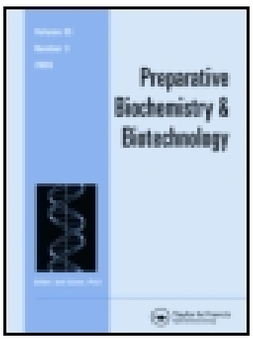

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Endoglucanase and xylanase production by *Bacillus* sp. AR03 in co-culture

Johan S. Hero[a], José H. Pisa[a], Nora I. Perotti[a,b], Cintia M. Romero[a,c], and María A. Martínez[a,b]

[a]PROIMI Planta Piloto de Procesos Industriales Microbiológicos, CONICET, Tucumán, Argentina; [b]Facultad de Ciencias Exactas y Tecnología, Universidad Nacional de Tucumán, Tucumán, Argentina; [c]Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, Tucumán, Argentina

ABSTRACT

The behavior of three isolates retrieved from different cellulolytic consortia, *Bacillus* sp. AR03, Paenibacillus sp. AR247 and *Achromobacter* sp. AR476-2, were examined individually and as co-cultures in order to evaluate their ability to produce extracellular cellulases and xylanases. Utilizing a peptone-based medium supplemented with carboxymethyl cellulose (CMC), an increase estimation of 1.30 and 1.50 times was obtained by the co-culture containing the strains AR03 and AR247, with respect to enzyme titles registered by their individual cultivation. On the contrary, the extracellular enzymatic production decreased during the co-cultivation of strain AR03 with the non-cellulolytic *Achromobacter* sp. AR476-2. The synergistic behavior observed through the combined cultivation of the strains AR03 and AR247 might be a consequence of the consumption by *Paenibacillus* sp. AR247 of the products of the CMC hydrolysis (i.e., cellobiose and/or cello-oligosaccharides), which were mostly generated by the cellulase producer *Bacillus* sp. AR03. The effect observed could be driven by the requirement to fulfill the nutritional supply from both strains on the substrate evaluated. These results would contribute to a better description of the degradation of the cellulose fraction of the plant cell walls in nature, expected to an efficient utilization of renewable sources.

KEYWORDS

Bacillus sp. AR03; biorefinery; cellulose degradation; co-culture; endoglucanase; xylanase

Introduction

Lignocellulosic biomass is the most abundant renewable feedstock in the world and could be used to provide energy and chemicals. Fermentable sugars and/or other products from lignocellulosic biomass are usually produced through the action of chemical pretreatment and catalytic degradation, which may be achieved via enzymatic hydrolysis. Enzymatic hydrolysis of pretreated lignocellulose with cocktails containing cellulase, xylanase, and accessory enzymes has aroused interest since it allows the use of agro-industrial residues and non-food plant biomass, thus contributing to the development of bio-based economies. However, the main impediment to lignocellulose biorefinery is the high cost of enzymes.[1,2]

Natural degradation of lignocellulose involves a complex multistep process requiring the joint action of several microorganisms that produce a variety of enzymes which act cooperatively to degrade plant biomass. Within these microbial consortia, non-cellulolytic species play an important role on its stabilization, consequently enhancing the efficiency of biomass hydrolysis.[3,4]

Numerous bacterial strains are able to produce a set of diverse biocatalysts. Particularly, members of the phylum Firmicutes are known to play a key role in biomass degradation in a variety of environments.[5] Because bacteria usually have high growth rates and short fermentation cycles, they are considered versatile and robust enzyme producers for microbial fermentations.[6] In this regard, most of the microbial cultures for enzyme production involve single strain isolates.[7] However, well-defined microbial consortia may contribute to assess their technological potential and to explain the possible role of the strains involved in the degradation of complex substrates as lignocellulose or xylans. Consequently, microbial consortia and co-cultures represent an approach to the understanding of the complex interaction of the pool of enzymes generated in nature and used in biodegradation of lignocellulosic biomass.[3–8]

The current study assessed the effects and interactions of the main medium components and bacterial strains that influenced the production of endoglucanase and xylanase in co-cultures.

Materials and methods

Bacterial strains and media

Bacterial strains used in this study, *Bacillus* sp. AR03, *Paenibacillus* AR247, and *Achromobacter* sp. AR476-2, were previously isolated as CMCases producers from different bacterial consortia collected from industrial samples and the gut of *Diatraea saccharalis* larvae.[9] All strains were grown in a modified nutritive medium, diluted (1:2) with Tryptic Soy Broth, DTSB Britania, (Buenos Aires, Argentina), and with the following final composition ($g\,L^{-1}$): 1.25 glucose;

CONTACT Cintia M. Romero cinromero78@gmail.com Planta Piloto de Procesos Industriales Microbiológicos (PROIMI-CONICET), Avenida Belgrano y Pasaje Caseros, San Miguel de Tucumán, Tucumán, C.P. 4000, Argentina.
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8.50 casein peptone; 1.50 soy peptone; 2.50 NaCl; and 1.25 K_2HPO_4. Media were supplemented with 1% (w/v) carboxymethyl cellulose (low viscosity CMC), Sigma Aldrich (St. Louis, MO USA). For hydrolysis halos, DTSB-CMC agar plates were stained with 0.1% (w/v) Congo Red.[10] Semi-quantitative assessment was performed over colonies showing significant hydrolysis halos according to the ratio between diameter of the halo plus the diameter of the colony and the diameter of the colony.

Culture conditions for growth and enzyme production

Cell cultures obtained after 24 h at 30°C and 200 rpm were inoculated in 125-mL flasks containing 20 mL of DTSB-CMC and incubated at 30°C under orbital shaking (200 rpm). All cultures were performed in triplicate. Cells were washed twice with saline solution (0.9% w/v NaCl) and resuspended at a concentration of c.a. 10^6 CFU mL^{-1}. Two milliliters aliquots of single strain cell suspensions and 1 mL of each strain in the case of co-cultures were added to 18 mL of fresh medium.

Assays were run for 96 h and sampled every 24 h. Culture supernatants were centrifuged at 10,000*g* for 10 min at 4°C and assayed for enzyme activity.

Enzyme activity assays

Endoglucanase (i.e., CMCase) activity was assayed with 0.45 mL of 2.0% (w/v) CMC in 100 mM sodium phosphate buffer (pH 6.0), and 0.05 mL of culture supernatant appropriately diluted. Similarly, xylanase activity was determined using 1.0% (w/v) birchwood xylan (Sigma) in 100 mM sodium phosphate buffer (pH 6.0). Reaction mixtures were then incubated at 50°C for 30 min and reducing sugars released were quantified using the dinitrosalicylic acid method.[9,11]

One international unit (IU) of enzyme activity was defined as the amount of enzyme that released 1 μmol of reducing sugars (measured as glucose or xylose, respectively) per mL and per min under the given assay conditions. All samples were analyzed in triplicate and mean values and standard deviations were calculated.

The extracellular enzymatic activities were tested from the culture supernatants for both individual and co-cultures. To that end, equal parts of the culture supernatants of each individual strain were mixed to be assayed and the results were subsequently corrected for the corresponding dilution.

Protein determination was performed by the Lowry method.[12] The specific enzyme activity was determined as IU per mg of protein.

The ratio between the specific activity of the culture supernatant of the co-culture—IU co-culture—and the specific activity of the sum of the corresponding single cultures simultaneously run—Σ IU monocultures—was utilized for the estimation of the degree of synergism (DS) as follows[13]:

$$DS = \frac{\text{IU co} - \text{culture}}{\Sigma\text{IU monoculture}}$$

Table 1. Factorial design matrix and the response evaluated, specific endoglucanase and xylanase activities, in the individual and co-culture of *Bacillus* sp. AR03 and *Paenibacillus* sp. AR247.

Duplicates for simple	Run	X1 AR03	X2 AR247	X3 CMC%	X4 Nitrogen sources	Specific endoglucanase activity (IU mg^{-1})	Specific xylanolytic activity (IU mg^{-1})	CFU mL^{-1} AR03	CFU mL^{-1} AR247
T1	1	+	−	+	−	3.62	3.61	3.0×10^{11}	*
T2	2	−	+	−	−	0.54	0.00	*	1.0×10^{11}
T3	3	−	−	−	+	0.00	0.00	*	*
T4	4	−	+	+	+	0.34	0.08	*	4.0×10^{11}
T4	5	−	+	+	+	0.35	0.10	*	4.4×10^{11}
T5	6	−	−	+	−	0.00	0.00	*	*
T6	7	+	−	−	−	4.93	3.98	3.0×10^{11}	*
T7	8	+	−	+	+	2.56	1.16	2.3×10^{12}	*
T8	9	+	−	−	+	4.22	1.35	2.7×10^{13}	*
T9	10	+	+	+	−	1.15	10.54	1.0×10^{11}	1.0×10^{12}
T10	11	−	+	+	−	1.87	0.01	*	9.4×10^{12}
T6	12	+	−	−	−	4.83	3.70	3.5×10^{11}	*
T1	13	+	−	+	−	3.76	3.42	3.3×10^{11}	*
T3	14	−	−	−	+	0.00	0.00	*	*
T11	15	+	+	−	+	3.49	5.27	9.0×10^{9}	1.0×10^{10}
T2	16	−	+	−	−	0.53	0.00	*	1.1×10^{11}
T12	17	−	−	+	+	0.00	0.00	*	*
T13	18	+	+	−	−	5.54	1.95	7.0×10^{11}	6.0×10^{11}
T14	19	−	−	−	−	0.00	0.00	*	*
T12	20	−	−	+	+	0.00	0.00	*	*
T11	21	+	+	−	+	3.36	5.34	9.2×10^{9}	1.5×10^{10}
T5	22	−	−	+	−	0.00	0.00	*	*
T14	23	−	−	−	−	0.00	0.00	*	*
T15	24	−	+	−	+	0.35	0.00	*	7.0×10^{11}
T16	25	+	+	+	+	5.51	8.16	3.0×10^{13}	2.0×10^{13}
T15	26	−	+	−	+	0.33	0.00	*	7.5×10^{11}
T8	27	+	−	−	+	3.99	1.11	3.0×10^{13}	*
T13	28	+	+	−	−	5.77	2.63	7.5×10^{11}	6.8×10^{11}
T9	29	+	+	+	−	1.24	10.79	1.4×10^{11}	1.5×10^{12}
T7	30	+	−	+	+	2.56	1.04	2.7×10^{12}	*
T16	31	+	+	+	+	5.82	8.78	3.4×10^{13}	2.3×10^{13}
T10	32	−	+	+	−	1.91	0.04	*	9.2×10^{12}

*, no corresponding data.

Evaluation of the interactions between *Bacillus* sp. AR03 and *Paenibacillus* sp. AR247 over the enzyme productions by a factorial design.

A complete factorial design was used to find significant interactions between the strains AR03 and AR247 that affected the endoglucanase and the xylanase production.

Medium components were selected on the basis of (g L^{-1}): 1.25 K_2HPO_4, 2.50 NaCl; and 10.0 glucose, according to Manfredi et al.[2] The nitrogen source was evaluated at 10% of total nitrogen as soy casein peptone or yeast extract, CMC concentration (0.5 or 3%) and presence or absence of *Bacillus* sp. AR03 and *Paenibacillus* sp. AR247 were evaluated as independent variables. Each of these variables was represented at two levels, high and low, which were denoted by (+1) and (−1), respectively. The coded level of each variable and the experimental 32 factorial design with 16 trials are shown in Table 1. Analysis of the effects of each independent variable was carried out with the aid of Minitab® 17 statistical software (Minitab Inc., State College, Pennsylvania, USA). 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 *t*-test and the *p* value at a 95% significance level.

Glycoside hydrolase encoding genes from Paenibacillus *sp. AR247 and* Achromobacter *sp. AR476-2*

The translated protein sequences from complete genome sequence of strains AR247 (unpublished data) and AR476-2 (LWDT00000000) were subjected to carbohydrate-active enzyme (CAZy) annotation using CAT[14] server, which is based on the CAZy database classification.[15] The search was further analyzed with the SignalP 4.1 server,[16] mainly targeted the primary sequences of representatives of cellulases belonging to GH6 and GH12 families.

Results

Strains growth and characterization

The CMSase and xylanase activities were studied in solid medium (Table 2). All the strains showed both activities; however, the strain *Bacillus* sp. AR03 showed the best enzyme activities in solid medium.

A qualitative evaluation of cellulolytic and xylanolytic activities of the selected strains in DTSB-CMC medium after 72 h of cultivation is shown in Table 2. *Bacillus* sp. AR03 has shown to be a strong endoglucanase and xylanase producer.[2] According to previous data, *Paenibacillus* sp. AR247 mainly provided the xylanolytic activity, yet reaching a better performance into a mineral-based media (data not shown). Despite the weak extracellular endoglucanase and xylanase activities detected in the culture supernatant of *Achromobacter* sp. AR476-2, this is the first report evaluating the growth of a member of this genus in a pure culture assessing its potential utilization of cellulose.

The individual behavior—monocultures—of the strains assayed showed that the availability of two carbohydrates in DTSB-CMC medium, glucose (1.25 g L^{-1}) and CMC (10.0 g L^{-1}), may explain the observation of two exponential growth phases in all cases, as the strains displayed a diauxic shift during a short period of 1–2 h as is shown in Figure 1.

Bacillus sp. AR03 showed a first period of exponential growth between 5 and 7 h of cultivation (Figure 1a) with a μ_{max} of 2.06 h^{-1} and a doubling time of 20.20 min. Then, a short lag phase between 8 and 11 h of cultivation was followed by a second exponential growth phase with a μ_{max} of 1.19 h^{-1} and doubling time of 34.80 min. Similarly, *Paenibacillus* sp. AR247 showed a first period of exponential growth with a μ_{max} of 1.50 h^{-1} and a doubling time of 27.70 min between 1 and 5 h of cultivation. A second exponential growth phase with a μ_{max} of 1.07 h^{-1} and a doubling time of 38.71 min between 7 and 9 h of cultivation was preceded by a short lag phase between 6 and 7 h (Figure 1b). Finally, the growth curve of *Achromobacter* sp. AR476-2 showed similar growth stages: an exponential growth with a μ_{max} of 1.72 h^{-1} and a doubling time of 24.20 min between 4 and 7 h of incubation, a short lag period and a second exponential growth phase between 8 and 10 h, with a μ_{max} of 1.09 h^{-1} and a doubling time of 38.22 min (Figure 1c).

Enzyme production by co-cultivation of Bacillus *sp. AR03 and* Paenibacillus *sp. AR247*

The strains AR03 and AR247, with similar growth rates, produced different titles of cellulolytic enzymes in DTSB-CMC. The co-culture of these two isolates showed that they coexisted with a similar development after 96 h of incubation, reaching 2.1×10^{12} CFU mL^{-1} (strain AR03) and 1.0×10^{11} CFU mL^{-1} (strain AR247) (Supplementary material.

Maximum endoglucanase production of ~3.4 IU mg^{-1} was detected from 48 h with no significant differences at 72 h. After 96 h, it declined to 2.92 ± 0.20 IU mg^{-1} (Figure 2a).

This endoglucanase production, mainly at the expense of the strain AR03, was improved as a whole with the presence of strain AR247 into a combined culture system. Additionally, this also accelerated the maximum output since the single culture of the strain AR03 registered its maximum production at 72 h (Figure 2a).

Table 2. Enzyme activities retrieved from liquid and solid media (DTS-CMC), at 50°C during 30 min for liquid media and 30°C 48 h for solid media, of individual cultures of *Bacillus* sp. AR03, *Paenibacillus* sp.247, and *Achromobacter* sp. AR 476-2.

	DTSB CMC 1%						
	Enzyme activity (IU mL^{-1})		Specific activity (IU mg^{-1})		DTSA CMC 1%		
Microorganism	CMase	Xylanase	CMase	Xylanase	Colony diameter (cm)	Halo diameter (cm)	Ratio halo/colony
Bacillus sp. AR03	1.12 ± 0.03	2.87 ± 0.26	2.62 ± 0.24	5.01 ± 0.51	0.7	2.2	3.1
Paenibacillus sp. AR247	0.13 ± 0.04	0.16 ± 0.03	0.49 ± 0.08	0.59 ± 0.09	0.4	0.7	1.7
Achromobacter sp. AR476-2	0.10 ± 0.03	0.24 ± 0.02	0.17 ± 0.02	0.34 ± 0.05	1.0	1.5	1.5

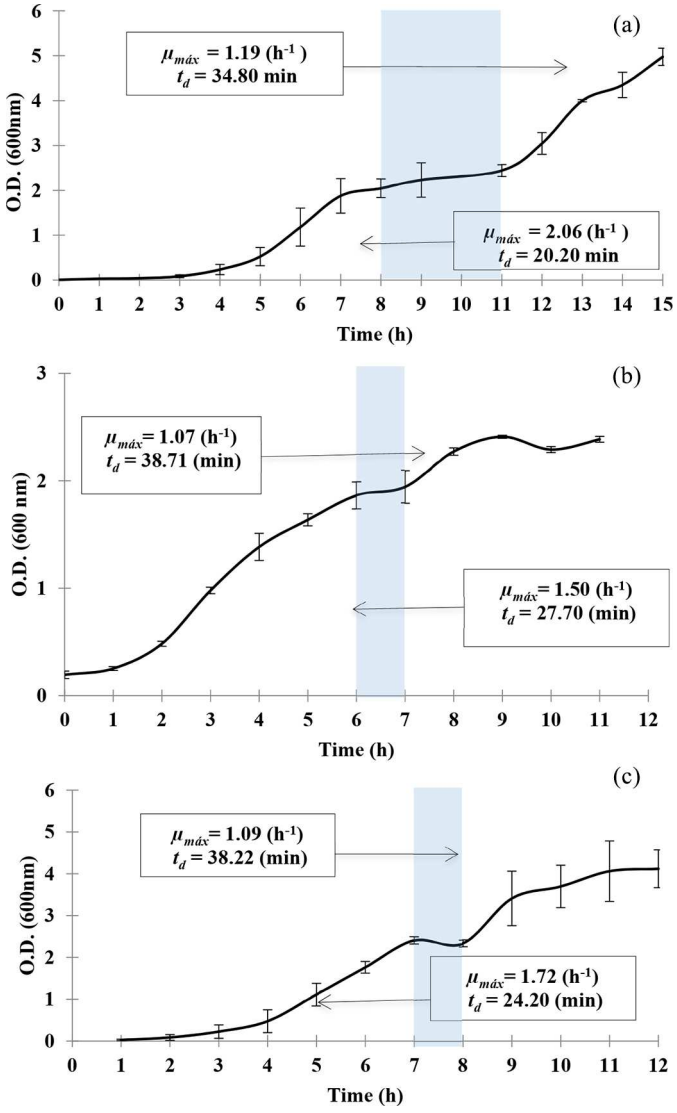

Figure 1. Growth of (a) *Bacillus* sp. AR03, (b) *Paenibacillus* sp. AR247, (c) *Achromobacter* sp. AR476-2 in DTSB-CMC medium containing 1.25 g L^{-1} glucose and 10 g L^{-1} CMC. *Note*: CMC, carboxymethyl cellulose.

On the other hand, the endoglucanase activity resulted from the sum of the enzyme produced by individual cultures was of 2.94 IU mg^{-1} at 72 h, whereas total CMCase was 1.30 times higher than the sum obtained at 48 h, thus suggesting a biological improvement for the substrate degradation (i.e., synergy) due the co-cultivation of both strains (Figure 2a).

Regarding the xylanolytic activity evaluated, its result also increased by this co-culture (strains AR03 and AR247), with an improvement of 1.50 times with respect to the sum of the individual enzymatic activities. As is shown in Figure 2b, the maximum values of the co-culture were of 8.39 ± 0.49 IU mg^{-1} at 72 h, while the corresponding sum of individual enzyme production was 5.40 IU mg^{-1}.

In order to further understand the enzymatic activities of the co-culture composed by strains AR03 + AR247, a mixture of equal parts of the culture supernatants of individual cultures was also assayed. Interestingly, the endoglucanase titles obtained were lower than the ones produced during the co-cultivation, supporting the synergy (Figure 2a and 2b).

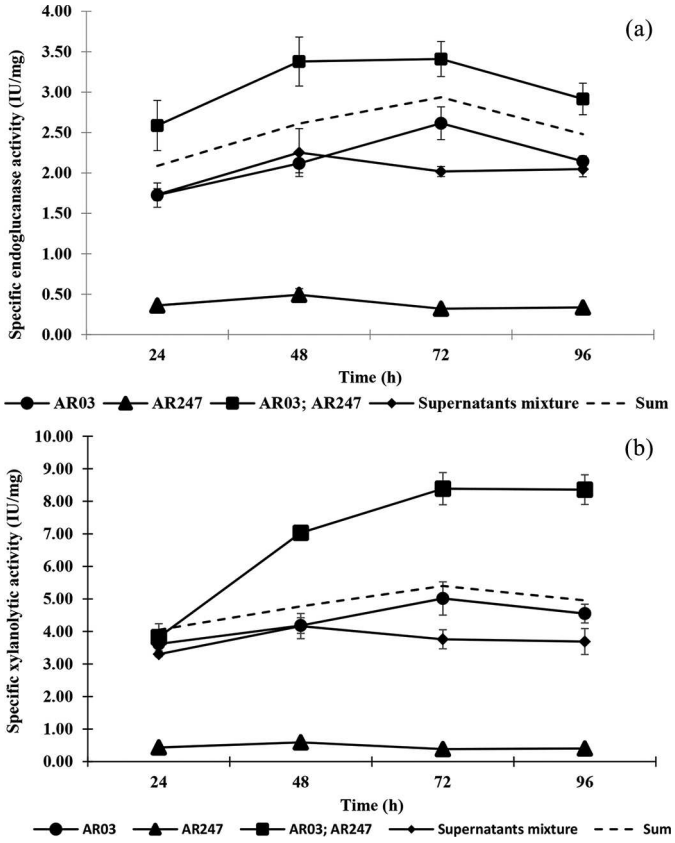

Figure 2. Endoglucanase (a) and xylanase (b) activity from individual cultures of *Bacillus* sp. AR03, *Paenibacillus* sp. AR247, and their co-culture in 1% (w/v) DTSB-CMC. The dotted line represents the expected value as the sum of the individual enzyme activities. The supernatant mixture corresponds to the enzyme activity with equal parts of individual culture supernatants. *Note*: CMC, carboxymethyl cellulose.

Enzyme production by co-cultivation of Bacillus *sp. AR03 and* Achromobacter *sp. AR476-2*

This strain combination resulted in a conspicuous drop of the growth and the enzyme production of *Bacillus* sp. AR03. Unexpectedly, final colony counts showed that the strain AR476-2 outgrew the cellulolytic strain AR03 under the assay conditions (Supplementary material). Correspondingly, the specific CMCase activity (0.65 ± 0.06 IU mg^{-1}) detected at 48 h was lower than the one obtained when *Bacillus* sp. AR03 was individually grown (Figure 3a). A similar conclusion was seen regarding the xylanolytic activity of this co-culture, which reached a specific xylanase activity of 1.43 ± 0.12 IU mg^{-1} (Figure 3b).

Endoglucanase and xylanase production by co-cultivation of Bacillus *sp. AR03 and* Paenibacillus *sp. AR247: Statistical analysis*

A complete factorial design approach was performed to better describe the increments of the enzyme production by the co-culture of the strains AR03 and AR247. The variables influencing the enzyme production were nitrogen and carbon sources, as well as the presence of each strain in the culture (Table 1). Both strains assessed as endoglucanase producers at a confidence level of 95% of ($p < 0.05$). As expected, *Bacillus*

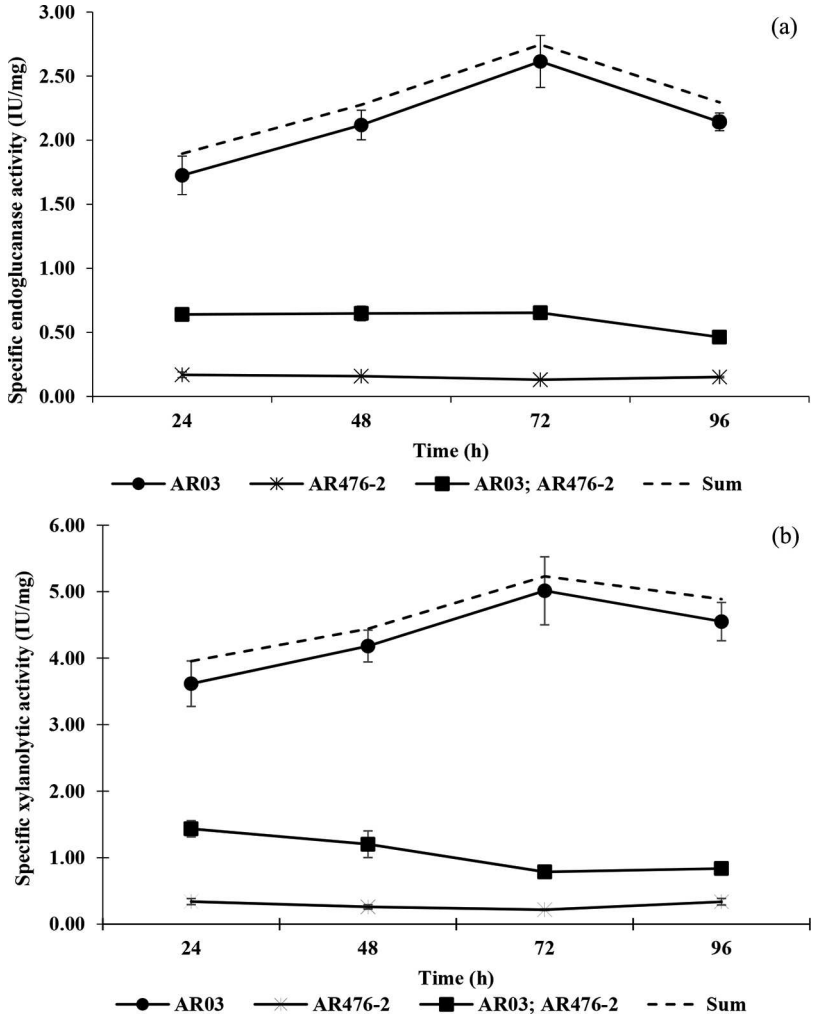

Figure 3. Endoglucanase (a) and xylanase (b) activity from individual cultures of *Bacillus* sp. AR03, *Achromobacter* AR476-2, and their co-culture in 1% (w/v) DTSB-CMC. The dotted line represents the expected value calculated as the sum of the individual enzyme activities. *Note*: CMC, carboxymethyl cellulose.

sp. AR03 showed a positive effect on endoglucanase activity (E-value = 3.98), whereas *Paenibacillus* sp. AR247 also showed a lesser, yet positive endoglucanase activity (E-value = 1.78). Interestingly, the simultaneous evaluation of the co-cultivation of both strains, AR03 and AR247, produced an improvement of 16.2% of the CMCase available into the culture broth (5.67 $\pm$ 0.22 IU mg^{-1}) with respect to the enzyme production of the monoculture of AR03 (4.88 $\pm$ 0.07 IU mg^{-1}). Therefore, the interaction of both strains led to a moderate positive effect on the response variable ($E = 1$) (Table 3), data that were supported by an $R^2 = 99.98\%$.

Individually, both strains showed xylanolytic activity in the conditions assayed, showing the significant factors at 95% of confidence level ($p < 0.05$), with an E value of 4.54 (for strain AR03) and 2.14 (for strain AR247) (Tables 1 and 3). Noteworthy, the interaction between both microorganisms showed a significant and positive effect ($p < 0.05$, $E = 2.12$), increasing the xylanolytic activity to 10.67 $\pm$ 0.18 IU mg^{-1},

Table 3. Effect of variables and statistical analysis of individual and co-culture of *Bacillus* sp. AR03 and *Paenibacillus* sp. AR247 using a factorial design for specific endoglucanase and xylanase activities.

			Specific endoglucanase activity (IU mg^{-1})			Specific xylanase activity (IU mg^{-1})		
	Level			Statistical significance			Statistical significance	
Variable	−1	+1	Effect (E)	Test t	p value	Effect (E)	Test t	p value
Bacillus sp. AR03	Absence	Presence	3.98	309.34	0.000	4.54	69.16	0.000
Paenibacillus sp. AR247	Absence	Presence	1.78	138.15	0.000	2.14	32.67	0.000
CMC (%)	0.5	3.0	0.17	13.51	0.000	1.40	21.32	0.000
Nitrogen sources (N)	Peptone	Yeast extract	−0.30	−23.25	0.000	−0.52	−7.89	0.000
AR03*AR247			1.00	77.96	0.000	2.12	32.24	0.000
AR03*CMC (%)			−0.17	−12.84	0.000	1.37	20.88	0.000
AR03*N			0.14	10.65	0.000	−0.53	−8.13	0.000
AR247*CMC (%)			0.19	14.49	0.000	1.51	23.06	0.000
AR247*N			−0.10	−7.83	0.000	0.74	11.26	0.000
CMC (%)*N			−0.15	−11.46	0.000	−0.62	−9.43	0.000

referred to strains AR03 and AR247 in single culture with xylanolytic activity of 3.84 ± 0.02 IU mg^{-1}, and 0.1 ± 0.01 IU mg^{-1}, respectively (Table 1), supported by an $R^2 = 99.68\%$.

In regard to the enzyme and biomass production by the co-cultures, the effect of the media components CMC and nitrogen sources was overall significant ($p < 0.05$). The highest enzymes production was attained utilizing CMC at 3% (w/v) and peptone as 10% of total nitrogen source (Table 3). Finally, the biomass production into the co-cultures reached $\sim 10^{13}$ CFU mL^{-1} for each strains when CMC was used at its highest level, whereas they produced $\sim 10^{11}$ CFU mL^{-1} into the media containing CMC at the lowest level assayed (Table 1).

Potential β-glucosidase encoding genes in Paenibacillus *sp. AR247 and* Achromobacter *sp. AR476-2 genomes*

The genome of *Paenibacillus* sp. AR247 was analyzed to search for sequences related to cellulose degradation. Although sequences encoding putative endoglucanase (EC 3.2.1.4) activities were detected, the main cellulases belonging to GH6 and GH12 were absent (data not shown). These observations could explain the low titles of enzymatic activity produced by this strain on CMC as substrate (Tables 1 and 2). On the other hand, the genome from *Achromobacter* sp. AR476-2 revealed the presence of catalytic modules of β-glucosidases although it does not contain genes encoding hydrolases responsible for the cellulose and/or xylan degradation.[17] Thus, this strain would not display a key meaning as part of a cellulolytic consortium, and its role in nature needs to be further studied.

Discussion

Bacillus sp. AR03, *Paenibacillus* sp. AR247, and *Achromobacter* sp. AR476-2 were assayed both as monocultures and co-cultures. Previous observations showed that several isolates from our laboratory collection reached OD values <0.9 when grown in minimal media.[18] Therefore, to assess their ability to produce extracellular cellulases and xylanases, a diluted nutrient broth was used to ensure the bacterial growth and the enzyme production. Comparable growth rates and a similar behavior in DTSB-CMC medium allowed us to evaluate them as co-cultures (Figure 1). The soluble substrate CMC was selected because it not only promoted the endoglucanase production by *Bacillus* sp. AR03, but also it did not affect significantly the system homogeneity. Then, the results observed here could be further used for the evaluation of insoluble substrates, such as xylan and even agricultural byproducts. These, on the other hand, are particulate and not only cause the adhesion of the cells, but also reduce the enzymes availability into the culture supernatant.[19,20]

In all the strains examined, a short diauxic lag period during cultivation was observed, probably due to the presence of glucose and CMC into the medium assayed. Our observations were in line with those described by Solopova et al.,[21] who revisited the diauxic growth.[22] The authors reported the coexistence of two stable cell types that coexisted in a pure culture of a *Lactobacillus* strain grown in a medium containing glucose and cellobiose, where most bacteria continued using the first substrate (glucose) while a sub-population of cells started to metabolize the second one (cellobiose). This conduct, described as a "bet-hedging" strategy, allows bacteria to adapt rapidly to unexpected changes in the environment. Accordingly, the authors proposed that a similar behavior might also exist for other carbon sources as was here observed.

Bacillus sp. AR03, described as a cellulolytic strain,[2] also exhibited xylanolytic activity when grown in DTSB supplemented with CMC (Table 2) as was extensively reported for several *Bacillus* species.[23,24] Also, the effect of the simultaneously cultivation of the xylanolytic isolate *Paenibacillus* sp. AR247 with a cellulolytic strain using CMC as the main carbon source was investigated since it was reported additive and/or synergistic effect of the variety of GHs produced by different strains over homopolysaccharides and heteropolysaccharides.[13]

The statistical analysis performed supported the synergy observed by means of the simultaneous cultivation of both strain significantly increased in both xylanase and CMCase production with respect to the individual cultures assayed (Tables 1 and 3). In fact, this effect was higher than the improvement obtained before varying the levels of the culture components CMC and nitrogen sources (Figure 2 and Table 1). While the highest activities of endoglucanase and xylanase were observed with CMC at its upper concentration assayed, the lowest substrate concentrations evaluated resulted in less production of the enzyme assessed.[25,26] Peptone was the nitrogen source that better promoted the growth and the enzymatic production; nevertheless, the use of yeast extract as nitrogen source was reported to be best for the cellulase production by a co-culture of two *Bacillus* spp. isolates strains.[25,27]

Interestingly, the augmentation of the biomass of the strains into the co-culture did not improve the endoglucanase production; in contrast, the xylanase produced increased with rising cell densities (Table 1).

No relevant cellulases were detected by means of an *in silico* analysis of the draft genome of *Paenibacillus* sp. AR247. However, several putative intracellular β-glucosidases were identified. Then, it is feasible to hypothesize that cellobiose, and even cello-oligosaccharides, might be further metabolized intracellularly by the strain AR247.[18–20] This would explain the overall outcome observed (Figure 2), which might be due to the utilization by *Paenibacillus* sp. AR247 of cello-oligosaccharides released by the CMCase produced by *Bacillus* sp. AR03. As a result, the strain AR247 might stimulate the endoglucanase production by its nutritional demand via the hydrolysis of CMC efficiently accomplished by the strain AR03.

During the time course of the co-cultivation of *Bacillus* sp. AR03 and *Achromobacter* sp. 476-2, an antagonistic compartment was observed due to an unexpected outgrew of the strain AR476-2 over *Bacillus* sp. AR03 in the co-culture (Supplementary material, Figure 1). This was correlated to a drop of enzyme production in DTSB-CMC when these strains were simultaneously cultivated under our assay conditions (Figure 3). Nevertheless, more research is required to unravel these observations.

Finally, the ability to use cellulose or hemicellulose has not been described for members of *Achromobacter* species.

However, the strain AR476-2 was able to grow with xylan (data not shown) and CMC as carbon sources, with barely detectable levels of extracellular GHs which might be due to nonspecific activities (Table 2).[9–17] Isolates belonging to this genus have been described as associated to cellulolytic bacteria, such as *Achromobacter* sp. CX2, which was reported to act synergistically with cellulolytic microbes. However, the contribution of this genus to lignocellulose degradation needs to be further examined.[8–28]

Conclusion

Endoglucanase and xylanase production was improved via the simultaneous cultivation of *Bacillus* sp. AR03 and *Paenibacillus* sp. AR247. Our observations might be the result of a boosting effect of the strain AR247 on the CMCase production by *Bacillus* sp. AR03, aimed to support the nutritional requirements of both microorganisms via CMC hydrolysis. The hypothesis here assayed is intended to contribute to the understanding of potential interactions between strains in co-cultures. This potential interaction and synergy may improve the overall performance of the enzymatic mixtures that are produced for different microorganisms to degrade the major carbohydrates from the plant cell walls.

On the contrary, a decrease in the extracellular enzyme production by *Bacillus* sp. AR03 became evident when it was co-cultivated with *Achromobacter* sp. AR476-2. This possible antagonism needs additional studies to be explained although some hints arise from this work, such as the fact that the strains AR03 and AR476-2 were isolated from different niches, and an apparent optimal development of *Achromobacter* sp. AR476-2 in the peptone-based broth used. These observations might explain the unexpected outgrew of *Achromobacter* sp. AR476-2 over *Bacillus* sp. AR03 growth and enzyme production.

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