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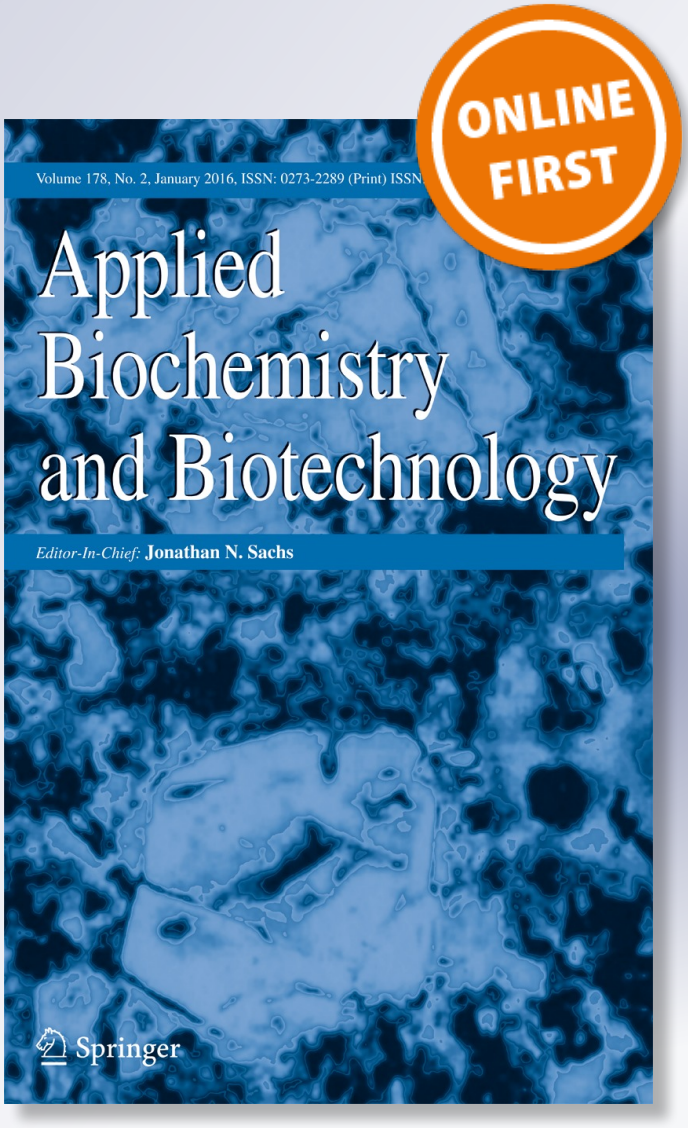

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Synergistic Effect of Simple Sugars and Carboxymethyl Cellulose on the Production of a Cellulolytic Cocktail from *Bacillus* sp. AR03 and Enzyme Activity Characterization

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Abstract A cellulase-producing bacterium isolated from pulp and paper feedstock, *Bacillus* sp. AR03, was evaluated by means of a factorial design showing that peptone and carbohydrates were the main variables affecting enzyme production. Simple sugars, individually and combined with carboxymethyl cellulose (CMC), were further examined for their influence on cellulase production by strain AR03. Most of the mono and disaccharides assayed presented a synergistic effect with CMC. As a result, a peptone-based broth supplemented with 10 g/L sucrose and 10 g/L CMC maximized enzyme production after 96 h of cultivation. This medium was used to produce endoglucanases in a 1-L stirred tank reactor in batch mode at 30 °C, which reduced the fermentation period to 48 h and reaching 3.12 ± 0.02 IU/mL of enzyme activity. *Bacillus* sp. AR03 endoglucanases showed an optimum temperature of 60 °C and a pH of 6.0 with a wide range of pH stability. Furthermore, presence of 10 mM Mn^{2+} and 5 mM Co^{2+} produced an increase of enzyme activity (246.7 and 183.7 %, respectively), and remarkable tolerance to NaCl, Tween 80, and EDTA was also observed. According to our results, the properties of the cellulolytic cocktail from *Bacillus* sp. AR03 offer promising features in view of potential biorefinery applications.

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Keywords *Bacillus* sp. AR03 · Factorial design · Endoglucanase production · Enzyme activity · Biorefinery

Introduction

Cellulosic biomass is an abundant and renewable resource for biorefinery with a great potential for bioconversion into value-added bioproducts. Its degradation needs the cooperative action of at least three types of enzymes: exoglucanases, endoglucanases, and β-glucosidases. These are frequently grouped as cellulases, enzymes produced by microorganisms to use the biomass surrounding their habitat [1]. To date, production of commercial cellulases is mainly carried with fungi owing to their high enzyme activity. However, there is a growing demand for bacteria as a source of novel cellulases as effective catalysts because of their diversity, the presence of multi enzyme complexes providing synergy, a high growth rate, and stability under extreme conditions [2].

Several culture-dependent studies on cellulose-degrading bacteria have reported the dominance of Firmicutes, since they play a key role in biomass deconstruction in a variety of environments, such as compost, soil, and the gut of xylophagous insects [3–5]. Among this phylum, members of the *Bacillus* genus are able to produce and secrete large quantities of extracellular enzymes. They usually have high growth rates and short fermentation cycles, and therefore, they are considered as one of the most versatile and robust enzyme producers for microbial fermentations [6–8].

Many taxonomically related bacterial isolates are originated from diverse habitats, thus frequently varying in growth capacity and requirements. This has challenged the assessment of the appropriate culture conditions for enzyme production. In this regard, the use of peptone-based media to improve bacterial growth and enzyme production has shown to be an efficient resource [9, 10]. In addition, the suitability of different carbon sources to efficiently promote cellulase and hemicellulose production has been reported [6, 11–13].

Considering this background, the current study examined the potential of *Bacillus* sp. AR03, previously isolated from the pulp and paper feedstock in the Northwest of Argentina [14] as cellulase producer for biorefinery technologies. Therefore, the significance of the medium components affecting both growth and cellulolytic cocktail production of strain AR03 were evaluated in order to improve, simplify, and even economize the fermentation process. Finally, the assessment of reaction conditions and stability properties of endoglucanase activity demonstrated that *Bacillus* sp. AR03 is capable to produce potentially robust biocatalysts for biorefinery applications, such as cellulosic ethanol.

Materials and Methods

Microorganism and Screening of Medium Components

The endoglucanase-producing bacterium *Bacillus* sp. AR03 was isolated from biological liquor from a bagasse processing mill in Tucuman, Argentina. As was reported before, its 16S ribosomal RNA (rRNA) gene sequence has been deposited at the European Nucleotide Archive (ENA) under accession number LN829557 [14].

A factorial design was used to find significant factors affecting endoglucanase production. Medium components were selected on the basis of the composition of tryptic soy broth-carboxymethyl cellulose (DTSB-CMC) medium, containing tryptone soy broth diluted 1:2, and amended with carboxymethyl cellulose, as previously defined by Manfredi et al. [14]. Components K_2HPO_4 and NaCl were as in DTSB, while glucose, CMC, soy peptone, and casein peptone were evaluated as four independent variables. Each of these variables was represented at two levels, high and low, which were denoted by (+1) and (−1), respectively. Low levels (−1) were defined as the absence of the components evaluated. The coded level of each variable and the experimental 2^4 factorial design with 16 trials are shown in Tables 1 and 2, respectively. All experiments were performed in duplicate and the mean values of the endoglucanase activity are given.

Analysis of the effects of each independent variable was carried out with the aid of Minitab® 17 statistical software (PA, USA, Minitab Inc.). The coefficient of determination, R^2, was used to see how well data fit the model. Significant variables were screened on the basis of the F test and the p value at a 95 % significance level.

Effect of CMC and Mono and Disaccharides on the Production of Endoglucanases

For enzyme production, *Bacillus* sp. AR03 was cultured in 125-mL flasks containing 20 mL of modified DTSB culture medium, which was composed of (g/L): casein peptone 8.5, soy peptone 1.5, NaCl 2.5, and K_2HPO_4 1.25. The medium was supplemented with different concentrations of CMC (5, 10, 20, 30, 40, and 60 g/L) and with 1.25 g/L glucose.

Inocula were grown in DTSB medium at 30 °C during 24 h. Then, cells were washed twice with saline solution (0.9 % (*w*/*v*) NaCl) and resuspended in saline solution at a concentration of ca. 10^6 colony forming units (CFU)/mL. Two-milliliter aliquots of these suspensions were added to the media assayed. Fermentations were performed in orbital shakers at 200 rpm at 30 °C and samples were taken each 48 h for periods up to 144 unless otherwise indicated. The culture supernatants used as cellulolytic cocktails were recovered by centrifugation at 4 °C and 8000 rpm for 10 min, and then used as an enzyme source for activity measurements. In order to determine the effect of different carbon sources on enzymatic production by *Bacillus* sp. AR03, modified DTSB culture medium was used with different carbohydrates: glucose, sucrose, maltose, fructose, and lactose, at two final concentrations, 2 and 10 g/L, and with 10 g/L CMC. The inocula and the culture conditions were the same as described earlier.

All experiments were carried out in triplicate. Growth was evaluated through colony forming units recounts (CFU/mL) on DTSB agar plates.

Table 1 The coded level and experimental design levels of independent variables for factorial design. K_2HPO_4 and NaCl media components were maintained at constant concentrations

Independent variable	Low level (−1) [g/L]	High level (+1) [g/L]
Soy peptone (A)	0	1.5
Casein peptone (B)	0	8.5
Glucose (C)	0	1.25
CMC (D)	0	10

Table 2 Factorial design matrix and the response evaluated for the screening of factors affecting endoglucanase production by *Bacillus* sp. AR03

Run	Coded values				Endoglucanase activity [IU/mL]
	A	B	C	D	
1	+1	−1	−1	+1	0.104
2	+1	−1	+1	+1	0.215
3	−1	+1	−1	+1	0.054
4	+1	+1	+1	−1	0.451
5	−1	+1	+1	+1	0.520
6	−1	−1	+1	+1	0.000
7	−1	+1	+1	−1	0.349
8	+1	−1	+1	−1	0.200
9	+1	+1	−1	+1	0.334
10	−1	−1	+1	−1	0.000
11	+1	−1	−1	−1	0.073
12	−1	+1	−1	+1	0.237
13	+1	+1	+1	+1	0.680
14	−1	−1	+1	−1	0.000
15	+1	+1	−1	−1	0.173
16	−1	−1	−1	+1	0.000

Enzyme Production in a 1-L Stirred Tank Reactor in Batch Mode

Enzyme production by *Bacillus* sp. AR03 was carried out in a 1-L stirred tank reactor (STR) in batch operation mode (New Brunswick Scientific, Discovery, Series 100, UK), using the following culture medium (in g/L): sucrose 10, CMC 10, casein peptone 8.5, soy peptone 1.5, NaCl 2.5, and K_2HPO_4 1.25. The reactor was inoculated with 10 % (*v*/*v*) of a 10^6 CFU/mL inoculum, grown as indicated before; fermentation conditions were 30 °C, 200 rpm and 1 vvm during 72 h. Growth and enzyme production were measured daily. Bacterial cells were harvested after fermentation by centrifugation at 8000 rpm during 30 min at 4 °C. The cell-free supernatant (cellulolytic cocktail) was stored with 3 mM sodium azide at 4 °C for subsequent assays.

Standard Endoglucanase Activity Assay and Native-PAGE Analysis

Endoglucanase (CMCase) activity was assayed with carboxymethyl cellulose as substrate and quantified by measuring the release of reducing sugars in the reaction mixture. Briefly, the reaction mixture contained 0.05 mL of a suitable dilution of the cellulolytic cocktail and 0.45 mL of 2.0 % (*w*/*v*) CMC solution in 100 mM sodium phosphate buffer (pH 6.0). After 30 min of incubation at 50 °C, reducing sugars were assayed using the dinitrosalicylic acid method [15]. One international unit (IU) of endoglucanase activity was defined as the amount of enzyme that releases 1 μmol of reducing sugars (measured as glucose) per milliliter per minute under the described conditions. All samples were analyzed in triplicate and mean values and standard deviations were calculated.

The corresponding controls (blanks) of enzyme without substrate and substrate without enzyme were performed simultaneously with all enzymes assays, thus sample values were corrected for any blank value.

Native-polyacrylamide gel electrophoresis (ND-PAGE) was performed on 10 % polyacrylamide gels according to Laemmli [16], at constant voltage (75 V) during 3 h. After electrophoresis, silver staining of proteins was carried out for their detection. Apparent molecular weight was estimated using a HMW- 669–66 kDa- marker kit (GE Healthcare Life Sciences). For zymographic profiles, gels were first submerged in 100 mM sodium phosphate buffer (pH 6.0), containing 1 % (*w*/*v*) CMC, at 30 °C for 30 min. Then, gels were stained with 0.1 % (*w*/*v*) Congo red solution. After staining, gels were rinsed two to three times with 1 M NaCl during 15 min until visualization of clear bands of enzyme activity [17].

Effect of Temperature and pH on Endoglucanase Activity and Stability

Endoglucanase activity was examined at temperatures ranging from 20 to 90 °C using buffer substrate as described in standard assay conditions. Evaluation of the optimum pH was assayed within a pH range of 2.2–10.0, employing the following buffering systems at a 100 mM final concentration and containing 2.0 % (*w*/*v*) CMC: glycine-HCl (pH 2.2–3.0), Na_2HPO_4–citric acid (pH 4.0–6.0), sodium phosphate (pH 6.0–7.5), Tris–HCl (pH 7.5–8.8), and glycine–NaOH (pH 8.8–10.6). Reactions were carried out in triplicate at 60 °C.

The effect of temperature on the enzyme stability was determined by pre-incubating the enzyme (in the absence of substrate) at a temperature range between 20 and 80 °C during 1 h. Residual activity was estimated with respect to control reactions simultaneously processed without pre-incubation and taken as 100 % reference value.

In order to determine the pH stability profile, aliquots of one volume of the cellulolytic cocktail were treated with two volumes of acetone and kept at −20 °C during 1 h. After centrifugation at 10,000 rpm for 5 min, the pellets were resuspended using different buffers and then maintained at room temperature for 1 h. Enzyme reactions were carried out in triplicate at 60 °C and residual activities were referred to a 100 % as a reference value obtained from a sample resuspended in buffer phosphate pH 6.0, assayed without pre-incubation.

Effects of Ions and other Additives on Carboxymethyl Cellulase Activity

The effect of the following metal ions on the enzyme activity was assayed at a final concentration of 5 mM in the reaction mixture: NaCl, KCl, $MgSO_4 \cdot 7H_2O$, $CaCl_2 \cdot 2H_2O$, $CuSO_4 \cdot 5H_2O$, $ZnSO_4 \cdot 7H_2O$, $FeSO_4 \cdot 7H_2O$, $MnSO_4 \cdot H_2O$, and $BaCl_2 \cdot 2H_2O$ (for analysis, without further treatment). Assays were performed under conditions determined as optimal, 60 °C and pH 6.0. The effect of Mn^{2+} was further evaluated at final concentrations of 2, 5, and 10 mM.

Similarly, the effect of EDTA, sodium dodecyl sulfate (SDS), and Tween 80 was assessed at a final concentration of 5 mM each in the reaction mixture, while PEG 4000 (molecular weight, MW) was examined at a final concentration of 0.25 g/L. Salt tolerance was assayed at increasing NaCl concentrations under standard assay conditions: final NaCl concentrations were 0.1, 0.5, 1.0, 1.5, and 2.0 M.

Controls were performed simultaneously running samples under identical conditions but in the absence of supplements. These were considered as 100 % reference values of enzyme activity.

Statistical Analysis

Data were analyzed using Minitab® 17 software (PA, USA, Minitab Inc.) by means of analysis of variance (ANOVA). All results are presented as the mean of three replicates ± SD. Differences were significant when $p < 0.05$. Associations between variables were assessed using Tukey's HSD test ($p < 0.05$).

Results

Bacillus sp. AR03; Taxonomic Affiliation and Morphology

The AR03 isolate is a spore forming and motile rod. The strain was previously isolated as a prominent CMCase producer and selected owing to its fast growth rate compared to other *Bacillus* spp. The isolate was identified as *Bacillus* sp. AR03 by means of 16S rRNA gene sequence analysis. An update of its taxonomic position indicated pairwise similarities of ≥99.71 % with *Bacillus subtilis* subsp. *inaquosorum* KCTC 13429$^{(T)}$ (accession number AMXN01000021), *Bacillus tequilensis* KCTC 13622$^{(T)}$ (accession number AYTO01000043) and *B. subtilis* subsp. *subtilis* NCIB 3610$^{(T)}$ (accession number ABQL01000001). This places the strain AR03 unequivocally within the *B. subtilis* group. Also, *Bacillus* sp. AR03 shows a colony growth pattern displaying flagellum-driven motility or swarming, property distinctive of many undomesticated *Bacillus* spp. strains [18] (Fig. 1).

Screening of Culture Media Components

A factorial design-based assay with four variables—soy peptone, casein peptone, glucose, and CMC—was performed in order to evaluate endoglucanase production by *Bacillus* sp. AR03. The zero concentration of each variable was used in preliminary one-factor-at-a-time experiments (data not shown). Table 2 shows the 2^4 factorial design. Enzymatic production varied considerably, ranging from 0.00 to 0.68 IU/mL in media with different compositions. The Pareto chart shows the media components with a significant effect on the enzyme production (Fig. 2). Statistical analysis of the data revealed that four main effects and three interaction effects were significant model terms at the 95 % level with p values ≤0.05 (Supplementary material). Accordingly, the highly significant medium components were casein peptone and

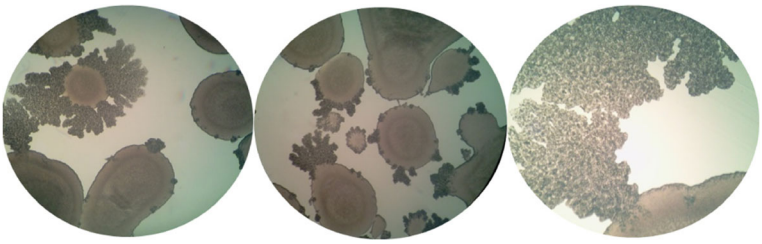

Fig. 1 Colony morphology showing swarming of *Bacillus* sp. AR03 on solid medium (×20 and ×40 magnification)

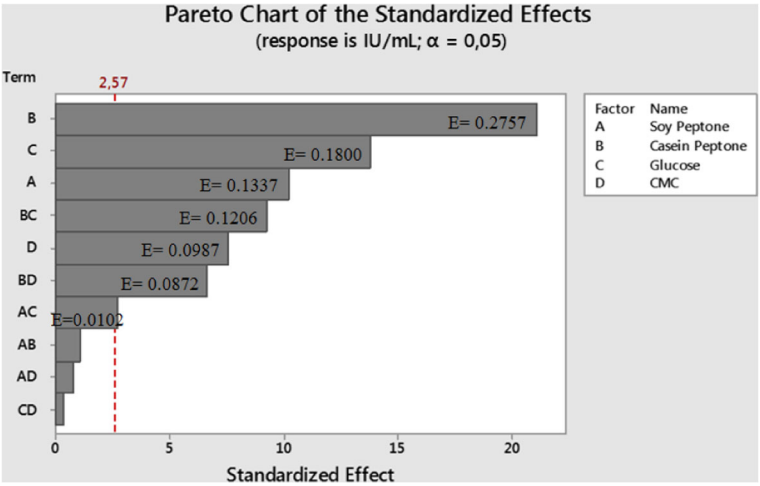

Fig. 2 Pareto chart with the values of the main effects of the medium components that affected CMCase production by *Bacillus* sp. AR03

glucose. These components were followed by (i) soy peptone, (ii) the interaction between casein peptone and glucose, (iii) CMC, and (iv) the interaction between casein peptone and CMC.

The R^2 value provides an estimation of variability in the observed response values that can be demonstrated by experimental factors and their interactions. In this study, the R^2 value was 0.995, indicating that 99.5 % of the variability in the response could be explained by the model. The F value of 93.67 % and the p value of 0.000 obtained indicate that the test model is statistically significant at the 95 % level of significance for enzyme production (Supplementary material).

Effect of CMC and Mono and Disaccharides on the Production of Endoglucanases

CMC was assayed for endoglucanase production by *Bacillus* sp. AR03 at concentrations between 0.0 and 6.0 % (*w*/*v*) during a fermentation period of 144 h (Fig. 3).

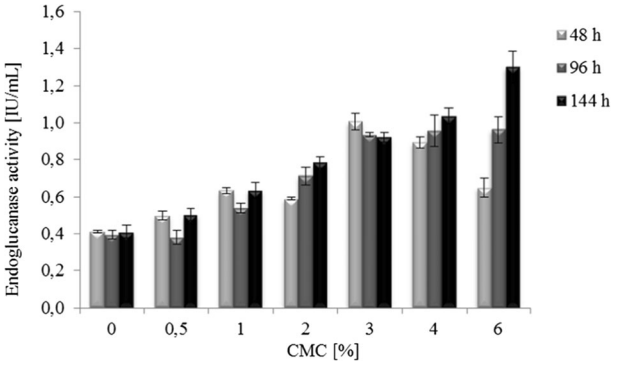

Fig. 3 Endoglucanase activity of the enzymatic cocktail produced by *Bacillus* sp. AR03 in the presence of increasing CMC concentrations. *Error bars* represent the standard deviation calculated from at least three independent experiments

The cultures reached an OD_{600nm} ≈2.0 units at CMC concentration between 0.0 and 1.0 %, whereas the biomass formation (OD_{600nm}) increased with ≈4 and 6 units when CMC was added at concentrations of 2.0 to 6.0 %. Increments in CMC clearly improved the enzyme production (Fig. 3).

All assayed conditions reached maximum enzyme production at 144 h of cultivation with the exception of the media containing 3 % CMC, which achieved the maximum production at 48 h. However, the enzyme titles obtained for each CMC concentration showed significant differences at 48 and 144 h in the case of media containing 2 to 6 % CMC (Fig. 3). When maximum enzyme production for all CMC concentrations evaluated was considered, according to Tukey's test no significant differences were observed between the culture conditions corresponding to 0.0 and 0.5 % CMC, nor between the cultures with 3.0 and 4.0 % CMC. Furthermore, 6.0 % CMC presented maximum endoglucanase production, which was 3.17 times the enzyme activity of medium without CMC and supplemented only with 1.25 g/L glucose as carbohydrate (Fig. 3).

Next, a screening of simple carbon sources, mono and disaccharides, was realized to assess their influence on the endoglucanase production by *Bacillus* sp. AR03. It can be observed in Fig. 4 that the addition of 10 g/L sucrose + 10 g/L CMC to the peptone-based broth produced a maximum enzyme title of 2.93 ± 0.01 IU/mL, leading to an increase of 21.6 times the cellulase production with respect to the base medium with peptone as the main nutrient. Presence of 10 g/L fructose + 10 g/L CMC provided an endoglucanase title of 2.41 ± 0.06 IU/mL, 17.7 times over the peptone-based medium. Interestingly, media only containing 10 g/L glucose produced 2.32 ± 0.03 IU/mL, meaning an increase of 17.1 times with respect to the peptone-based medium, whereas 10 g/L glucose + CMC reached 2.1 ± 0.02 IU/mL of enzyme activity, an increment of 15.4 times.

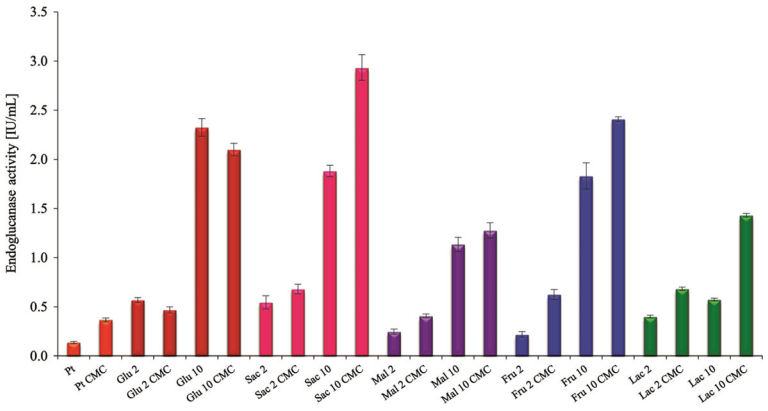

Fig. 4 Effect of different carbon sources in the culture medium on endoglucanase production by *Bacillus* sp. AR03. Data were obtained after 96 h of incubation at 30 °C and 200 rpm. *Error bars* represent the standard deviation calculated from at least three independent experiments. *Pt* Casein peptone, *Glu* glucose, *Sac* sucrose, *Mal* maltose, *Fru* fructose, *Lac* lactose

In most of the conditions assayed, CMC produced a positive effect when combined with simple sugars. Endoglucanase production by stain AR03 was enhanced 1.1 and 2.9 times with 10 g/L maltose + 10 g/L CMC and 2 g/L fructose + 10 g/L CMC, respectively. However, the CMC enhancing effect was not observed in media supplemented with 2 and 10 g/L glucose, 2 g/L sucrose, and 10 g/L maltose.

Enzyme Production in a 1-L STR in Batch Operation Mode

A 1-L batch reactor was used to produce a cellulolytic cocktail from *Bacillus* sp. AR03. A peptone-based medium supplemented with 10 g/L sucrose + 10 g/L CMC was selected for STR batch production. The inoculum, 10 % of the reactor volume, was composed of a cell suspension of c.a. 10^6 CFU/mL. Culture conditions were at uncontrolled pH and aeration was of 1 vvm. The strain reached an enzyme production of 3.12 ± 0.04 IU/mL and a population of 10^9 CFU/mL after 48 h of incubation, results that were not significantly modified after 72 h.

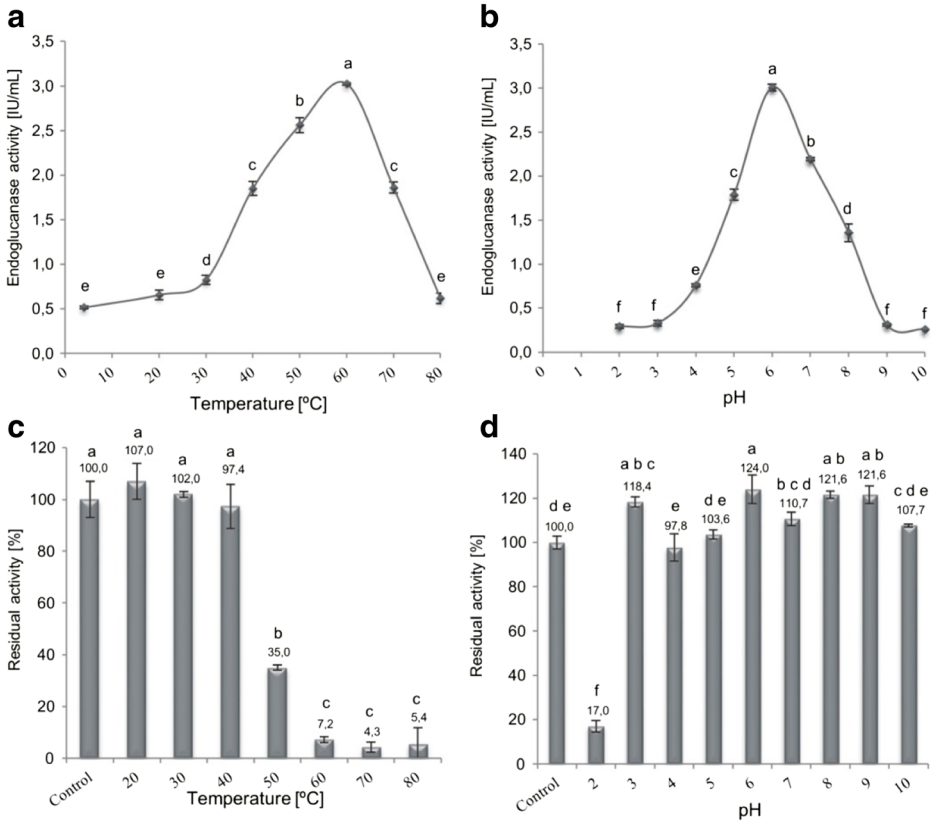

Fig. 5 Effect of temperature (**a**) and pH (**b**) on enzyme activity of the cellulolytic cocktail from *Bacillus* sp. AR03. Enzyme stability at different temperatures (**c**) and at various pH values (**d**) is shown. Residual activity for stability assays was estimated using as a control a reaction without pre-incubation that was simultaneously assayed and taken as 100 %. *Error bars* represent the standard deviation calculated from at least three independent experiments. Similar *letters* are not significantly different from each other (Tukey test, $p < 0.05$)

The culture broth was then recovered by centrifugation and used for further enzyme characterization.

Effect of Temperature and pH on Endoglucanase Activity and Stability

The *Bacillus* sp. AR03 cellulolytic cocktail activity was assayed over a range of temperatures between 0 and 80 °C. The optimum temperature of the endoglucanases was 60 °C and activity declined thereafter (Fig. 5a). Over 61 % of the maximum enzyme activity was retained between 40 and 70 °C. In addition, maximum endoglucanase activity for hydrolysis of CMC was found at pH 6.0, and the enzyme retained nearly 60 % of its activity within a pH range of 5.0–7.0 (Fig. 5b).

The thermal stability profile of the cellulolytic cocktail from *Bacillus* sp. AR03 showed total preservation of activity when samples were kept at 20, 30, and 40 °C before enzymatic assays. Enzyme activity decreased considerably at higher pre-incubation temperatures, and was almost completely lost after pretreatments between 60 and 80 °C (Fig. 5c). However, the enzyme cocktail was remarkably stable between pH 3.0 and 10.0 (Fig. 5d).

Effects of Ions and other Additives on Carboxymethyl Cellulase Activity

Addition of the metal ions Na^{+}, K^{+}, Ca^{2+}, Ba^{2+}, Fe^{2+}, and Zn^{2+} to the reaction mixture showed no substantial effect on the cellulase activity from *Bacillus* sp. AR03, while Mg^{2+} slightly improved the enzyme activity. Presence of Cu^{2+} had an inhibitory effect, retaining a relative activity of 70.5 ± 7.5 %. Remarkably, endoglucanase activity was strongly stimulated by Mn^{2+} and Co^{2+}, reaching relative activities of 229.4 ± 6.4 and 183.7 ± 7.2 %, respectively (Fig. 6a).

The manganese salt was further examined at final concentrations of 2, 5, and 10 mM. The results shown in Fig. 6b indicate that increases of the Mn^{2+}

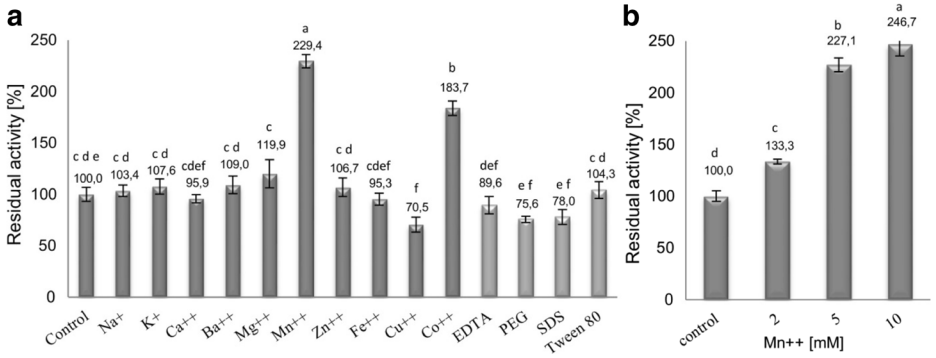

Fig. 6 **a** Effect of different metal ions, inhibitors, and surfactants on the cellulolytic cocktail. Enzymatic activity was performed at 60 °C and pH 6 in the presence of various supplements: metal ions, EDTA, SDS, and Tween 80 at a final concentration of 5 mM, and PEG 4000 at 0.25 g/L. **b** Effect of different concentrations of Mn^{2+} on the endoglucanase activity. A control, considered as 100 % enzyme activity, was run simultaneously under the same conditions but in the absence of supplements. *Error bars* represent the standard deviation calculated from at least three independent experiments. Similar *letters* are not significantly different from each other (Tukey test, $p < 0.05$)

Table 3 Effect of increasing NaCl concentration on the cellulolytic cocktail from *Bacillus* sp. AR03. Enzyme reactions were performed at 60 °C for 30 min. The reference value without NaCl represents 100 %. Assays were performed in three independent replicates and results are represented by mean values ± standard deviation

NaCl [M]	Residual activity [%]
0	100.0 ± 1.1
0.1	100.2 ± 4.8
0.5	81.5 ± 2.9
1	70.2 ± 1.3
1.5	62.2 ± 1.7
2	61.6 ± 2.8

concentration raised the relative enzyme activity up to 246.7 ± 11.5 % with respect to the control where manganese was absent.

The effect of increasing NaCl concentrations was also evaluated; enzyme activity was unaffected in the presence of 0.1 M NaCl and it retained over 60 % of activity in assays containing up to 2 M NaCl (Table 3).

Finally, the cellulolytic enzymes from *Bacillus* sp. AR03 were not affected by addition of Tween 80, whereas moderate effects were observed after addition of EDTA (89.6 ± 8.5 %), SDS (78.0 ± 7.3 %), and PEG 4000 (75.6 ± 3.1 %) (Fig. 6a).

Native-PAGE Protein and Zymographic Analysis

The crude cellulolytic extract from strain AR03 was subjected to native-PAGE. Silver staining revealed multiple protein bands; more than 10 bands were observed in a range of 50 to 500 kDa. On the other hand, the zymographic analysis revealed

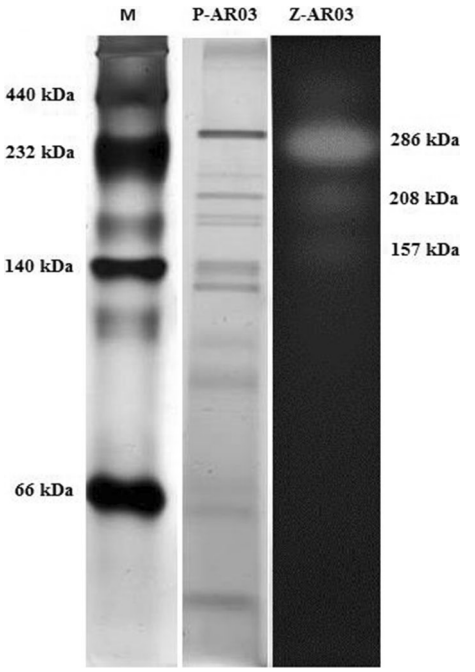

Fig. 7 Protein and zymographic profile of the cellulolytic cocktail from *Bacillus* sp. AR03 developed on 10 % ND-PAGE. *Line M* molecular weight markers; *line P* AR03, proteins visualized by silver staining; *line Z* AR03, cellulases visualized after Congo red staining

three bands with cellulase activity with a molecular mass of around 286, 208, and 157 kDa (Fig. 7).

Discussion

Culture Medium Components Affecting Endoglucanase Production by *Bacillus* sp. AR03

Strain AR03, a cellulase-producing bacterium isolated from pulp and paper feedstock from a paper mill in Argentina, was selected on the basis of its significant cellulase activity and growth rate [14]. As was discussed earlier, strain AR03 belongs to the *B. subtilis* group.

Because many bacterial isolates originate from a wide range of habitats, they may vary in growth capacity and nutrient requirements even if they are taxonomically related. This frequently challenges the assessment of culture conditions. *Bacillus* sp. AR03 was able to use both the inorganic and organic nitrogen sources for cellulase production (data not shown). However, we have previously observed that growth was improved using a peptone-based medium. Other authors have also shown that peptone was an efficient resource to improve bacterial growth and enzyme production [9, 10]. Therefore, a modified DTSB medium supplemented with CMC was selected as the starting point to evaluate the enzyme production by means of a factorial design to independently assess medium components.

Both peptone and carbohydrates significantly influenced the enzyme production according to statistical analysis of the results (Fig. 2). Furthermore, a peptone-based media could support endoglucanase production by strain AR03 in the absence of carbohydrates in the medium (Table 2), which might be the result of an improvement in bacterial growth. Similarly, casein peptone was found to be the optimal nitrogen source for endoglucanase production by *Bacillus amyloliquefaciens* DL-3 [19] and *Bacillus* sp. BCCS A3 reached maximum cellulase production in a medium including tryptone [8]. Moreover, considering the benefits of the use of peptone, it was suggested that growth and enzyme production of some *Bacillus* species could be negatively affected by acidification of the medium when using an inorganic nitrogen source [20].

It has been extensively demonstrated that addition of CMC to the culture medium promotes cellulase production. Although most of the research on CMC has analyzed the use of concentrations close to 1 % (*w*/*v*) [2, 8], certain authors examined the effect of elevated concentrations of CMC. *Bacillus* sp. MTCC 10046 reached an optimum cellulase production with 8 % (*w*/*v*) CMC [13], while optimum cellulase production by *Microbacterium* sp. Z5 was obtained with 3 % (*w*/*v*) CMC after 10 days of cultivation [6]. The present study assayed the effect of CMC concentrations from 0.0 to 6.0 % on cellulase production during a fermentation period of 144 h, in order to compensate a possible slow growth because of the high viscosity of the medium. Interestingly, it was observed that the initial viscosity, which strongly diminished after 24 h of cultivation, did neither prevent bacterial growth nor enzyme production. A maximum of 1.30 IU/mL was obtained with 6.0 % (*w*/*v*) CMC after 144 h. Taking into account the relation between substrate concentration and fermentation period, modified DTSB + 3 % (*w*/*v*) CMC medium producing 1.01 IU/mL of cellulases after 48 h of cultivation, was considered the most favorable medium in these assays (Fig. 3).

Regarding the influence of simple sugars on endoglucanase production, addition of mono and disaccharides to DTSB medium, with and without CMC, it was observed that strain AR03 was able to grow and to produce endoglucanases in all conditions examined, allowing to

significant improvements of endoglucanases production, particularly by using sucrose, fructose, and glucose. It is worth mentioning that CMC combined with simple carbohydrates had a synergistic effect in most of the combinations assayed; the enzyme production was raised between 1.1 and 2.9 times after the addition of CMC to mono or disaccharides (Fig. 4). Maximum endoglucanase production was obtained in media supplemented with sucrose combined with CMC, showing an increase of 1.6 times with respect to the use of sucrose only and reaching enzyme titles near 3.0 IU/mL. These observations confirm that the presence of different carbohydrates in combination with peptone strongly affects the cellulolytic cocktail production by *Bacillus* sp. AR03.

Other authors observed that CMCase production by different microorganisms in fermentation media could be improved by adding simple sugars as additional carbon source. Bano and coworkers [10] found that endoglucanase production by *B. subtilis* KIBGE HAS was favored when sugarcane bagasse was supplemented with lactose, xylose, maltose, and sucrose, whereas cellobiose and fructose constrained enzyme production. Likewise, lactose as the sole carbon source promoted cellulase production in *Bacillus* sp. K1 [20] and in *Microbacterium* sp. Z5. In the latter case, a synergistic effect on cellulase production was also observed in the presence of lactose and CMC in the culture medium. Nevertheless, when individually assayed, the lactose components, D-galactose and D-glucose, had no effect on Z5 CMCase production [6].

The effect of simple sugars on cellulase production has been observed in bacteria and fungi. It has been hypothesized that the lactose uptake and the effect on CMCase production could be explained by mechanisms described in fungi [6, 20, 21]. Our observations made consider that the use of simple carbon sources seems to improve, simplify, and even economize fermentation processes for cellulase production. Agro-waste materials are cost-effective substrates; yet, the advantages for submerged fermentation of simple sugars and/or soluble substrates favor operative culture conditions and downstream processing of the enzyme production.

It is important to take into account that the substrate may be strain-dependent and related to the original niche of a particular isolate. Hence, substrates should always be examined even for closely related isolates. *Bacillus* sp. AR03 was isolated from sugar cane-associated processes, and consequently, it is not surprising that sucrose, glucose, and fructose proved to be suitable substrates for this strain. On the other hand, further analysis should be performed to explain gene induction and regulation.

Enzyme Production and Characterization

All assays to improve enzyme production by *Bacillus* sp. AR03 were performed in shaken flasks and fermentation periods ranging from 48 to 144 h. Maximum cellulase values combining 10 g/L sucrose + 10 g/L CMC in the peptone-based broth were obtained after 96 h of cultivation (Fig. 4). By using this medium composition, the production period of strain AR03 was significantly reduced by using a 1-L STR in batch operation mode, probably due to a better transference of nutrients offered by the submerged fermentation system. This allowed obtaining 3.12 IU/mL of endoglucanases in 1 L of broth at 48 h, which may improve potential productivity and costs [22].

The optimum temperature of the cellulolytic cocktail from *Bacillus* sp. AR03 was 60 °C, a value comparable to those observed for *Bacillus* sp. M-9 [23], *Bacillus pumilus* S124A [2], and *Bacillus* sp. C1 [13]. Cellulase from strain AR03 was completely stable after pretreatments at temperatures ≤40 °C, yet they did not show thermal stability after preheating at 60 °C for 1 h.

The enzyme activity showed an optimum at pH of 6.0 (Fig. 5b), similar to that found in *Bacillus* sp. BG-CS10 [24], in *B. pumilus* S124A, and in *Bacillus* sp. K1 [20]. Many endoglucanases from *Bacillus* species are frequently stable over a wide pH range as was observed for the cellulolytic cocktail of strain AR03. A carboxymethyl cellulase purified from *B. pumilus* S124A showed to be stable within a pH range between 4.0 and 8.0 [2], while endoglucanase activity produced by *B. subtilis* KIBGE HAS retained nearly 70 % of its maximum activity in a pH interval between 3.0 and 8.0 [10].

The zymographic profile of strain AR03 was related to that of various cellulolytic *Bacillus* spp. Singh et al. [25] reported a carboxymethyl cellulase from *Bacillus sphaericus* JS1 with a molecular weight (MW) of 181 kDa, while Shoseyyov and Doi [26] isolated a cellulase with a MW ≥ 272 kDa from *Clostridium cellulovorans*. In contrast, ten bands ranging from 80 to 216 (MW) with CMCase activity were described for the culture supernatant from *Paenibacillus curdlanolyticus* strain B-6 after a Native-PAGE zymographic analysis [27].

The cellulolytic cocktail produced by strain AR03 displayed a remarkable tolerance and even activation in the presence of various ions and additives included into the reaction mixture. Interestingly, the cellulolytic enzymes from *Bacillus* sp. AR03 were not affected by Tween 80, while moderate effects were observed with EDTA, SDS, and PEG 4000 (Fig. 6a).

Metal ions may be required as cofactors for enzyme activity or as components of the enzyme complex. They have also been reported to enhance substrate binding and stability of the catalytic site [13, 28]. Most of the salts evaluated on *Bacillus* sp. AR03 did not affect its enzymatic activity; however, clear positive effects were observed in the presence of Mn^{2+} and Co^{2+} (Fig. 6a). Similar results have been described for *Streptomyces misionensis* PESB-25 endoglucanase that reached relative activities of 161.2 and 201.5 % in the presence of Co^{2+} and Mn^{2+} (2 mM), respectively [29]. Also, a positive effect on carboxymethyl cellulases from *Bacillus mycoides* S122C and *B. pumilus* S124A was observed with Mn^{2+} and Co^{2+}, yet the enzyme activities reported were similar to their respective controls [2, 30]. Cellulolytic activity from a *Bacillus* spp. was enhanced (124 %) in the presence of 5 mM Mn^{2+} [31]. Similarly, Gaur and Tiwari [28] reported enzyme activation after the addition of Ca^{2+} and Mg^{2+} (10 mM) to the reaction mixture of a purified thermostable cellulase from *Bacillus vallismortis* RG-07. However, manganese produced a reduction in this enzyme activity of 76.9 and 61.9 % in the presence of Mn^{2+} 5 and 10 mM, respectively, while cobalt was not evaluated. In contrast, Zn^{2+} and Mn^{2+} (10 mM) strongly reduced the CMCase activity of *Bacillus* sp. C1 [13], whereas manganese also produced a negative effect on cellulases produced by *B. amyloliquefaciens* DL-3 [19] and on xylanases from *Bacillus halodurans* S7 [32].

The protein structure and the subsequent protein function could be adversely affected by high salt concentrations, fact that is particularly important for non-halophilic proteins [33]. Nevertheless, the cellulolytic cocktail activity produced by *Bacillus* sp. AR03 proved to be highly tolerant to concentrations up to 2.0 M of NaCl, and unaltered or enhanced in the presence of several ions. It is important to remark that agro-industrial wastes contain numerous impurities like metal ions, which can potentially inhibit enzyme activity. Hence, in view of biorefinery applications, the tolerance of the enzymatic cocktail from *Bacillus* sp. AR03 to different metal ions is a promising feature.

Concluding Remarks

Bacillus sp. AR03 was selected as a cellulase producer. The examination of the culture components influencing bacterial growth and enzyme production demonstrated that mono

and disaccharides are useful substrates for endoglucanases production. Moreover, combined with CMC, presented a synergistic effect on enzyme production which could be further optimized. As a result, a peptone-based medium including soluble substrates, sucrose and CMC, was utilized to efficiently produce a cellulolytic cocktail in a 1-L STR in batch operation mode, reaching a maximum of 3.12 IU/mL of endoglucanases within 48 h. Compared to agro-wastes as substrates, it is important to point out that the use of simple sugars for submerged fermentation production favors both operative culture conditions and downstream processing. Interestingly, the cellulolytic cocktail from *Bacillus* sp. AR03 was not affected by addition of Tween 80, while moderate effects were observed with EDTA, SDS, and PEG 4000. It was highly tolerant to concentrations up to 2.0 M of NaCl, and also remained unaltered or even enhanced in the presence of various metal ions. Hence, the enzymatic cocktail from *Bacillus* sp. AR03 presents promising features in view of biorefinery applications.

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Compliance with Ethical Standards The manuscript does not contain human studies or experiments using animals.

Conflict of Interest The authors declare that they have no competing interests.

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