Biotechnology Reports 29 (2021) e00599

Contents lists available at ScienceDirect

Biotechnology Reports

journal homepage: www.elsevier.com/locate/btre

Research Article

Optimization of the cultivation conditions of *Bacillus licheniformis* BCLLNF-01 for cellulase production

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ARTICLE INFO

Article history:
Received 27 April 2020
Received in revised form 12 November 2020
Accepted 4 February 2021

Keywords:
Carboxymethylcellulases
Rotational central composite design
Bacillus licheniformis

ABSTRACT

The objective of this study was to optimize the production of CMCase by *Bacillus licheniformis* BCLLNF-01, a strain associated with the mucus of the zoanthid *Palythoa caribaeorum* (Cnidaria, Anthozoa). Production of total cellulase and CMCase was investigated in the supernatant, intracellular content and wall content. Cultivation was carried out in BLM medium supplemented with 1.5 % (w/v) CMC, 5.5 % (v/v) inoculum, 40 °C, pH 6.5, 500 rpm for 72 h, and the highest activity was recorded in the supernatant. A Rotational Central Composite Design (RCCD) 2^3 was used to investigate the influence of the carbon source concentration (CMC-0.5 to 1.5 % w/v), inoculum concentration (1–10 % v/v) and temperature (35–45 °C) on CMCase production. The maximum enzyme activity was achieved for a CMC concentration of 1.5 % w/v at 40 °C, attaining 0.493 IU/mL after 96 h of cultivation.

1. Introduction

Cellulases play an important role in the degradation of lignocellulosic biomass, cleaving the 1,4-ß-D-glycosidic bonds of cellulose, a biopolymer abundantly found in nature. The term cellulase is commonly used to describe the enzyme complex formed by the endoglucanases (endo-1,4-ß-glucanase EC 3.2.1.14), also known as carboxymethylcellulases (CMCase), which act on the inner region of the cellulose fiber releasing oligosaccharides of varying sizes; exoglucanases (exo-1,4-ß-D-glucanase EC 3.2.1.91), enzymes that act on the ends of the cellulose fiber resulting in the release of cellobiose; and ß-glucosidases (1,4 ß-D-glucosidase EC 3.2 .1.21), responsible for the breakdown of cellobiose bonds, which results in free glucose molecules [1].

These enzymes are applied in juice extraction processes, pulp and paper, textile industry, secondary metabolites, animal feed, extraction of vegetable dyes and in obtaining fermentable sugars used in the production of biofuels [2], and the demand for this enzyme is exponentially increasing [3].

The advantage of the industrial application of cellulases is that these enzymes remain stable under various physical and chemical conditions [2]. Representatives of the genera *Trichoderma*,*Aspergillus* and *Bacillus* are already used in the commercial production of these enzymes by companies such as Novozymes® and DuPont®. There are several reports of cellulases produced by strains of fungi; however, bacteria have good potential to be used in cellulase production because its higher growth rate as compared to fungi. In addition, bacterial cellulases are often more effective catalysts and may also be less inhibited by the presence of material hydrolyzed [4]. Due to the high cost of production, there is a constant search for new producers of cellulases and improvement of culture conditions to maximize enzymatic synthesis [5–8]. The prospection of microbial enzymes in the marine environment has been an important strategy in the search for new cellulase producers. The different environmental conditions (salinity, pressure, temperature) of this habitat reflect on the metabolic apparatus of its microbiota, making it interesting from the biotechnological point of view [9–11]. Another strategy that stands out in the field of enzymatic production is the optimization studies that allow the design of culture conditions with an optimal combination of factors such as pH, temperature, carbon source, nitrogen source and their interaction effects which promoting a maximum enzyme production and minimizing the production cost [12,13].

The cellulase production by genus *Bacillus* has been studied extensively, and many researches has reported the production of cellulases by strains of *B. licheniformis*, such as KIBEG-IB2 [14], K-3 [15], NCIM 5556 [12] and CGMCC 2876 [16].

In recent years, we have been exploring the biodiversity of the zoanthid *Palythoa caribaeorum* [17] by isolating new strains of

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https://doi.org/10.1016/j.btre.2021.e00599

culturable marine heterotrophic bacteria associated with its mucus. A new marine strain identified as *Bacillus licheniformis* BCLLNF-01 was the best cellulase producer in enzymatic screening (unpublished data). In this paper, we report the optimization of the cultivation conditions of *B. licheniformis* BCLLNF-01, isolated from the mucus of colonies of *P. caribaeorum*, for cellulase production.

2. Material and methods

2.1. Strain isolation, microscopic analysis, and molecular identification

Samples of mucus from healthy exposed colonies of *P. caribaeorum* were collected during low tide in Porto de Galinhas beach (8°30′20″ S; 35°00′34″ W), South coast of Pernambuco, Brazil. All samples were collected in sterile 50 mL conical vials, kept on ice, and transported to the Federal University of Pernambuco, Academic Center of Vitória. In the laboratory, mucus samples were homogenized, and aliquots of 1 mL were dispensed aseptically into Petri plates containing Zobell Marine Agar 2216 (HiMedia Laboratories, Mumbai, India) using the Pour Plate method. After incubation at 30 °C for five days, selected colonies were streaked on the same media used in the former step to obtain pure bacterial cultures, which were then photographed using a Canon EOS 70D attached to a 100 mm macro lens. Pure cultures were cultured in the Zobell Marine Broth 2216 (HiMedia Laboratories, Mumbai, India) on an orbital shaker (Orbital Shaker MA563, Marconi, São Paulo, Brazil) at 500 rpm for 18 h at 30 °C and samples were prepared for Gram's stain test for the observation of microscopical aspects (Eclipse 80i coupled to DS Ri1 camera, Nikon; 1000× magnification). Bacterial isolates were also cultured in MBS medium (30 °C, 500 rpm, for up to five days) to evaluate their ability to form spores. Fresh mounts immobilized on agar pads (3 % aqueous agar solution) were prepared using an aliquot of the culture and the slide was examined under a phase contrast microscope (Ph2 filter; 800× magnification). Molecular identification was performed by PCR amplification and sequencing of the bacterial genes 16S rRNA (primers fD1/1492R) [18,19], and rpoB (primers rpoB-F/rpoB-R) [20,21], at Macrogen Inc. (Seoul, South Korea). The sequence similarities were determined by the BLASTn algorithm [22] against the GenBank database, available at NCBI server.

2.2. Microorganism and conditions of preservation

Strain was preserved in the form of spores impregnated in paper discs [23]. Initially, microorganism was grown in MBS broth sporulation medium (composition, in g/L: meat peptone 10.0; yeast extract 1.0; KH_2PO_4 1.0; $MgSO_4.7H_2O$ 0.1; $CaCl_2.2H_2O$ 0.1 ; $FeSO_4.7H_2O$ 0.01; $MnSO_4.H_2O$ 0.01; $ZnSO_4.7H_2O$ 0.01), pH 7.2, in a shaker (MARCONI®) at 200 rpm, 30 °C for 5 days. A volume of 30 mL of the culture was centrifuged at 6000 rpm (VISION®) for 20 min and the supernatant was discarded. Then, the sample was resuspended in 3 mL saline solution (NaCl 0.85 % w/v), incubated at 80 °C for 12 min, and subsequently immersed in an ice bath for 5 min. For spores quantification, serial decimal dilutions of cell suspension in saline solution (NaCl 0.85 % w/v) were made, and a 100-μL aliquot was evenly distributed over Petri dishes containing nutrient agar medium using a Drigalski spreader. Plates were incubated for 48 h at 30 °C until bacterial colonies were visually observed, and the results were expressed in spores mL^{-1}.

Blotting paper 250 g m^{-2} and a 7.0 mm hole punch was used to prepare the discs. Twenty discs were distributed per Petri dish (90 × 15 mm), which were individually packed, autoclaved at 121 °C in three 1-hour cycles with 24-hour intervals and then oven-dried at 60 °C. After the sterilization and drying process, discs were impregnated with 20 μL aliquots of the cell suspension (5.8 × 109 spores mL^{-1}), dried in an oven at 35 °C for 24 h and stored in a refrigerator at 4 °C.

2.3. Culture medium for the development of the inoculum

Basic Liquid Media (BLM) medium was tested (composition, g L^{-1}): KH_2PO_4, 1.36; $(NH_4)_2SO_4$, 1.0; $MgSO_47H_2O$, 0.2; $FeSO_4$, 0.01; NaCl, 2.0; yeast extract, 1.0 [12]. The medium was supplemented under three different conditions: Condition 1 (0.5 % w/v high viscosity carboxymethylcellulose-CMC-Sigma-Aldrich®); condition 2 (1 % w/v glucose) and condition 3 (1 % w/v glucose + 1 % w/v casein). *B. licheniformis* BCLLNF-01 was cultured in 500 mL Erlenmeyer flasks containing 150 mL of medium at pH 6.5. A paper disc containing the sporulated culture (1.16×10^9 spores per disc) was added to each flask and culture was incubated in a shaker (MARCONI®) at 200 rpm at 30 °C for 24 h. Aliquots of each culture were taken from the flasks every 30 min and the optical density was determined by spectrophotometry at 600 nm. The cell growth curve was plotted as absorbance versus time, using cell-free culture medium as blank for analysis.

2.4. Distribution of enzymatic activity of B. licheniformis *BCLLNF-01*

For determination of enzymatic activity distribution of *B. licheniformis* BCLLNF-01, cells were cultured in BLM medium supplemented with 1.5 % w/v CMC (Carboxymethylcellulose sodium salt, degree of substitution 0.65–0.90, high viscosity 1500–3000 cps), pH 6.5, at 40 °C and inoculum size of 5.5 % v/v. 50 mL tubes received a volume of 18.9 mL of the culture medium, and were incubated for 72 h at 500 rpm. Every 24 h, aliquots were collected and centrifuged at 6000 rpm for 20 min at 4 °C. Activities of total cellulase and CMCase were analyzed from three distinct fractions of the culture (supernatant, intracellular and cell wall contents). For analysis of cellulase produced in the extracellular extract was used the supernatant obtained from the centrifugation step. Intracellular and cell wall contents were obtained according to Kupletskaya and Netrusov [24], with modifications. Pellet was resuspended in 0.05 M sodium citrate buffer (pH 4.8). Then, cell rupture was performed by abrasion with the use of glass beads, being stirred in cycles of five minutes in a vortex mixer (BIOMIXER QL - 901) with intervals of two minutes in an ice bath, in order to avoid enzymatic denaturation. At the end of 30 min, samples were centrifuged in the same conditions as described before, and the new supernatant was used in the analysis of the intracellular portion, while the pellet was resuspended in sodium citrate buffer and used in the analysis of the cell wall content. Experiments were performed in duplicate.

2.5. Determination of enzymatic activities of total cellulase and CMCase

Total cellulolytic activity was assessed using the 3,5-dinitrosalicylic acid (DNS) method [25], as described by Ghose [26]. 0.5 mL volume of crude enzyme extract was incubated with 20 mg Whatman No. 1 filter paper (Whatman Inc., Florham Park, NJ, USA) and 1 mL 0.05 M sodium citrate buffer (pH 4.8) at 50 °C for 60 min. As a control, a sample was prepared under the same conditions described except for the replacement of the enzymatic extract by distilled water. For the spectrophotometer calibration blank, only sodium citrate buffer was used. After incubation, 0.5 mL of each sample was transferred to tubes with the same proportion of DNS and the mixture was boiled for 5 min. Then, the samples were cooled in an ice bath and had their volume adjusted to 5 mL by the addition of distilled water. The reducing sugars released were quantified by UV-Vis spectrophotometry at 540 nm using glucose (2 g L^{-1}) as standard. An International Unit (IU) of cellulolytic activity

was expressed as the amount of enzyme required to release 1 μmol of glucose per minute under standard reaction conditions. Standard glucose curves were performed for each analysis and assays were performed in triplicate. To determine CMCase activity, CMC substrate solubilized at 2 % w/v in 50 mM sodium citrate buffer (pH 4.8) was used. The assay consisted of incubating 0.5 mL of crude enzyme extract diluted in 0.5 mL of sodium citrate buffer and 0.5 mL of the substrate (w/v). For the control sample the enzyme was replaced by distilled water and for the calibration blank, sodium citrate buffer was used. All samples were incubated at 50 °C for 30 min [26] and the enzyme activity was measured using DNS method [25]. Standard glucose curves were performed for each analysis and assays were performed in duplicate. The enzymatic activities (A) of total cellulase and CMCase were determined according to Eq. (1) [27], where *D* is dilution factor, C is concentration of glucose released in the enzymatic reaction (μmol mL^{-1}), Vt is total reaction volume (mL), *T* is reaction time (min) and *Ve* is volume of the enzymatic solution (mL).

$$A = \frac{D \times C \times Vt}{T \times Ve} \quad (1)$$

2.6. Optimization of fermentation conditions for CMCase production

In order to optimize the parameters that influence the production of CMCase, three independent variables (concentration of CMC, concentration of the inoculum and temperature) and the response variable (enzymatic activity of CMCase - *EA*) were evaluated using a full 2^3 factorial design, with three central points (level 0) and six axial points (levels ± α, where α = 1.68), totaling seventeen experiments (Rotational Central Composite Design – RCCD). Table 1 shows the experimental conditions investigated. CMC, inoculum concentrations and temperature values were selected based on data obtained in the literature [14,12,28].

The tests were performed randomly, and the data were subjected to analysis of variance (ANOVA) for analysis of regression coefficient, prediction equations and case statistics. Statistica® software (version 7.0) was used for regression and graphical analysis of the data obtained using a 95 % confidence level. Experimental error was obtained from the mean and standard deviation of the central points.

The medium used in the inoculum and in the optimization tests was the BLM medium at pH 6.5. For the inoculum, 500 mL Erlenmeyer flasks containing 100 mL of medium supplemented with 0.5 % CMC (w/v) were used. A paper disc containing the sporulated culture (1.16 × 109 spores per disc) was added to each flask and culture was incubated in a shaker (MARCONI®) at 200 rpm at 30 °C for 16 h.

In the optimization tests, *B. licheniformis* BCLLNF-01 was cultured in 50 mL Falcon tubes containing 20 mL of medium at pH 6.5, and culture was incubated in a tube shaker at 500 rpm for 96 h. Samples were taken at 24, 48, 72 and 96 h and centrifuged at 6000 rpm for 20 min at 4 °C. The cell-free supernatant (crude enzyme) was used in the enzyme activity assays.

3. Results and discussion

3.1. Molecular and biological characterization of strain

The current study is a part of a larger project that aims to investigate the bacterial diversity associated with colonies of *P. caribaeorum*, as well as the biotechnological potential of such microorganisms. In the first step of this project, we employed a general method to isolate different bacterial species from mucus samples. The microscopic analyzes were then used as a first screening to select endospore-forming bacilli. After the molecular identification and pilot experiments of enzymatic screening (unpublished data), we selected the isolate BCLLNF-01 to further our studies. Fig. 1 shows the morphological aspect of the bacterial culture isolated from the mucus of *P. caribaeorum*. The isolate showed white-beige and slimy colonies, with Gram-positive rods in single/short chains, which were endospore-forming, and was identified as *B. licheniformis*.

3.2. Inoculum conditions of B. licheniformis *BCLLNF-01*

The growth kinetics of *B. licheniformis* BCLLNF-01 in the three conditions tested for the development of the inoculum is shown in Fig. 2. The results show that when the microorganism was grown in the medium supplemented with 0.5 % w/v CMC, the exponential growth phase started after 14 h of cultivation, differing from the other conditions (glucose and glucose + casein), where the log phase started after approximately 17 h. On the other hand, media supplemented with glucose and glucose + casein induced to a higher specific growth rate. CMC has been used as a carbon source in cellulase production media, promoting both microbial growth and induction of these enzymes [12,29,30].

Nutritional factors have a great influence on microbial growth, especially preferences regarding the consumption of carbon and

Table 1
Codified levels and actual values of the variables studied in the optimization of the CMCase production.

Assays	CMC concentration (% w/v)	Temperature (°C)	Inoculum concentration (% w/v)
1	0.7 (-1)	37 (-1)	2.8 (-1)
2	1.3 (+1)	37 (-1)	2.8 (-1)
3	0.7 (-1)	43 (+1)	2.8 (-1)
4	1.3 (+1)	43 (+1)	2.8 (-1)
5	0.7 (-1)	37 (-1)	8.2 (+1)
6	1.3 (+1)	37 (-1)	8.2 (+1)
7	0.7 (-1)	43 (+1)	8.2 (+1)
8	1.3 (+1)	43 (+1)	8.2 (+1)
9	1.0 (0)	40 (0)	5.5 (0)
10	1.0 (0)	40 (0)	5.5 (0)
11	1.0 (0)	40 (0)	5.5 (0)
12	0.5 (-α)	40 (0)	5.5 (0)
13	1.5 (+α)	40 (0)	5.5 (0)
14	1.0 (0)	35 (-α)	5.5 (0)
15	1.0 (0)	45 (+α)	5.5 (0)
16	1.0 (0)	40 (0)	1.0 (-α)
17	1.0 (0)	40 (0)	10.0 (+α)

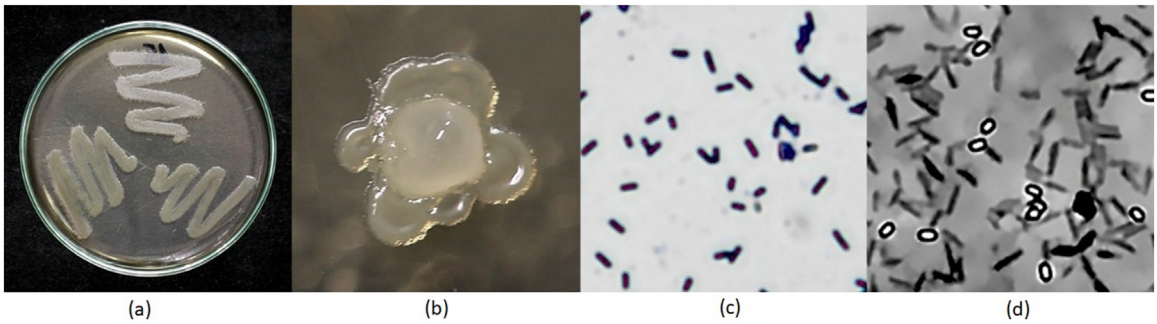

Fig. 1. Morphological aspect of the bacterial culture isolated from the mucus of *Palythoa caribaeorum*. Culture on nutrient agar after 24 h of incubation (a); detail of an isolated colony on nutrient agar (b); Gram staining of culture grown in nutrient broth (c) and photomicrography with phase contrast showing spores from the cultures grown in MBS medium after 5 days of incubation (d).

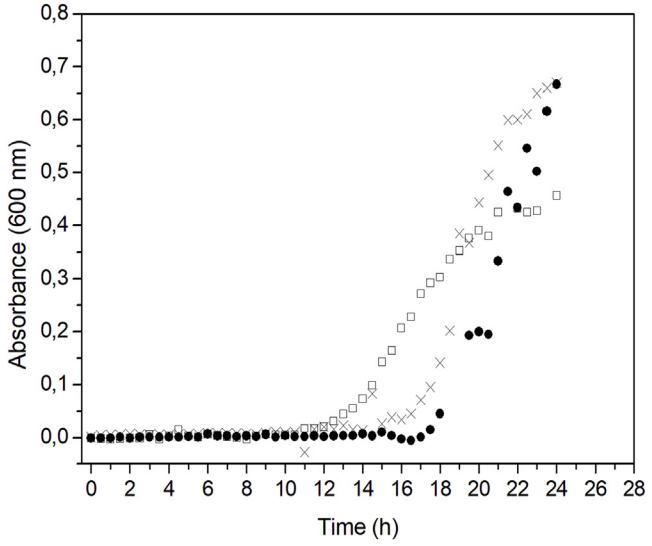

Fig. 2. Cell growth kinetics of *B. licheniformis* BCLLNF-01 in BLM + 0.5 % w/v CMC (□) (μ = 0.06 h^{-1}), BLM + 1 % w/v glucose (●) (μ = 0.11 h^{-1}) and BLM + 1 % w/v glucose + 1 % w/v casein (×) (μ = 0.11 h^{-1}) (μ = maximum specific growth rate).

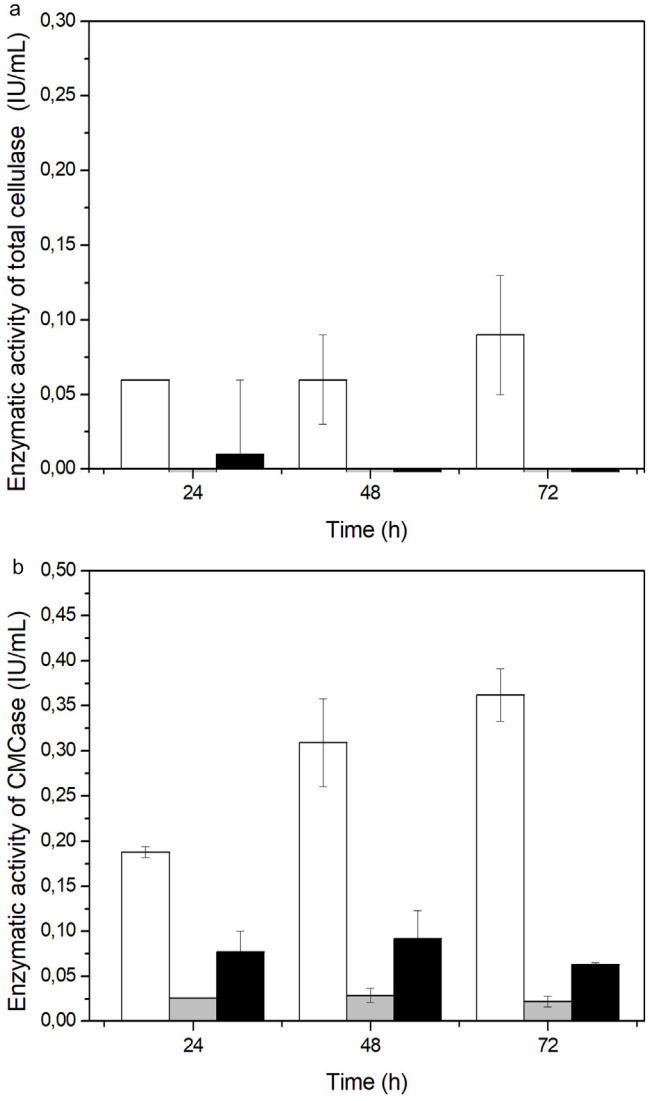

Fig. 3. Distribution of total cellulase (a) and carboxymethylcellulase (CMCase) (b) activity of *B. licheniformis* BCLLNF-01 (inoculum 5.5 % v/v; CMC 1.5 % w/v; 40 °C; 500 rpm) according to the cultivation time. (□ Supernatant; ▒ Intracellular; ■ Cell wall).

nitrogen sources, which vary according to the microorganism and the complexity of the substrate [31–33]. Glucose is a simple sugar which is more quickly assimilated, resulting in a higher growth rates [34,35]. As for the use of casein, it can be used both as a carbon and nitrogen source as it provides essential amino acids, carbohydrates and inorganic elements such as calcium and phosphorus [36].

Although in media supplemented with glucose and glucose+casein have been observed a higher specific growth rate, BLM + 0.5 % w/v CMC medium was selected for the inoculum development, because in this condition the time required to start the log phase has been reduced. This is relevant when considering the cost-benefit ratio for enzymatic production. In addition, CMC has been widely reported as a good inducer in the synthesis of enzymes of the cellulolytic complex [12,29], and its use can be advantageous since the inoculum preparation.

3.3. Enzymatic activities of total cellulase and CMCase

In order to analyze the distribution pattern of cellulases synthesized by *B. licheniformis* BCLLNF-01, the enzymatic activity of total cellulase (Fig. 3a) and CMCase (Fig. 3b) were investigated in the supernatant, intracellular content and wall content over 72 h of culture. The highest enzymatic activities were observed in the supernatant samples, revealing that the cellulases synthesized by *B. licheniformis* BCLLNF-01 are mainly excreted in the extracellular medium.

Cellulases activities have been commonly reported from the extracellular content [37–40]. In addition, although CMC is used as an inducer of total cellulases and CMCase [41,42,12], the results suggest that CMC mainly favored the synthesis of CMCase. These findings are corroborated by studies of Abu-Gharbia et al. [43], who evaluated, among other factors, the effect of supplementing the culture medium with various carbon sources (glucose, fructose, sucrose, lactose and CMC). The authors observed that CMCase activity was higher than that of total cellulase, cellobiase and hemicellulases when CMC was incorporated into the culture.

3.4. Effect of CMC concentration, temperature and inoculum concentration on CMCase production

Fig. 4 shows the CMCase activity for all assays performed in the experimental design after 24, 48, 72 and 96 h of cultivation. The higher CMCase activity was observed in assay 13 (5.5 % v/v inoculum, 40 °C and 1.5 % w/v CMC), reaching 0.5 IU mL^{-1} after 96 h of incubation. The optimum time for enzymatic synthesis can vary depending on the species and growing conditions [44–46]. The effects of some nutritional and environmental factors on the production of CMCase and exoglucanases (FPase) from *B. licheniformis* MVS1 were studied by Acharya and Chaudhary [47]. Maximum CMCase production was detected after 60 h incubation period, reaching 0.12 and 0.099 IU mL^{-1} for wheat and rice straw, respectively.

A Rotational Central Composite Design (RCCD) (23) was employed to optimize the CMCase synthesis by *B. licheniformis* BCLLNF-01 in relation to concentration of CMC, concentration of inoculum and temperature. The adequacy of the model was checked using ANOVA (Table 2), and the second-order polynomial model for the enzymatic activity (EA) of CMCase was expressed by Eq. (2). p-values less than 0.05 for the effects CMC (first-order effect) and temperature (T) (second-order effect) indicate these model terms are significant at the 95 % confidence level.

The *lack-of-fit* F-value of 2.37 implies that this parameter is not significant and there is a 32.24 % chance that this occurrence is due to noise. Fig. 5 shows predicted and experimental values to show the correlation; the predicted R^2 value was 0.9137 and the adjusted R^2 was 0.8028.

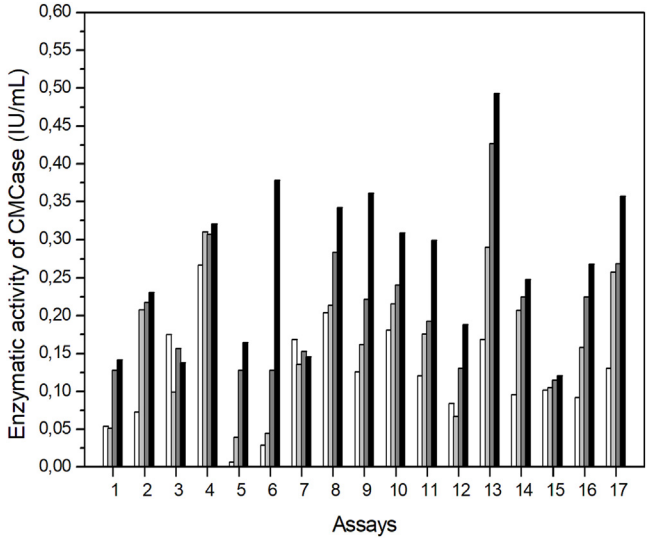

Fig. 4. CMCase activity for all assays performed in the experimental design after 24, 48, 72 and 96 h of cultivation of *B. licheniformis* BCLLNF-01 (□ 24 h; ■ 48 h; ■ 72 h; ■ 96 h).

Table 2
Analysis of variance (ANOVA) of the quadratic model of Rotational Central Composite Design – RCCD.

Source	SS	df	MS	F-value	p-value
CMC	0,104349	***1***	0,104349	94,17,814	0,010452
Temperature (T)	0,002408	*1*	0,002408	2,17,320	0,278369
Inoculum	0,008900	*1*	0,008900	8,03,222	0,105214
CMC^2	0,000143	*1*	0,000143	0,12885	0,753984
$\boldsymbol{T^2}$	0,039338	***1***	0,039338	35,50,347	0,027029
$Inoculum^2$	0,002061	*1*	0,002061	1,86,048	0,305788
CMC by T	0,000741	*1*	0,000741	0,66889	0,499377
CMC by Inoculum	0,002346	*1*	0,002346	2,11,744	0,282880
T by Inoculum	0,002485	*1*	0,002485	2,24,289	0,272935
Lack of Fit	0,013154	*5*	0,002631	2,37,441	0,322417
Pure Error	0,002216	*2*	0,001108		
Total	0,178128	*16*			

SS = sum of squares; df = degree of freedom; MS = mean square; F = F-statistic; $p<0.05$ (significant for a 95% confidence level). The values in bold are statistically significant. Predicted R^2 = 0.9137; Adjusted R^2 = 0.8028.

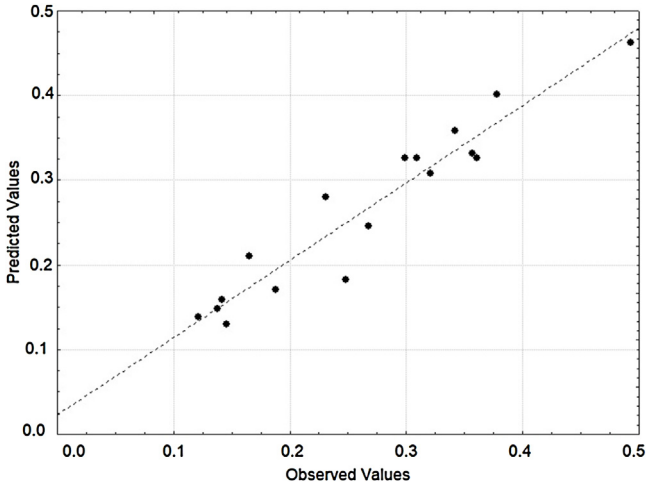

Fig. 5. Observed and predicted values for the enzymatic activity (EA) of CMCase expressed by Equation (1).

$$EA = -10,496 - 0,171 \times T - 0,040 \times \boldsymbol{T}^2 + 0,528 \times \boldsymbol{CMC} - 0,007 \times CMC^2 + 0,011 \times T \times CMC + 0,021 \times 5,5 \times T - 0,002 \times 5,5 \times CMC + 0,471 \quad (2)$$

The Pareto graph (Fig. 6) revealed that the first-order effect (CMC concentration) affected posivively the CMCase activity. In contrast, the significant second-order effect (temperature) was negative. The results indicate that did not occur interaction between the parameters evaluated in the study.

The inoculum concentration has been reported as one of the factors that influence cellulase synthesis. Different sizes of inoculum are evaluated in optimization studies and the reports described that the optimal concentrations are in the range of 1–5 % v/v [12,28,47,48]. However, results show that this variable did not significantly influence the synthesis of CMCase by *B. licheniformis* BCLLNF-01.

The response surface curve of the relationship between temperature and CMC concentration while the inoculum concentration was kept at 5.5 % (v/v) is shown in Fig. 7. CMCase formation was increased with increasing CMC concentration in the experimental range studied (0.5–1.5 % w/v). Among carbon sources such as glucose, lactose, rice bran, sugar cane bagasse and filter paper, CMC has stood out as an efficient substrate in the synthesis of cellulolytic

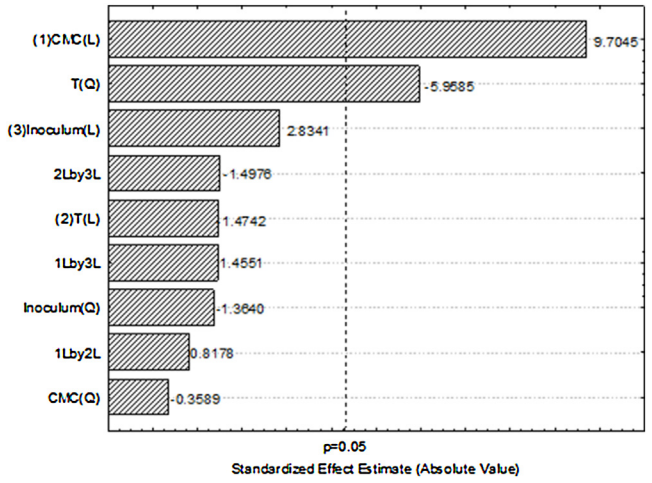

Fig. 6. Pareto graph for enzymatic activity of CMCase as a function of the temperature, CMC and inoculum concentrations for the Rotational Central Composite Design (RCCD) after 96 h of cultivation of *B. licheniformis* BCLLNF-01. Linear term (L) and Quadratic term (Q) of the independent variables (temperature, CMC and inoculum concentrations).

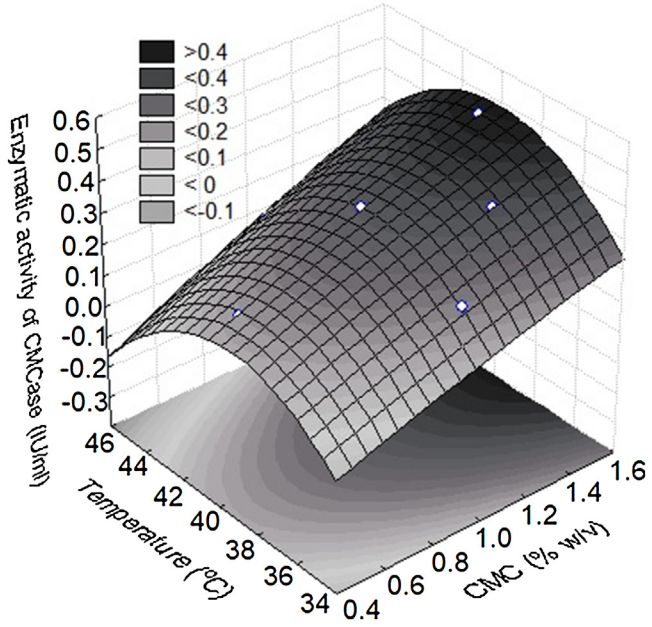

Fig. 7. Three dimensional response surface curve for enzymatic activity of CMCase as a function of the temperature and CMC concentration, for the Rotational Central Composite Design (RCCD) after 96 h of cultivation and 5.5 % v/v inoculum.

complex enzymes [41,46]. However, it is a highly specific substrate for endo-acting cellulases [49], this because endoglucanase or carboxy methyl cellulase (CMCase) act specifically in the disruption of the internal amorphous regions of cellulose chains [50], and CMC structure is ideal for endoglucanase action since it is an amorphous cellulosic substrate, and has been used extensively to characterize enzyme activity from endoglucanases.

Contrary to the results of this study, Karim et al. [14] observed a decreased in enzyme production when CMC concentration was increased. The authors reported an optimum value for CMC of 0.5 % w/v at 37 oC when studied the synthesis of endo-1,4-β-D-glucanase by *B. licheniformis* KIBGE-IB2. These results could be attributed to the increase in the viscosity of the culture medium caused by high concentrations of CMC, hindering the distribution of nutrients and oxygen. Although high CMC concentrations may be a limiting factor, Shajahan et al. [12] optimized the cultivation conditions of *B. licheniformis* NCIM 5556 for cellulase production and did not report a reduction in enzyme production, finding an optimum value of CMC of 19.21 g L^{-1} at 43.35 °C. Thus, the ideal concentration of CMC can vary depending on the microorganism studied, temperature and its chemical characteristics, considering that the market offers this substrate in different degrees of viscosity.

Temperature is a factor that has a great influence on enzyme production. Microorganisms belonging to the genus *Bacillus* are generally reported to produce cellulases over a wide temperature range, which varies from 30 to 60 °C [14,48,51]. In the present study, the optimal temperature for the production of CMCase by *B. licheniformis* BCLLNF-01 was 40 °C, with no significant interaction effect between this variable and the CMC concentration (Fig. 7). This same temperature has been reported as ideal for production of CMCase by *B. subtillis* MU S1 [7], *Bacillus* sp. JS14 [52], *B. thuringiensis* ABS 125 [53] and *Paenibacillus* sp. [54].

4. Conclusion

Experimental planning and response surface methodology are statistical tools enabling the planning and analysis of the interaction between variables that influence different types of processes, being widely used for the optimization of enzymatic processes. BLM medium supplemented with 0.5 % w/v CMC was considered more appropriate for inoculum development of *B. licheniformis* BCLLNF-01. Analysis of the *B. licheniformis* BCLLNF-01 cellulases distribution evidenced the production of extracellular enzymes, and CMCases was the enzymatic group chosen to be investigated in the optimization assays. A Rotational Central Composite Design (RCCD) 2^3 was used to optimize the production of CMCase, and results show that the optimal temperature for the CMCase production by *B. licheniformis* BCLLNF-01 was 40 °C. The higher CMCase activity was observed using 5.5 % v/v inoculum, 40 °C and 1.5 % w/v CMC, reaching 0.5 IU mL^{-1} after 96 h of incubation. This was the first study to optimize the production of CMCase by the new strain *B. licheniformis* BCLLNF-01 associated with mucus from the cnidarian *Palythoa caribaeorum*.

Funding source

This work was supported by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brazil) through the Master's degree scholarship.

CRediT authorship contribution statement

Raquel Nascimento da Silva: Investigation, Methodology, Writing - original draft. **Liany Figuerêdo de Andrade Melo:** Investigation, Methodology. **Christine Lamenha Luna Finkler:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Software, Supervision, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors thank the Pró-Reitoria de Pesquisa e Pós-Graduação (Propesq) of the Universidade Federal de Pernambuco (UFPE, Brazil) for the financial support throughout this research.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.btre.2021.e00599.

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