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Bacillus licheniformis SMIA-2 protease used as an additive in detergent formulations

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ABSTRACT. Microbial proteases, especially from *Bacillus* spp., have been widely exploited for a broad variety of applications, such as the improvement of the cleaning efficiency of conventional detergents. In this work, the statistical design of the experiment was used to optimize the concentrations of a three-component mixture: *Bacillus licheniformis* SMIA-2 protease, Linear Alkylbenzene Sulphate and hydrogen peroxide, in an attempt to prepare an environmentally correct cleaning formulation. The results demonstrated that the combination of 1% (w/v) protease with 1.5% (w/v) LAS and 1% (w/v) H_2O_2 was effective in removing blood from cloth pieces and that a protease concentration decrease from 1.0% to 0.5% (w/v) would not have a significant impact on percent blood removal if LAS concentrations between 1.5-2.0% (w/v) in combination with lower (<0.5%, w/v) concentrations of H_2O_2 were used. Thus, the protease from *Bacillus licheniformis* SMIA-2 can be effectively incorporated into cleaning formulations together with LAS and hydrogen peroxide to formulate more sustainable detergents.

Keyword: *Bacillus;* protease; enzymatic detergent; linear alkylbenzene sulphate; hydrogen peroxide; statistical design.

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Introduction

Proteases or peptidases constitute the class of enzymes most used worldwide, which account for 65% of the present cost for the sale of industrial enzymes (Barzkar, Homaei, Hemmati & Patel, 2018; Benmrad et al., 2016). As crude or purified preparations, proteases have diverse applications in a wide variety of industries, including leather, silk manufacturing, food and detergent industry (Freitas-Junior et al., 2012; Nasri, Abed, Karra-châabouni, Nasri, & Bougatef, 2015; Singh & Bajaj, 2017).

The demand for microbial proteases to be used in the detergent industry has steadily increased, since they are environmentally friendly and completely biodegradable natural surfactants capable of improving washing performance. Protease supplementation in detergents can reduce the amount of other hazardous chemical-based detergent components, which makes them more eco-friendly (Niyonzima, 2018; Baweja, Tiwari, Singh, Nain & Shukla, 2016; Jegannatha & Nielsen, 2013). However, protease performance is affected by several factors, such as the detergent pH and composition, ionic strength, bleach systems, mechanical handling and long-term storage capacity (Rekik et al., 2019; Niyonzima & More, 2015; Barberis, Quiroga, Barcia, & Liggieri, 2013).

Bacillus licheniformis SMIA-2 is a Gram-positive, aerobic, thermophilic, spore-forming bacterium capable of producing thermostable enzymes, such as proteases (Silva, Delatorre, & Martins, 2007), amylases (Carvalho, Corrêa, Silva, Vianna, & Martins, 2008a; Carvalho, Correa, Silva, Mansur, & Martins, 2008b), pectinases (Andrade, Delatorre, Ladeira, & Martins, 2011) and cellulases (Oliveira, Barbosa, Martins, & Martins, 2014; Ladeira, Cruz, Delatorre, Barbosa, & Martins, 2015; Costa et al., 2017; Cruz, Moraes, Costa, Barbosa, & Martins, 2019). The bacterium was isolated from a soil sample collected in Campos dos Goytacazes City, Rio de Janeiro, Brazil. A phylogenetic analysis demonstrated that this strain is a member of the *Bacillus* rRNA group 5 (Souza & Martins, 2001). Bernardo et al. (2020) have recently revealed that SMIA-2 is 100% similar to the type strain *Bacillus licheniformis* Gibson 46 (ATCC 14580^T). According to these authors, the thermostable enzymatic activities of SMIA-2 could be supported by gene inventories, including six amylase genes; 13 loci for xylose metabolism, 55 protein degradation-associated loci and three cellulolytic enzyme loci (e.g. endoglucanase) under a putative cellulosome complex.

Bacillus licheniformis SMIA-2 was able to express a promising level of proteases in submerged cultures employing agricultural byproducts (Ladeira, Andrade, Delatorre, Pérez, & Martins, 2010; Corrêa, Moutinho, Martins, & Martins, 2011; Barbosa, Gentil, Ladeira, & Martins, 2014), which opened perspectives to generate high-value products from sustainable production processes. The proteases were capable of functioning at high temperatures and pH levels, retained good stability in the presence of several surfactants, oxidizing agents and locally available detergents (Nascimento & Martins, 2006), which indicates the potential of this bacterium and its protease for use in various industrial applications, including the detergent industry.

In this article, we examine the cleaning efficiency of protease recovered from a thermophilic *Bacillus licheniformis* SMIA-2 in the presence of the surfactant LAS (linear alkylbenzene sulfonate) and the oxidant hydrogen peroxide. In order to obtain the optimal enzyme cleaning solution, the concentrations of these three ingredients were optimized, using response surface methodology (RSM), considering the washing performance in removing blood stains.

Material and methods

Organism and culture conditions

The thermophilic *Bacillus licheniformis* bacterial strain SMIA-2, previously isolated from a soil sample collected in Campos dos Goytacazes city, Rio de Janeiro, Brazil (Souza & Martins, 2001), was used in the present study.

The protease production was carried out in a medium containing (g L^{-1} of distilled water): KCl-0.3, $MgSO_4$-0.5, K_2HPO_4-0.87, $CaCl_2$-0.29, ZnO-2.03x10^{-3}, $FeCl_3.6H_2O$-2.7x10^{-2}, $MnCl_2.4H_2O$-1.0x10^{-2}, $CuCl_2.2H_2O$-8.5x10^{-4}, $CoCl_2.6H_2O$-2.4x10^{-3}, $NiCl_3.6H_2O$-2.5x10^{-4}, H_3BO_3-3.0x10^{-4}, commercial corn steep liquor (Sigma Aldrich)-3.0, whey protein-1.0 and soluble starch-2.5 (Corrêa et al., 2011).

The pH was adjusted to 7.2, with 1.0 M NaOH, and the medium was sterilized by steam-autoclaving at 121ºC, 1 atm, for 15 min. The medium (50 mL in 250 mL Erlenmeyer flasks) was inoculated with 1 mL of a standard overnight culture (initial number of cells 10^4) and incubated at 50ºC, in an orbital shaker (Thermo Forma, Ohio, USA) operated at 150 rpm, for 36 hours. Thereafter, the cell-free enzyme supernatant was obtained by centrifugation (Hermle Z 382K), at 15,500 g, for 15 min, at 4ºC and then spray-dried, using a Model SD-04 LAB-PLANT, (inlet air temperature = 70+2ºC, outlet air temperature = 100+1ºC, pressure 5 Kgf/cm^2 and flow rate = 4 mL min^{-1}). Maltodextrin (1%, w/v) was incorporated into the crude enzyme solution prior to spray drying (Rodrigues, Andrade, & Martins, 2013). The spray-dried particles were stored at 5º C.

Enzyme assay

The protease activity was assessed in triplicate by measuring the release of trichloroacetic-acid soluble peptides from 0.2% (w/v) azocasein in 0.2 M Tris-HCL buffer (pH 8.5), at 70ºC, for 10 min (Jenssem et al. 1994). The 1 mL reaction was terminated by the addition of 0.5 mL of 15% (w/v) trichloroacetic acid and then centrifuged at 15,000 g, for 5 min, after cooling. One enzyme activity unit (U) was defined as the amount of enzyme required to increase absorbance equal to 1.0, at 420 nm, in 60 min.

The concentration of total soluble exocell protein was determined by Bradford (1976), using bovine serum albumin as standard.

Compatibility and stability of protease with surfactants and oxidants

The surfactants (Triton X-100, Renex 60, Renex 95 and Linear Alkylbenzene Sulfonate), oxidants (hydrogen peroxide and sodium perborate) and bleaching agents (Ethylenedi-aminetetra acetic acid and sodium carbonate) were incorporated into the reaction mixture (0.25-0.75%, w/v), and the reaction was carried out under standard assay conditions. The activity of the enzyme assayed in the absence of surfactants, oxidizing and bleaching agents was taken as 100%. The stability of protease was determined by incubating the enzyme solution (1.0 mg mL^{-1}), at 45°C, in the presence of 0.75% (w/v) Linear Alkylbenzene Sulfonate (LAS), hydrogen peroxide (H_2O_2) and sodium carbonate (Na_2CO_3). Aliquots (0.5 mL) were taken at different time intervals, and the residual activity was determined under standard assay conditions and compared with the control sample incubated at 45ºC, without any surfactant or oxidant. The results were represented as mean ± S.D. of triplicate determinations.

Cleaning solution

The cleaning solutions were prepared by mixing the components in the following order: hydrogen peroxide, Na_2CO_3/glycine (30:70), LAS, protease from *Bacillus* sp SMIA-2 and water (up to 100 %, w/v). The Na_2CO_3/glycine was used at a fixed concentration of 1% (w/v) of the total formulated cleaning solution volume. The cleaning solution components and their quantities are summarized in Table 1.

Washing performance of cleaning solution

The cleaning solution (25mL) containing H_2O_2, LAS, Na_2CO_3/Glicyne and protease was added to 125mL Erlenmeyer flasks at the required concentration in each assay. A small blood-soaked cloth (3.0 x 3.0 cm - EMPA 111, TEXCONTROL) was added to the flasks containing the cleaning solution, and the flasks were incubated at 45°C, under 150 rpm shaking on a Thermo Forma orbital shaker (Ohio, USA), for 60 minutes. After incubation, the pieces of cloth were taken out, rinsed three times with water (5 min each) in a mechanical stirrer, at 45°C and 150 rpm and subsequently dried.

The washing performance (ΔE) was evaluated using a colorimeter Miniscan XE Plus (HUNTER Lab), CIELAB scale (L*, a *, b *). On this scale, the L* parameter indicates color variation from black to white on a 0 to 100 scale, where 0 indicates black, and 100 is white. The parameter a* varies from red (+a*) to green (-a*). The value b* varies from yellow (+b*) to blue (-b*). The color measurements were expressed in colorimetric difference, before and after the washing of the cloth pieces.

$$\Delta E = [(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2]1/2 \quad (1)$$

Where ΔL = L before − L after, Δa = a before − a after and Δb = b before − b after.

Experimental Design and Statistical Analysis

The response surface methodology (SRM) was used to obtain a model for washing performance of cleaning solution *(*ΔE). A central composite design (CCD) 2^3 was constructed to optimize the concentration of the three components LAS, H_2O_2 and protease in the cleaning solution. The factorial planning had three central points and yielded a total of 17 treatments. Table 1 describes the factors studied and their levels.

Table 1. Factors and Levels studied in CCD.

Factors	Factor Level				
	-1.68	-1	0	+1	+1.68
LAS (%, w/v)	0.00	0.60	1.50	2.40	3.00
H_2O_2 (%, w/v)	0.00	0.40	1.00	1.60	2.00
Protease (%, w/v) /v)	0.00	0.20	0.50	0.80	1.00

The results were evaluated using Statistica version 7.0 software. In this context, the F test was used as a validation criterion of statistical significance of the models obtained at a confidence level of 90 %. The optimization of condition was performed using CCD, and surface-response was produced for both experiments. The experimental model can be expressed as follows:

$$Y = b_o + \sum_{i=1}^{n} b_i x_i + \sum_{i=1}^{n} b_{ii} x_i^2 + \sum_{i \neq j=1} \sum b_{ij}\, x_i x_j \quad (2)$$

Where b_o, b_i, b_{ii} and b_{ij} are the intercept terms, linear, quadratic coefficient and interactive coefficient, respectively, and *xi* and *xj* are coded independent variables.

Results

Compatibility and stability of protease with surfactants and oxidants

The compatibility of the protease with surfactants, oxidants and bleaching agents is presented in Table 2. The protease was compatible with Renex 60, Renex 95 and Triton X-100 for all concentrations tested (0.25% - 0.75%, w/v). In the presence of 0.75% (w/v) LAS, the protease activity was around 17% higher, compared to the control

(enzyme without the presence of any surfactant). The protease from *Bacillus licheniformis* SMIA-2 was stimulated in the presence of 0.75% (w/v) hydrogen peroxide, but lost about 20% of its activity in the presence of sodium perborate. Protease activity decreased in the presence of EDTA and sodium carbonate. The enzyme maintained about 20% and 44% of its activity in the presence of 0.75% (w/v) of EDTA and sodium carbonate, respectively.

Table 2. Compatibility of protease from *Bacillus licheniformis* SMIA-2 with some detergent components.

Agents	Relative activity (%)		
	0.25% (w/v)	0.5% (w/v)	0.75% (w/v)
Surfactants			
Triton X-100	102.29 ± 0.64	96.97 ± 0.65	97.38 ± 0.39
Renex 95	101.53 ± 0.59	95.14 ± 0.33	103.69 ± 0.43
Renex 60	96.05 ± 0.70	86.81 ± 0.45	92.9 ± 0.61
LAS	132.9 ± 0.12	120.57 ± 0.21	117.7 ± 0.19
Oxidizing agents			
H_2O_2	86.96 ± 0.32	108.8 ± 0.23	111.9 ± 0.24
Sodium perborate	112.44 ± 0.21	94.55 ± 0.32	80.49 ± 0.21
Bleaching agents			
EDTA	42.93 ± 0.13	37.35 ± 0.18	19.55 ± 0.25
Sodium carbonate	92.91 ± 0.13	63.38 ± 0.14	43.52 ± 0.09

The activity is expressed as a percentage of the activity level in the absence of surfactants or oxidizing agents (100% of enzyme activity = 11.46 U mg^{-1} Protein).

The stability of protease in the presence of LAS, H_2O_2 and Na_2CO_3 was examined by incubating the enzyme containing the solution with 0.75% (w/v) of each detergent component, at 45°C. As observed in Table 3, the protease from *Bacillus* sp SMIA-2 was stable when incubated for 2 hours, at 45°C, in the presence of H_2O_2 and Na_2CO_3. The enzyme activity increased about 44% in the presence of LAS.

Table 3. Stability of protease from *Bacillus licheniformis* SMIA-2.

	Residual activity (%)			
Agents (0.75 %, w/v)	15 min	30 min	60 min	120 min
Control	102.0± 0.09	100.3± 0.21	97.5 ± 0.38	95.8 ± 0.18
LAS	133.4± 0.79	120.8± 0.59	134.1± 0.43	143.8± 0.82
H_2O_2	88.0 ± 0.22	125.3 ± 0.45	123.0 ± 0.51	119.9 ± 0.65
Na_2CO_3	107.2 ± 0.58	102.2 ± 0.32	102.9 ± 0.41	103.5 ± 0.28

The activity is expressed as a percentage of the activity level in the absence of any detergent component (100% of enzyme activity = 11.46 U mg^{-1} protein).

Washing performance of cleaning solution containing protease

A central composite design (CCD) 2^3 was constructed to evaluate the washing performance (ΔE) of cleaning solutions containing LAS, H_2O_2 and protease in the removal of blood from test fabric (EMPA 111). A mixture containing Na_2CO_3 and glycine (30:70) was prepared and added at 1% (w/v) of the total formulated solution volume (Table 4). All solutions presented pH levels higher than 8.5.

Table 4. Matrix of CCD 2^3 (real and coded values) used and its response (ΔE).

Assays	LAS (%, w/v)	H_2O_2 (%, w/v)	Protease (%, w/v)	ΔE
F1	(-1)	(-1)	(-1)	15.37
F2	(+1)	(-1)	(-1)	14.27
F3	(-1)	(+1)	(-1)	13.93
F4	(+1)	(+1)	(-1)	12.88
F5	(-1)	(-1)	(+1)	19.10
F6	(+1)	(-1)	(+1)	18.84
F7	(-1)	(+1)	(+1)	19.11
F8	(+1)	(+1)	(+1)	18.28
F9	(-1.68)	(0)	(0)	12.29
F10	(+1.68)	(0)	(0)	17.65
F11	(0)	(-1.68)	(0)	17.92
F12	(0)	(+1.68)	(0)	16.50
F13	(0)	(0)	(-1.68)	7.91
F14	(0)	(0)	(+1.68)	19.60
F15	(0)	(0)	(0)	17.69
F16	(0)	(0)	(0)	17.61
F17	(0)	(0)	(0)	17.37

According to the results presented in Table 4, variation from 7.91 (Treatment 13) to 19.60 (Treatment 14) was observed in ΔE. The comparison of the two treatments showed that, in the solution of treatment 13, no protease was added; and in treatment 14, the enzyme was added at its highest concentration tested.

The statistical significance of the model equation was assessed by an F-test (ANOVA), and the data are presented in Table 5. The outcome of ANOVA analysis revealed that the adjusted model was significant, according to the analysis of the F test ($p<0.1$). The determination coefficient (R^2) of the model was 0.8527, which indicates that 85.27% of the variability in the ΔE response could be accounted by the model and that it was suitable to represent the real relationship among the independent variables studied.

Table 5. ANOVA for the variables of response surface quadratic model for ΔE.

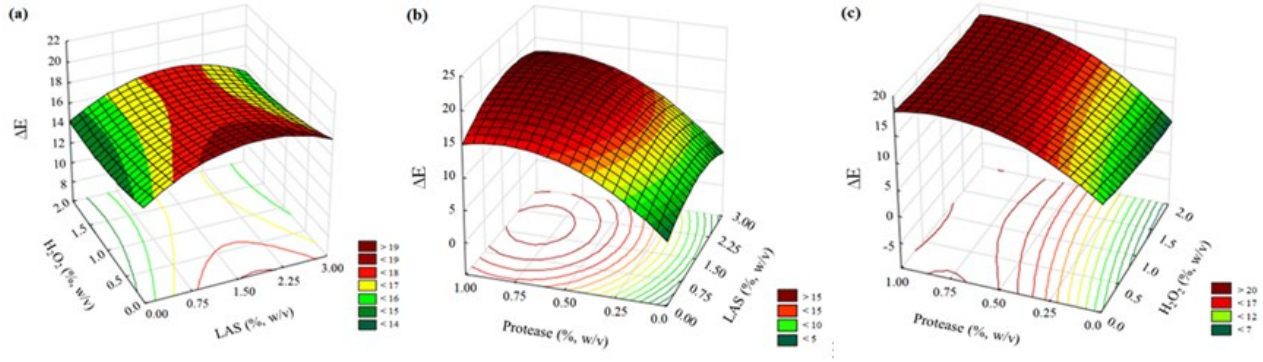
Variable	Sum of squares	(Degrees of freedom)	Mean square	Fcal	Ftab
Regression	131.7727	9	14.64141	4.490404	2.72
Residues	22.8242	7	3.2606		
Lack of Adjustment	22.7687	5	4.55374	164.0987	9.29
Pure Error	0.0555	2	0.02775		
Total error	154.5969	16			
				R^2 =	85.27%

An equation for ΔE (Eq. 3) was developed based on a regression analysis of the following experimental data:

$$W = 17.47307 + 0.42282x_1 - 0.62658x_1^2 - 0.42236x_2 - 0.16538x_2^2 + 2.82204x_3 - 1.05615x_3^2 - 0.65000x_1.x_2 + 0.13250x_1.x_3 + 0.28500x_2.x_3 \quad (3)$$

Where W is ΔE, x_1 is the LAS concentration, x_2 is the H_2O_2 concentration and x_3 is the protease concentration.

The effect of the interaction between H_2O_2 and LAS concentrations when the protease concentration remained constant at level 0 (0.5%, w/v) on ΔE was depicted in the response surface plot of Figure 1a. The best performance for blood removal from test fabric was observed when the cleaning solution contained between 1.5-2.0% LAS and lower concentrations of H_2O_2. Fig. 1b shows the response surface plot of the effect of LAS concentration and protease concentration and their mutual effect on the removal of blood from test fabric, with H_2O_2 concentration constant at level 0 (1.0%, w/v). Higher performance in the removal of blood from test fabric was found when concentrations of LAS between 1.4 and 2.3% (w/v) and protease concentrations between 0.7 and 1.0% (w/v) were used to formulate the cleaning solution. Figure 1c) presents the interactive effect between protease and H_2O_2 concentrations, when the LAS concentration remained constant at level 0 (1.5%, w/v). Higher ΔE values were found when lower and higher concentrations of H_2O_2 and protease were used in the cleaning solution, respectively.



(a)·Effect·of·LAS·and·H_2O_2·concentration·on·ΔE·at·a·fixed·protease·concentration·(0.5%,·w/v)¤

(b)·Effect·of·protease·and·LAS·concentration·on·ΔE·at·a·fixed·H_2O_2·concentration·(1%,·w/v)¤

(c)·Effect·of·protease·and·H_2O_2·concentration·on·ΔE·at·a·fixed·LAS·concentration·(1.5%,·w/v)¤

Figure 1. Three-dimensional response surface plot for ΔE. Dark red color indicates high activity, while green and yellow colors indicate low protease activity.

Images of fabric soiled with blood (EMPA 111) washed with the cleaning solution containing 1.5% (w/v) LAS and 1.0% (v/v) H_2O_2 and 1% (w/v) protease from *Bacillus licheniformis* SMIA-2 (Figure 2) confirmed its performance in the removal of blood from test fabric.

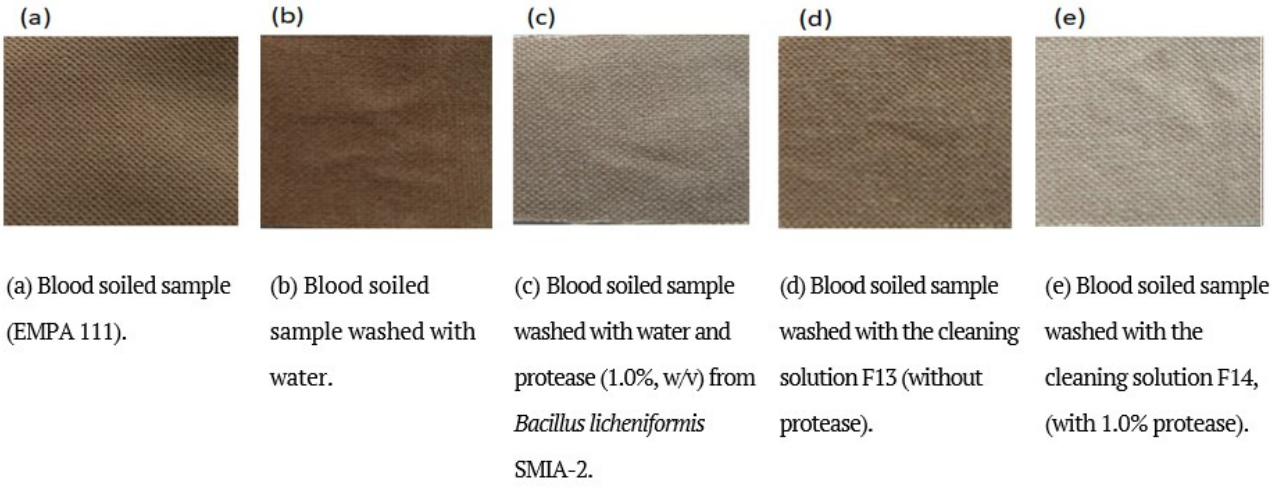


Figure 2. Images of samples of clothes soiled with blood (EMPA 111).

Discussion

The search for new sources of proteases is continuous, due to the increased demand for these enzymes by various industrial sectors, concerned with environmental issues, product quality and cost reduction (Singh & Bajaj, 2017). Whereas it is mainly applied as an additive for laundry detergents, the suitability of the *Bacillus licheniformis* SMIA-2 crude protease to be incorporated in a detergent formulation was investigated by testing its cleaning capability in the removal of blood from test fabric (EMPA 111). The use of proteases in cleaning solutions does not require the enzyme to be in pure form (Niyonzima & More, 2015). Thus, the use of pure *Bacillus licheniformis* SMIA-2 protease was disregarded due to its high cost. In addition, during the purification process, ligand and/or proteins with a protective effect on the crude enzyme could be removed and, consequently, the enzyme properties could be modified in such a way that their kinetic and thermodynamic behaviour could also be different (Braga et al., 2013).

Protease should be active and stable with surfactants, oxidizing and bleaching agents to produce an efficient detergent. In this context, *Bacillus licheniformis* SMIA-2 protease was compatible with Renex 60, Renex 95 and Triton X-100. Protease activity was stimulated in the presence of LAS. Surfactants are known to enhance the activity of enzymes (Barberis et al., 2013). They facilitate enzyme reactivity by increasing the solubility of the reaction components (Buxbaum, 2011). The effect of surfactants on enzyme activity varies according to the type of enzyme. However, in general, nonionic surfactants are more benign to an enzyme than anionic surfactants (Saraswat, Verma, Sistla, & Bhushan, 2017), despite some indication of enzyme activity loss caused by nonionic surfactants as well (Hilton, Tachikawa, Hildebrand, & Kolano, 2014). The protease from *Bacillus subtilis* B22 was unaffected by nonionic surfactants and tolerated an anionic surfactant (Elumalai et al., 2020), while the protease from *Bacillus cereus* SIU1 increased its activity in the presence of sodium lauryl sulphate, Triton X100 and Tween 80 (Singh, Singh, Tripathi, & Garg, 2011).

The protease from *Bacillus licheniformis* SMIA-2 was compatible with 0.75% (w/v) hydrogen peroxide, which usually inactivates proteins by the oxidation of a methionine residue present in the enzyme (Siezen & Leunissen, 1997). Hydrogen peroxide significantly increased the activity of protease from *Bacillus cereus* SIU1 (Singh et al., 2011). EDTA, a chelating agent, inhibited *Bacillus licheniformis* SMIA-2 protease activity and, in the presence of sodium carbonate (bleaching agent), protease activity was partially reduced. The role of the bleaching and oxidizing agents is usually to discolor the stain molecules during washing. As *Bacillus* SMIA-2 protease showed better compatibility with LAS, H_2O_2 and Na_2CO_3, its stability with each of these ingredients was investigated in the following experiments. Protease displayed good stability when incubated in the presence of 0.75% (w/v) LAS for 2 hours, at 45ºC. LAS is an anionic surfactant that plays a significant role in modern cleaning products, due to its efficiency in removing dirt (Liley et al., 2017; Cornwell, Radford, Ashcroft, & Ault, 2018). The protease (namely SAPB) from *Bacillus pumilus* strain CBS I 52 exhibited high

stability against strong anionic surfactants, particularly sodium dodecyl sulphate (SDS) and LAS (Jaouadi, Ellouz-Chaabouni, Rhimi, & Bejar, 2008). The enzyme retained its activity upon treatment with 0.8% SDS and 0.5% LAS. In addition, 80% and 65% residual activities were obtained after incubation with 1.5% SDS and 1.0% LAS, respectively. The protease from *Bacillus licheniformis* SMIA-2 also displayed good stability when incubated for 2 hours, at 45ºC, in the presence of 1.0% (w/v) H_2O_2 and Na_2CO_3. It corroborates its potential for application in the laundry detergent industry, since detergents may contain these ingredients for improving its washing performance (Rai, Konwarh, & Mukherjee, 2009). The protease from *Bacillus invictae* AH1 presented a quasi total stability against H_2O_2 (5% for 1 h) (Hammami et al., 2020), while the protease from *Bacillus pumilus* strain CBS I 52 retained 110% of its initial activity after treatment with 15% hydrogen peroxide (Jaouadi et al., 2008). The protease from *Bacillus* sp. B001 (designed AprB) retained 99% of its original activity after incubation with 1% H_2O_2, at 30ºC, for 1 hour (Deng, Wu, Zhang, Zhang, & Wen, 2010).

The washing performance *(*ΔE) of cleaning solutions formulated with LAS, H_2O_2 and *Bacillus licheniformis* SMIA-2 protease was investigated in the removal of blood from test fabric - EMPA 111. EMPA 111 is an artificially blood-soiled cotton commonly used to determine the cleaning performance of detergents and washing processes. A central composite design (CCD) 2^3 was constructed to optimize the concentrations of LAS, H_2O_2 and *Bacillus licheniformis* SMIA-2 protease in order to obtain a balance between them, so as to enhance their washing performance. A mixture of Na_2CO_3 and glycine (30:70) was added at 1% (w/v) of the total formulated solution volume to stabilize the protease, besides adjusting the pH value of the cleaning solution to the optimal activity (pH=8.5-9.0) of the protease (Silva et al., 2007; Barbosa et al., 2014.). Glycine is an amino acid that has been used to stabilize protein formulations (Platts & Falconer, 2015). In the comparison of the performance of the cleaning solutions, a relatively weak stain removal performance was observed in formulations with lower levels of protease, while formulations with higher levels of protease presented the best overall performance, which demonstrates its efficacy as an additive in laundry detergent. The optimal combination for maximal washing performance of LAS, H_2O_2 and protease from *Bacillus licheniformis* SMIA-2 on the removal of blood from test fabric (EMPA 111) was 1.5% (w/v), 1.0 (w/v) and 1.0 (w/v), respectively. A protease concentration decrease from 1.0% to 0.5% (w/v) did not have a significant impact on percent blood removal when LAS concentrations between 1.5-2.0% (w/v) were used in combination with lower concentrations of H_2O_2. However, protease concentration decrease to below 0.5% (w/v) reduced the washing performance of the cleaning solutions. Ideally, proteases or other hydrolytic enzymes used in detergent formulations should be effective at low levels, from 0.4% to 0.8% (Rai et al., 2009). Therefore, the experimental design approach used in this study was efficient to evaluate the effectiveness of protease from *Bacillus licheniformis* SMIA-2 with any chosen combination of LAS and H_2O_2 for the removal of blood from test fabric, thus preventing unnecessary addition of these three components in the cleaning solution. The visual examination of fabric soiled with blood washed with water and protease (1.0%, w/v) produced by *Bacillus licheniformis* SMIA-2 demonstrated that the enzyme was capable of cleaning stains. However, its mixture with LAS and H_2O_2 proved to be even more effective, which corroborated its performance in the removal of blood from test fabric.

Conclusion

In conclusion, the protease recovered from a thermophilic *Bacillus licheniformis* SMIA-2 can be combined with LAS and H_2O_2 into a single composition so that the benefits of these three components on stain-removing mechanisms could be perceived.

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