of the contrast coefficient (b) obtained from the difference between the measurements average made at the high (+) and the low (-) levels of the variable.

Table 1 Levels of independent variables on the protease production of *Lysinibacillus fusiformis* strain C250R.

Code of variables	Variables	Levels	
		Low (-1)	High (+1)
X1	Temperature (°C)	30	37
X2	pH	7	9
X3	Agitation (rpm)	150	180
X4	Gruel (g/L)	5	10
X5	Sucrose (g/L)	0	5
X6	Lentil flour (g/L)	0	5
X7	Wheat bran (g/L)	0	5
X8	Yeast extract (g/L)	1	3
X9	Urea (g/L)	0	2
X10	NaNo$_3$ (g/L)	0	2
X11	Crude oil (%, v/v)	1	4
X12	NaCl (g/L)	20	50
X13	CaCl$_2$ (g/L)	0	2
X14	FeSO$_4$ (g/L)	1	3
X15	MnSO$_4$ (g/L)	0	2
X16	Trace elements (%, v/v)	1	3
X17	KH$_2$PO$_4$ (g/L)	1	2

2.3.3. Response surface methodology using box–Behnken design

Based on the results of the Plackett–Burman design, four variables (gruel (**X1**), wheat bran (**X2**), yeast extract (**X3**), and FeSO$_4$(**X4**)) were found to have a greater influence on protease production by the *Lysinibacillus fusiformis* strain C250R. Then, the Box–Behnken experimental design was performed to determine the level of the selected variables that influence protease production. These variables were prescribed into three levels, coded −1, 0, and 1. The realized modeling was based on the second order polynomial equation:

$$y = b_0 + \sum_{i=1}^{n} b_i x_i + \sum_{i=1}^{n} b_{ii} x_i^2 + \sum_{i=j}^{n-1} \sum_{j=i+1}^{n} b_{ij} x_{ij}$$

Where y is the protease activity, b_0 is the offset term, b_i is the linear effect, b_{ii} is the squared effect, b_{ij} is the first order interaction effect and x_i is the independent variable.

2.4. Assay of proteolytic activity

The protease activity was assayed by a modified caseinolytic Peterson's method protocol using Hammerstein casein (Merck, Darmstadt, Germany) as a substrate (Peterson 1977). Enzyme solution (0.5 mL) suitably diluted was mixed with 0.5 mL 100 mM glycine-NaOH buffer supplemented with 2 mM CaCl$_2$ at pH 10 (Buffer A) containing 10 g/L of casein, and incubated for 15 min at 70°C. The reaction

was stopped by the addition of 0.5 mL TCA 20% (w/v). The mixture was allowed to stand at room temperature for 15 min and then centrifuged at 10,000g for 15 min to remove the precipitate. The acid soluble material was estimated spectrophotometrically at 280 nm. A standard curve was generated using solutions of 0-100 µg/L tyrosine. One unit (U) of protease activity was defined as the amount of enzyme, which liberated 1 mg tyrosine per minute under the experimental conditions used. The proteolytic activity in presence of the laundry detergent solution was evaluated by the method suggested by Touioui Boulkour et al. (Touioui et al. 2015) using *N,N*-dimethylated casein (DMC) as a substrate. Unless otherwise stated, a suitably diluted enzyme solution (0.5 mL) was mixed with 1 mL laundry detergent, 2 mL 100 mM borate-NaOH buffer (pH 9) containing 10 g/L DMC, and 0.25 mL of 10 g/L 2,4,6-trinitrobenzene sulfonic acid (TNBSA) as a color indicator. The mixture was shake-incubated at 70 °C for 25 min, and the reaction was stopped by the addition of a 2.5 mL of cold water for 15 min. The precipitate was then removed by centrifugation at 10,000g for 15 min. Absorbance was measured at 450 nm. One unit of protease activity was defined as the amount of enzyme required to catalyze the liberation of 1 µmole of product from DMC per min under the experimental conditions used.

2.5. Statistical analyses

The data were analyzed using SPSS (Version 11.0.1 2001, LEAD Technologies, Inc., and USA). The response surface was plotted using Microsoft Excel software (Version 2007, Microsoft Inc., USA). All determinations were performed at least three independent replicates, and the control experiment without protease was carried out under the same conditions. The experimental results were expressed as the mean of the replicate determinations and standard deviation (mean ± SD). The statistical significance was evaluated using t-tests for two-sample comparison and one-way analysis of variance (ANOVA) followed by t-test. The results were considered statistically significant for P values of less than or equal to 0.05. The statistical analysis was performed using the R package Version 3.1.1 (Vanderbilt University, Nashville, TN, USA).

3. Results and discussion

3.1. Screening of protease-producing strains

The bacterial strain used for this study was isolated from the production water of the Ramoura oil field (onshore) at Tunisia (Mnif et al. 2011). It exhibited an interesting proteolytic activity by hydrolyzing casein with a zone of diameter on skimmed milk agar plates (SMAP) **(Figure 1)**. The optimum production of protease is 680 U/mL achieved at pH 10 and 70°C in an initial media composed per Litre from 5 g gelatin, 1 g yeast extract, 50 g NaCl, 2 g CaCl$_2$, 1 g MgSO$_4$.7H$_2$O, 1 g K$_2$HPO$_4$, and 1 g KH$_2$PO$_4$ at pH 7.4.

Fig. 1. Detection of protease activity on SMAP medium by *Lysinibacillus fusiformis* strain C250R.

3.2. Effect of carbon and nitrogen sources

Protease production was tested in the initial medium containing (g/L): yeast extract (as nitrogen source), 1; CaCl$_2$, 2; KH$_2$PO$_4$, 1; FeSO$_4$, 10; trace elements 1%; and 10 g/L of different carbon and energy sources: casein, gelatin, gruel, starch, glucose and maltose. As shown in table 1, the best carbon source for protease production was gruel, (680 U/ml); followed by gelatin (659 U/ml). In general, both organic and inorganic nitrogen sources were used efficiently by *Bacillus* species, for protease production. In the present study, different organic (yeast extract, meat extract, beef extract, and soya flour) and inorganic [ammonium sulphate, (NH$_4$)$_2$SO$_4$ and ferric ammonium sulphate, NH$_4$Fe(SO$_4$)$_2$] nitrogen sources, at a concentration of 2 g/L, were also tested in a medium containing gruel at 10 g/L as a sole carbon and energy source. As shown in table 2, the best nitrogen source for protease production was yeast extract (750 U/ml) followed by meat extract (400 U/ml) and beef extract (304 U/ml). In the light of these results, the gruel and yeast extract turns out to be more adequate for the production of protease by *Lysinibacillus fusiformis* strain C250R. In fact, the gruel and yeast extract are two sources of amino acids and growth factors for the bacterium. They also contain vitamins and cofactors essential for the development of the bacterium and for the synthesis of metabolites as well.

Table 1: Effect of different carbon (5 g/L) **(A)** and nitrogen (2 g/L) **(B)** on proteases production by *Lysinibacillus fusiformis* strain C250R.

A

Carbon source	Control	Casein	Gelatin	Gruel	Glucose	Starch	Maltose
Protease activity (U/mL)	114	200	659	**680**	478	191	250
Absorbance at 600 nm	0.49	1.8	0.68	**3**	1.43	1.43	0.6

B

Carbon source	Control	(NH4)$_3$FeSO$_3$	(NH4)$_2$SO$_4$	Soy flour	Yeast	Beef	Malt
Protease activity (U/mL)	91	241	300	182	**680**	304	400
Absorbance at 600 nm	2.23	2.8	6.15	5.77	**3.5**	3.17	4.12

3.3. Screening of significant variables using Plackett-Burman design

The Plackett-Burman design is widely used in biotechnological production because it allows the screening of main factors from a large number of variables that can be retained in the further optimization process (Sathish Kumar et al. 2014). In this study, we first used the Plackett–Burman design to screen the most influential variables on *Lysinibacillus fusiformis* strain C250R protease production. Seventeen different variables including some operational parameters and medium components were screened using a 19 runs matrix. Levels of the factors tested for the production of proteases by strain C250R using Plackett-Burman methodology are presented in Table 1. The data showed a wide variation of protease activity production ranging from 680 to 3045 U/ml. This variation proved the importance of this step in selecting the most influent factors and the level of the others.

The main effect of each variable was estimated by evaluating the contrast coefficient (*b*). Result analysis showed that gruel, wheat bran, yeast extract and FeSO$_4$ were the most significant variables that

present the largest contrast coefficient. In fact, wheat bran, yeast extract and FeSO₄ concentration had a positive effect on protease production, while the gruel concentration exhibited a negative effect. The design matrix with the corresponding results of Plackett–Burman experiments is presented in Table 3. For further optimization, the level of the most significant factors was optimized by RSM using Box-Behnken design while insignificant ones were used in all trials at their optimum level. The other variables with positive effect (speed of agitation, crude oil, $MnSO_4$, trace elements and KH_2PO_4) were fixed at high level while variables with a negative effect (temperature, initial pH, sucrose, lentil flour, urea, $NaNO_3$, NaCl, and $CaCl_2$) were maintained at low level.

Table 3 Experimental design using the Plackett-Burman method for screening of factors affecting protease production.

Run	Global	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	X_{12}	X_{13}	X_{14}	X_{15}	X_{16}	X_{17}	Protease activity (U/ml)[a]
1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	0
2	+	-	+	-	-	+	+	+	+	-	+	-	+	-	-	-	-	+	1137
3	+	-	-	+	-	-	+	+	+	+	-	+	-	+	-	-	-	-	1682
4	+	+	-	-	+	-	-	+	+	+	+	-	+	-	+	-	-	-	1182
5	+	+	+	-	-	+	-	-	+	+	+	+	-	+	-	+	-	-	0
6	+	-	+	+	-	-	+	-	-	+	+	+	+	-	+	-	+	-	1182
7	+	-	-	+	+	-	-	+	-	-	+	+	+	+	-	+	-	+	728
8	+	-	-	-	+	+	-	-	+	-	-	+	+	+	+	-	+	-	0
9	+	+	-	-	-	-	+	+	-	-	+	-	-	+	+	+	+	-	910
10	+	-	+	-	-	-	-	+	+	-	-	+	-	-	+	+	+	+	3045
11	+	+	-	+	-	-	-	-	+	+	-	-	+	-	-	+	+	+	1500
12	+	-	+	-	+	-	-	-	-	+	+	-	-	+	-	-	+	+	0
13	+	+	-	+	-	+	-	-	-	-	+	+	-	-	+	-	-	+	364
14	+	+	+	-	+	-	+	-	-	-	-	+	+	-	-	+	-	-	0
15	+	+	+	+	-	+	-	+	-	-	-	-	+	+	-	-	+	-	0
16	+	+	+	+	+	-	+	-	+	-	-	-	-	+	+	-	-	+	546
17	+	-	+	+	+	+	-	+	-	+	-	-	-	-	+	+	-	-	637
18	+	-	-	+	+	+	+	-	+	-	+	-	-	-	-	+	+	-	0
19	+	+	-	-	+	+	+	+	-	+	-	+	-	-	-	-	+	+	319
B	696.42	-188.9	-7.263	2.4211	-337.3	-437.8	-88.42	318.32	260.63	-12	-117.2	74.105	-93.37	-289.5	131.58	21.474	35.789	107.68	

[a] Values represent means of three replicates.

B: Contrast coefficient.

### 3.4. Level optimization of the main factors using response surface methodology (RSM)

Based on the results given above, four factors namely gruel, wheat bran, yeast extract and FeSO₄ were chosen as critical variables affecting protease production. The design matrix with the corresponding results of Box-Behnken experiments, as well as the predicted results is presented in Table 4.

The model was established after the regression analysis and was predicted by the following equation:

$$Y = 8991.906 - 2239.733 \times X_1 + 147.683 \times X_2 + 348.229 \times X_3 - 2419.875 \times X_4 + 173.160 \times X_1 \times X_1 + 12.739 \times X_1 \times X_2 + 77.699 \times X_1 \times X_4 - 4.264 \times X_2 \times X_2 + 12.5 \times X_2 \times X_3 - 40.900 \times X_2 \times X_4 - 63.468 \times X_3 \times X3 + 39.874 \times X_3 \times X_4 + 273.437 \times X_4 \times X_4$$

When **Y** is the protease activity (U/ml), X_1, X_2, X_3 and X_4 are respectively gruel (g/L), wheat bran, (g/L), yeast extract (g/L) and $FeSO_4$ (g/L). The regression analysis result showed that the *F*-value was 3.417 with a very low probability value ($P < 0.001$) indicating the significance of the model. The closeness of experimental and predicted protease activity can be expressed by the determination coefficient of $(R^2 = 0.88)$ which stipulates that only 12% of the total variation could not be explained by the model. Adjusted R Square (predicted R_2) of 0.774 explains the good agreement between the experimental and the predicted results. The model equation related to protease production shows an important negative linear effect for gruel (X_1) and $FeSO_4$ (X_4) and significant interactions between gruel and $FeSO_4$ (X_1X_4), wheat bran and $FeSO_4$ (X_2X_4) and yeast extract and $FeSO_4$ (X_3X_4). These results were confirmed by the Student's *t*-test ($\alpha = 0.05$).

Response surface plot was generally the graphical representation of the regression equation, from which the response (protease production) is plotted against any two variables, while other variables were fixed at their middle levels. Response surface plots can directly reflect the impact of various factors on the response. Contour map traits can reflect the strength of the interaction between the two factors: an oval contour indicates that the interaction between the two factors is strong, whereas a circular contour indicates that the interaction between the two factors is weak (Wang et al. 2015). Fig. 2 shows the mutual interaction between the gruel and $FeSO_4$ ($X_1 X_4$) and yeast. An increase in protease production was recorded in the minimum levels of the two factors. Thus, the protease activity variation was observed with the simultaneous decrease or increase of these two factors.
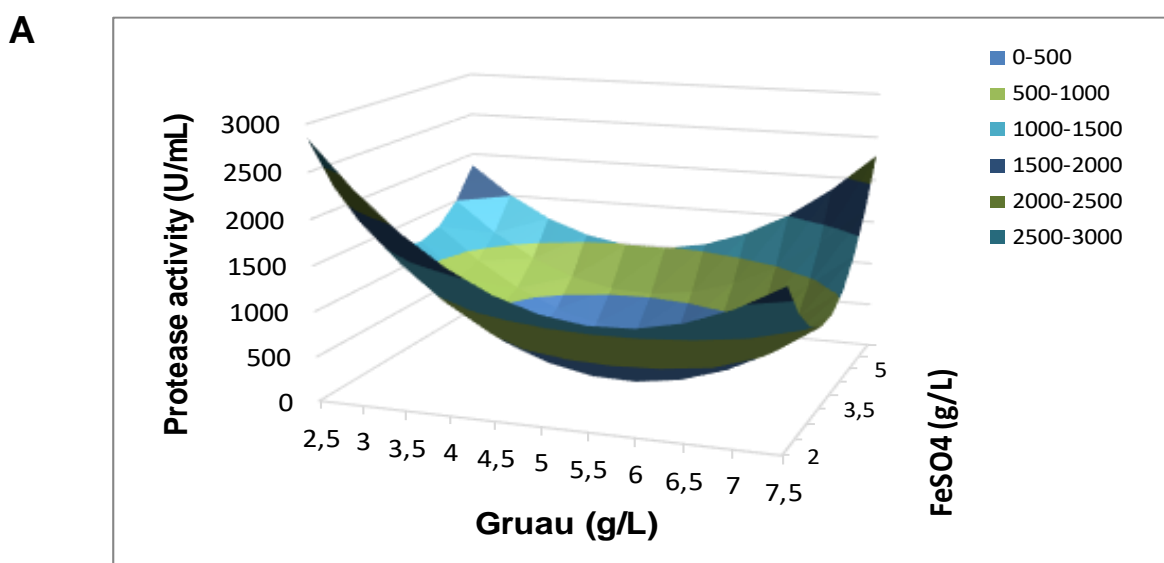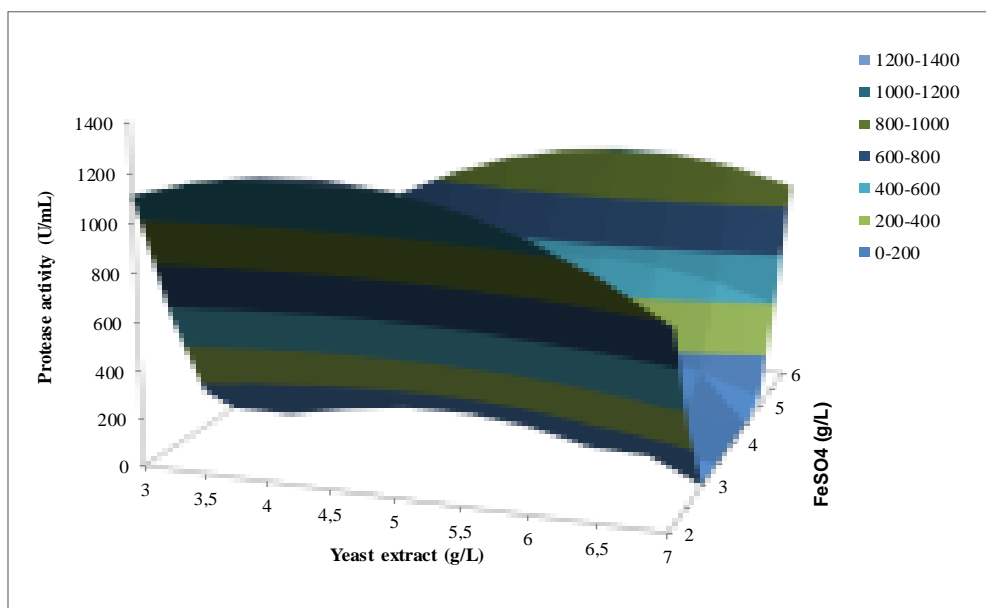
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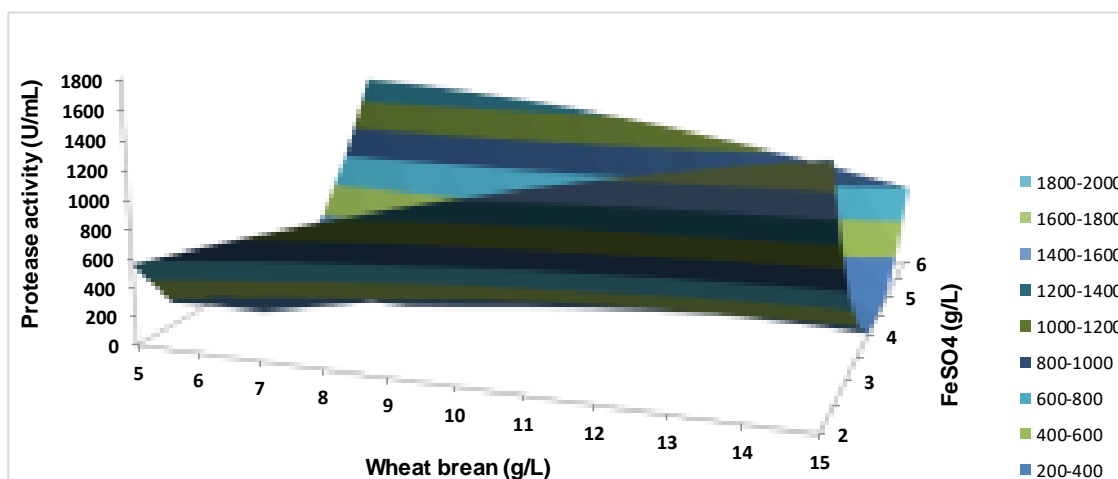
C

Fig. 2. Response surface plot of proteolytic enzyme production showing the interactive effects of the FeSO$_4$ and gruel concentrations (A), FeSO$_4$ and wheat bran concentrations (B) and FeSO$_4$ and yeast extract concentrations (C).

3.5. Model validation

The model validity was examined by additional experiments using the optimal culture conditions: temperature 30 °C, initial pH 7, speed of agitation 180 rpm, gruel (5 g/L), wheat bran (15 g/L), yeast extract (5 g/L), crude oil (4%, v/v), NaCl (20 g/L), FeSO$_4$ (2 g/L), MnSO$_4$ (2 g/L), trace elements (3%, v/v), and KH$_2$PO$_4$ (2 g/L). Protease production yield (3100 U/ml) was absolutely more important than that obtained during the preliminary study (680 U/ml). Thus, protease activity was multiplied by a factor of 4.55 fold. The expected result (3114.75 U/ml) was very close to the experimental result (3100 U/ml). By optimizing the medium composition and the culture conditions, not only the production of proteases

was enhanced but also the cost of enzyme production was reduced, since two cheap and readily available complex substrates (gruel and wheat bran) were used.

3.6. Performance evaluation of protease activity from strain C250R

3.6.1. Stability and compatibility with laundry detergents

The use of proteases as detergent additives represents a major application of industrial enzymes. To be suitable, they must be active under thermophilic (60 °C) and alkalophilic (pH 9-11) conditions, as well as in the presence of the various laundry detergents (Adrio and Demain 2014). According to Fig. 5C, this protease was extremely stable and compatible with the commercial solid detergents used, retaining 84% of its initial activity with Ecovax and 85% with Alyss even after 1 h incubation at 40 °C. However, Alcalase Ultra 2.5 L was noted to be less stable in the presence of Eovax and Alyss, retaining respectively only 75% and 77% of its initial activity. In addition, the protease from *Lysinibacillus fusiformis* strain C250R was highly stable in the presence of liquid laundry detergents at a concentration of 7 mg/mL (Fig. 3). The alkaline protease exhibited higher stability in Ariel, Dixan, than in Nadhif and Skip. However, Alcalase Ultra 2.5 L was noted to be less stable in the presence of Nadhif, retaining only 80% of its initial activity. Detergent stable proteases with variable stability in the presence of different detergents have been studied by several other researchers (Singh et al. 2012, Deng et al. 2010). Mechri et al. (Mechri et al. 2017) reported an alkaline protease from *Aeribacillus pallidus* strain VP3 with 60–100% residual activity when incubated with 7 mg/ml commercial detergents for 1 h at 45 °C. These results support the potential of protease from only 75% and 77% of its initial activity. In addition, the protease from *Lysinibacillus fusiformis* strain C250R as an ideal candidate for use in laundry detergent.
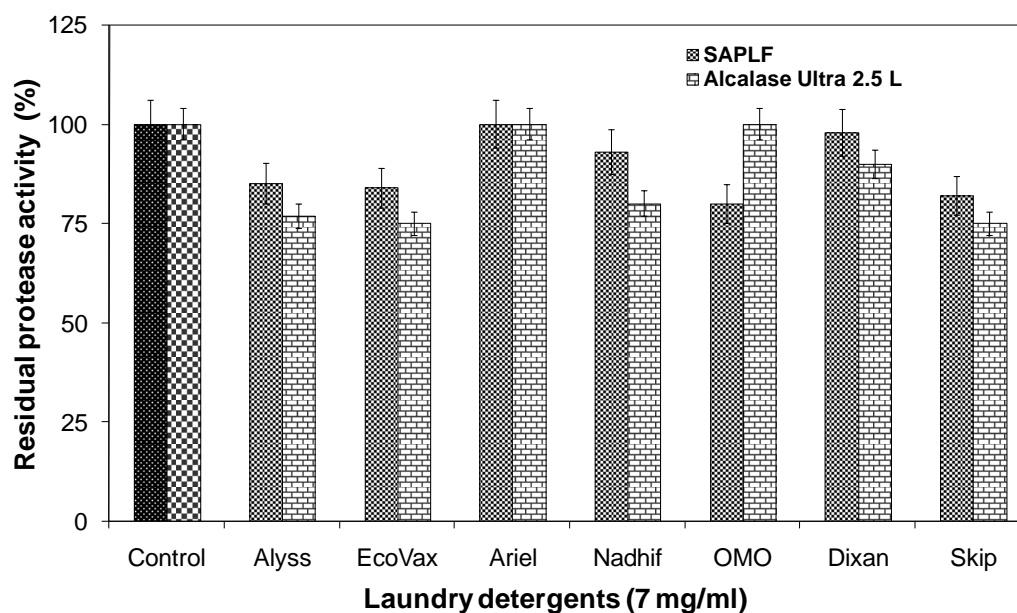

Fig. 3. Stability of the protease from strain C250R and Alcalase Ultra 2.5 L in the presence of liquid and solid laundry detergents. Enzyme activity of the control sample, which contained no additive and incubated under similar conditions, was taken as 100%. Each point represents the mean of three independent experiments.

3.6.2. Removal of blood stains from cotton fabrics

With the progress of urbanization, there is growing interest in detergent industries, especially to prepare bio-formulations effective for removal of various types of stains on the fabric (Hemlata et al. 2016). Removal of proteinaceous stains such as that of blood, grass, and sauces always remained a challenge to laundry workers in addition to households. The role of protease as an additive in detergent formulations has long been maintained. In laundry detergents, they account for approximately 25% of the total worldwide sales of enzymes (Adrio and Demain 2014). The success of subtilisins in the field of industrial detergents is based on their stability and high substrate specificity. The application of protease from *Lysinibacillus fusiformis* strain C250R in detergents was represented in the Fig. 4. The stained cloth was washed with prepared detergent solutions without and with the protease from *Lysinibacillus fusiformis* strain C250R.
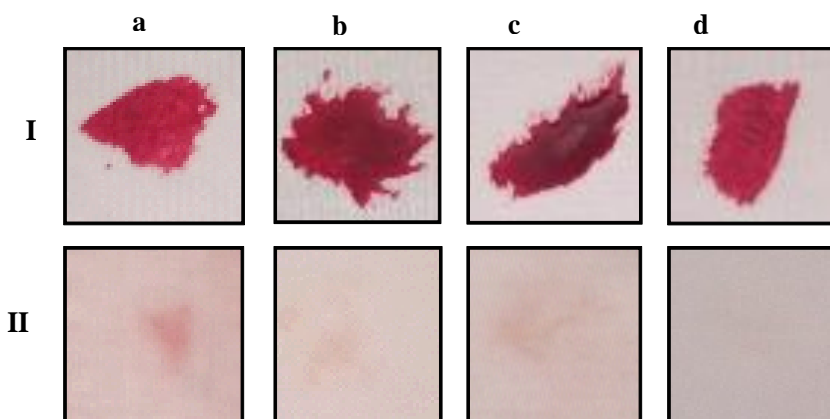

Fig. 4. Washing performance analysis test of our protease in the presence of the commercial detergent Ariel. (a) Cloth stained with blood washed with tap water; (b) blood-stained cloth washed with Ariel detergent (7 mg/ml), (c) blood-stained cloth washed with Ariel added with Alcalase Ultra 2.5 L (commercial enzyme, 500 U/ml), (d) blood-stained cloth washed with Ariel added with C250R protease (500 U/ml). I: untreated cloths (control) and II: treated cloths.

We noticed a limited washing performance with detergent (Ariel) only. The supplementation of our enzyme or commercial protease Alcalase Ultra 2.5 L in detergent seems to improve the cleansing process as evidenced by rapid blood stain removal when compared to detergent alone. In fact, our protease facilitated the release of proteinacious materials in a much easier way than the currently used Alcalase Ultra 2.5 L protease. Furthermore, the combination of protease from strain C250R with the Ariel detergent resulted in the complete stain removal (Fig. 4). These results confirm the newly isolated alkaline protease SAPLF from strain C250R as an effective cleaner of stains.

Alkaline proteases have been used in the detergents to hydrolyze and clean proteinaceous stains on garments (Choudhary 2012). Jaouadi et al. (Jaouadi et al. 2009) reported the stain removal using protease in cotton fabric to remove blood stains completely. The alkaline SAPB protease produced by *Bacillus pumilus* strain CBS was capable of cleaning stains but its mixture with detergent was proved to be more effective. Additionally, the protease isolated from mutant *Bacillus licheniformis* used in stained cloth increased ability to remove blood stain from cotton cloth (Kumari and Rani 2013).

4. Conclusions

In this research, Plackett-Burman and Box-Behnken designs were employed to optimize the medium and culture conditions for the production of detergent stable protease by strain C250R. Compared to other proteases and the commercial Alcalase Ultra 2.5 L enzyme, the protease from this strain showed high stability and compatibility with a wide range of commercialized laundry detergents, making it particularly suitable for detergent formulations. Accordingly, further studies, some of which are currently underway in our laboratories, are needed to clone the encoding gene and to explore the structure-function relationships of the enzyme.

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