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RESEARCH PAPER

Control of phenotypic diversification based on serial cultivations on different carbon sources leads to improved bacterial xylanase production

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Abstract

Thermobacillus xylanilyticus is a thermophilic and hemicellulolytic bacterium of interest for the production of thermostable hemicellulases. Enzymes' production by this bacterium is challenging, because the proliferation of a cheating subpopulation of cells during exponential growth impairs the production of xylanase after serial cultivations. Accordingly, a strategy of successive cultivations with cells transfers in stationary phase and the use of wheat bran and wheat straw as carbon sources were tested. The ratio between subpopulations and their corresponding metabolic activities were studied by flow cytometry and the resulting hemicellulases production (xylanase, acetyl esterase and β-xylosidase) followed. During serial cultivations, the results pointed out an increase of the enzymatic activities. On xylan, compared to the first cultivation, the xylanase activity increases by 7.15-fold after only four cultivations. On the other hand, the debranching activities were increased by 5.88-fold and 57.2-fold on wheat straw and by 2.77-fold and 3.34-fold on wheat bran for β-xylosidase and acetyl esterase, respectively. The different enzymatic activities then stabilized, reached a plateau and further decreased. Study of the stability and reversibility of the enzyme production revealed cell-to-cell heterogeneities in metabolic activities which could be linked to the reversibility of enzymatic activity changes. Thus, the strategy of successive transfers during the stationary phase of growth, combined with the use of complex lignocellulosic substrates as carbon sources, is an efficient strategy to optimize the hemicellulases production by *T. xylanilyticus*, by preventing the selection of *cheaters*.

Keywords Xylanases · *Thermobacillus xylanilyticus* · Successive cultivations · Lignocellulosic substrates · Phenotypic heterogeneity · Sporulation

Background

Hemicellulases, and notably xylanases, are widely used in various industrial processes (bread-making, pulp bleaching in paper process, fruit juice clarification, improvement of digestibility for animal feeding, …) [1, 2]. More recently, an increase of interest for hemicellulases occurred in the context of the development of lignocellulosic substrates biorefining for biofuels and biomolecules production [3–5]. In this context, *Thermobacillus xylanilyticus* is a Gram-positive and thermophilic bacterium exhibiting interesting features as a natural producer of hemicellulases [6, 7]. Its hemicellulolytic enzymes' arsenal includes two xylanases (from glycoside hydrolase families GH 10 and 11, EC 3.2.1.8), one arabinofuranosidase (GH 51, EC 3.2.1.55) and one feruloyl esterase (Carbohydrate esterase family CE1, EC 3.1.1.73) [8–10]. Moreover, its genome sequence pointed out the presence of several GH3, GH43 GH51 and GH120 annotated as β-xylosidases [11]. Previous studies indicated that the xylanase GH11 is secreted by the strain during the microbial growth whereas the other produced enzymatic activities are intracellular. Interestingly, *T. xylanilyticus* can adapt its complex enzymatic arsenal according to the lignocellulosic biomass used for its microbial growth. Debranching activities, such as xylosidases and esterases, are stimulated in presence of complex substrates [12, 13]. Esterase activity is promoted by growth with wheat straw and wheat bran

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as carbon source probably due to the high content in ferulic acid and acetate groups in xylans from these substrates, respectively [12, 14]. The use of the enzymes produced by this bacterium in various enzymatic processes is promising due to their thermostability, their activity over a wide range of pH values and their ability to release sugars and phenolic compounds from complex lignocelluloses [9, 10, 15].

In a recent study, we identified a significant physiological bottleneck impacting the production of hemicellulases by *Thermobacillus xylanilyticus* [16]. Indeed, this bacterium exhibited a decrease of xylanase production caused by phenotypic instabilities due to the presence of a phenotypically distinct subpopulation made of cells displaying a cheater phenotype, i.e., taking benefit from the monomers released but not producing the associated enzymes. These differences were easily observable since cheaters exhibited a different morphology [16]. Accordingly, in exponential phase, two subpopulations exhibiting different FSC properties and different levels of xylanase production were observed. More precisely, we observed *high* population i.e., displaying a high Forward Scatter Signal (FSC-A) in flow cytometry (superior to 30,000) and a *low* population, displaying a low FSC-A signal (inferior to 30,000). We hypothesized that, for *T. xylanilyticus*, premature sporulation of the producing cells can be induced because of the metabolic burden associated with enzymes productions. The other subpopulation, composed of cheaters, can avoid this metabolic burden but is taking benefit from the monomers released by the active members of the population (Fig. 1) [16]. This phenomenon was previously described and this kind of subpopulation can be recognized as cheaters and can appear when microbial population relies on an extracellular enzymatic production for degrading complex substrates [17, 18]. In *T. xylanilyticus* cultivation, this diversification occurred during exponential phase, allowing the selection of cheater cells over multiple generations (Fig. 1). We also showed, based on cell sorting, that the two subpopulations exhibited the same level of xylanase activity in stationary phase, opening new possibilities for the optimization of hemicellulases production based on the mitigation of the phenotypic diversification mechanism involved in the generation of cheaters.

With the aim to control the phenotypic population diversity within *T. xylanilyticus* during growth on xylan, we tried to alternate between the utilization of glucose and xylan as carbon source. By this way, it was possible to obtain an improvement of xylanase activity until to ~ 15-fold by comparison with continuous cultivation on xylan only [16]. The aim of this study was to further investigate the effect of phenotypic diversification on the production of hemicellulases by *T. xylanilyticus*. More specifically, the goals were: 1) to prevent the selection of cheaters by keeping the phenotypic diversification process under control. Our strategy was based

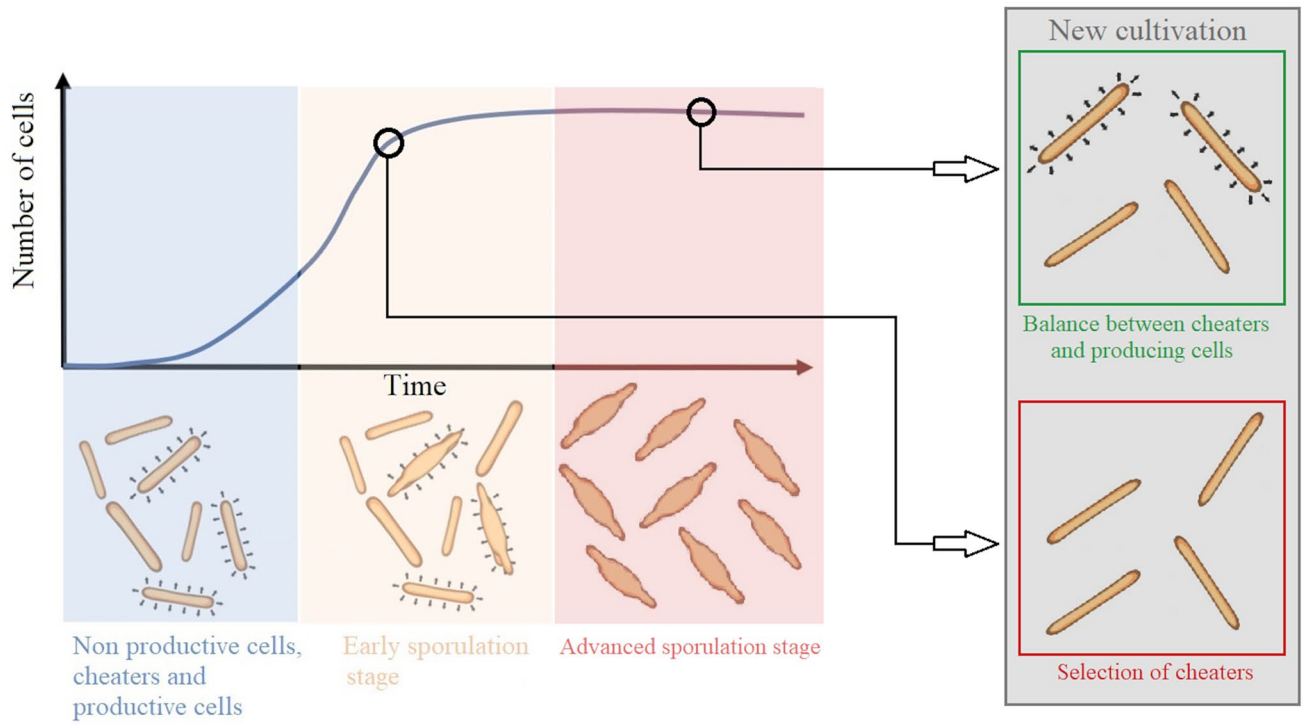

Fig. 1 Population dynamics during cultivation of *T. xylanilyticus* with the hypothesis of cheaters selection along the successive cultivations on exponential phase, which could be prevented by successive transfers during stationary phase (with advanced sporulating cells) adapted from [16]

on successive cultivations with serial transfers in stationary phase in presence of complex lignocellulosic biomasses; 2) to study the reversibility of the phenotypic states obtained during serial transfers experiments.

Materials and methods

Strains and media

Thermobacillus xylanilyticus XE9/11/91, isolated from a farm soil under a manure heap in northern France, was used in this study. The bacterium was cultivated on basal medium composed by three different solutions, a macro-mineral solution, a vitamin solution, and a metallic trace solution complemented with NH_4Cl (1 g/L), yeast extract (2 g/L) and $KHCO_3$ (5 g/L). The medium was supplemented with 10% (v/v) CO_2 as previously described by [7]. Cultivation volumes are 10 mL of media in sealed contents (100 mL bottles). Various carbon sources were used: glucose 5 g/L (Sigma Aldrich), xylan from beechwood 5 g/L (Roth), destarched wheat bran 10 g/L, and wheat straw 15 g/L, (ground to 1–2 mm) (ARD Pomacle-Bazancourt, France) (Table 1).

The complementation of xylan medium with glucose was done by reducing the xylan concentration from 5 to 4 g/L (ratio 80/20) or 2.5 g/L (ratio 50/50) before completing with glucose. The final glucose concentration was 1 g/L or 2.5 g/L for the ratios 80/20 and 50/50, respectively, to reach the total carbon source concentration of 5 g/L. The addition of glucose was realized after sterilization of the medium.

Successive cultivations of *Thermobacillus xylanilyticus*

The strain was regenerated from a glycerol stock and an overnight non-sporulated preculture was prepared in glucose basal medium at 50 °C and 150 RPM with Multitron shakers (Infors). The xylan, wheat bran or wheat straw media were inoculated (1% v/v) with the preculture diluted 100-fold. The cultivation was done at 50 °C, 150 RPM for 15 h to reach stationary phase. The transfers were then done after dilution of 100-fold for xylan cultivations, whereas there was no dilution between each wheat bran and wheat straw cultivation.

For each cultivation, when the stationary phase was reached (OD_{600nm} between 2.5–4, 0.75–1.6 and 2.2–3.2 for xylan, wheat bran and wheat straw cultures, respectively), the transfers of the cells in fresh media for starting new cultivations were done. For each new set of cultivations, a measurement of the xylanase activity, β-xylosidase and esterase activities were performed.

Each cultivation and measurements were performed in triplicate.

Table 1 Chemical composition of destarched wheat bran and wheat straw [12]

	Wheat bran	Wheat straw
Total sugar (% DM)	86	84
Glucose	22	44
Arabinose	21	4
Xylose	31	28
Phenolic compounds (mg/g DM)		
p-Coumaric acids	0.2 ± 0.0	5.7 ± 0.3
Ferulic acids	6.8 ± 0.1	3.2 ± 0.1
Diferulic acid	0.9 ± 0.0	0.3 ± 0.0
Acetyl esters	8 ± 0.9	31.0 ± 0.1

Measurements of the enzymatic activities

To evaluate the xylanase activity secreted by *T. xylanilyticus*, for each cultivation, samples were centrifuged at 3354 × g for 10 min (Sorvall ST 8R centrifuge, Thermo Fisher Scientific) at 4 °C and the supernatants were recovered. The xylanase activity was quantified by using the reducing end sugars measurement according to the procedure described by [19]. Briefly, 0.1 mL of the supernatants (diluted or not with 50 mM sodium acetate buffer pH 5.8) were incubated in 0.9 mL beechwood xylan (Roth) at 0.5% w/v homogeneously suspended in 50 mM sodium acetate buffer (pH of 5.8) at 60 °C for 10 min. Each 2 min, the hydrolysis reaction was stopped by transferring 100 µL of the supernatant-substrate mix in 1.5 mL of reagent composed of 0.3 g/L of potassium ferricyanide and 10 g/L of sodium carbonate. The ferricyanide was reduced by the liberated reducing sugars which resulted in a discoloration which could be tracked by a decrease of the absorbance. The reducing sugars were then measured by following the evolution of absorbance at 420 nm on a Specord 200 Plus uv/vis spectrophotometer (Analytik Jena) and by comparing the values with a standard curve done with varying concentrations of xylose. The activity was expressed in IU/mL. One international unit (IU) of enzyme activity was defined as the quantity of enzyme (xylanase) required to liberate one µmol of equivalent xylose per minute at 60 °C.

For the evaluation of the intracellular enzymatic activities, cells were first concentrated fivefold by centrifugation at 12,108 × g (Sorvall ST 8R centrifuge) and resuspended in 1 mL of a lysis buffer, TRIS HCl 25 mM (pH of 7). The cells were then lysed with FastPrep-24™ 5G (MP Biomedicals™) and 0.25 mL of lysis matrix beads (0.25 mm diameter). The lysis program was four cycles of 30 s at 6.5 m/s with break of 30 s between each cycle. The supernatant (intracellular fractions) was recovered after a centrifugation step at

12,108×*g* (Sorvall ST 8R centrifuge). The samples were kept at 4 °C until analyses.

For the evaluation of the β-xylosidase and esterase activities, the rate of hydrolysis of *p*-nitrophenyl β-xylopyranoside (Carbosynth) and *p*-nitrophenylacetate (Sigma) to *p*-nitrophenol was determined spectrophotometrically by measuring the absorbance at 401 nm. The experiments were performed in a volume of 1 mL containing 450 µL of TRIS HCl 25 mM (pH 7), 450 µL of the substrates (final concentration of 0.5 mM) and 100 µL of the intracellular protein fractions for 10 min at 50 °C.

Study of strain stability

To study the stability and reversibility of the production modifications during serial transfers in stationary phase, the strain obtained after six and ten successive cultivations on xylan medium, the conditioned strain C6 (corresponded to the strain with stabilized xylanase production peak) and the strain C10 (with decreased production of xylanase activity) were kept at 4 °C for 2 months. Then, new successive cultivations were started with serial transfers in the stationary phase on xylan medium as described above. Measurements of xylanase activity productions and the metabolic activities of the populations were followed.

Population analyses by flow cytometry

Population analyses at the different cultivations during the reversibility and stability study, were done by flow cytometry with a BD Accuri™ C6 (BD Biosciences) using the MOBICYTE core facilities (University of Reims Champagne Ardenne). The analytical parameters were flow rate of 35 µL/min, core size of 16 µm and the threshold was down to 30,000 in FSC-H. 20,000 cells were collected for each sample as previously described in [16].

Samples were first filtered with 5 µm cellulose syringe filters and diluted with PBS 1 × to reach maximum 2500 detected events by second (and events/µL) to prevent doublet reading. Samples were analyzed first unstained, and populations were detected by the forward and scatter signals (respectively, FSC-A and SSC-A) to determine cell percentage of each detected population with a gating at 30,000 in FSC-A as previously described in [16]. The BD CSampler Software was used to acquire and treat the cytometric data.

Samples were also analyzed after staining with BacLight™ RedoxSensor™ Green (RSG, 1 µM) or propidium iodide (PI, 10 mg/L) (Invitrogen) to detect the metabolic activity and the membrane permeability, respectively [20, 21]. After 488 nm laser excitation, green fluorescence was collected at 525 nm ± 30 nm for RSG (FL1-A) and the red fluorescence signal at 670LP (FL3-A) for PI. For the study of metabolic activity, a gating at 50,000 in FL1-A was used to detect some subpopulations such as for the gating at 30,000 in FSC-A described above and in [16].

Statistical analyses

The data of enzymatic activities and metabolic activities were acquired for each replicate of cultivation. FL1-A means data of each replicate were used for metabolic activities. The calculation of the means and standard deviations (STDEVA) were performed for each cultivation from the data acquired for each replicate. All the means and STDEVA were then compared for each successive cultivation by statistical analysis with Student's test from MATLAB statistics and machine learning toolbox. Differences were considered significant for p value ≤ 0.05.

Results

Serial cultivations on xylan with transfer of cells collected during stationary phase led to improved xylanase activity.

To avoid the selection of cheaters during successive cultivations, serial transfers in stationary phase have been considered, by systematically starting new cultivations with sporulating cells (Fig. 1). In presence of xylan as the only carbon source, xylanase production in the cultivation medium of *T. xylanilyticus* was increased over the successive cultivations (Fig. 2). During the first cultivation, the enzymatic activity was found to be low (4.91 ± 0.03 IU/mL). After only four successive cultivations, it increased significantly and reached 37.78 ± 9.70 IU/mL (p value < 0.05). A stabilization around this maximal activity was observed during five transfers. The increasing factor between the first cultivation and the stabilization phase was around 7.15-fold. After this stabilization and until the end of the cultivations, the xylanase activity significatively decreased to a value of 11.60 ± 1.17 (p value < 0.05). However, the activity remained 2.36-fold higher, which was significatively higher (p value < 0.01) than for the beginning of the cultivation. When xylanase enzymatic activity was normalized with the secreted protein concentrations (IU/mg), the peak of xylanase activity was observed at the seventh cultivation. At this level, the xylanase enzymatic activity reached 2820.81 ± 408.48 IU/mg corresponding to a significant 20.11-fold increase of xylanase activity by comparison with the first cultivation on xylan (p value < 0.01) (data not shown).

During the three first successive cultivations on xylan, the optical densities (OD_{600nm}) after the 15 h of cultivation were around 2.43 ± 0.10. When xylanase production increased, the growth increased accordingly (mean value of 3.40 ± 0.37). However, when xylanase production began to decrease, the growth remained at the same level (mean value of 3.21 ± 0.34).

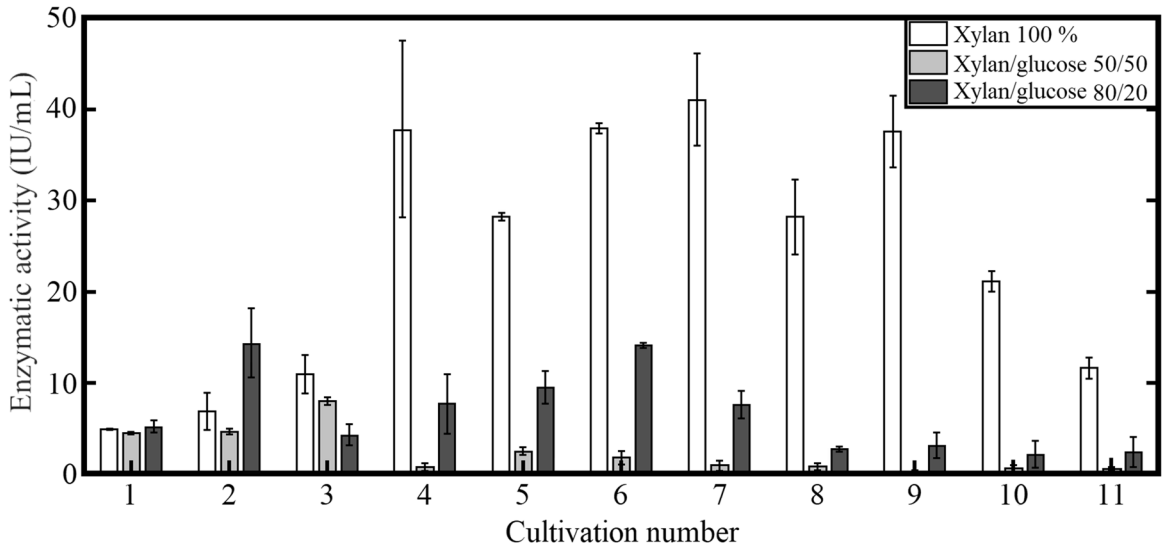

Fig. 2 Evolution of the xylanase activity over eleven successive cultivations during stationary phase on xylan or with different ratios of xylan and glucose. Xylanase activity is expressed in IU/mL

During the experiment, the complementation of xylan with glucose with various proportions (mixtures of xylan and glucose with ratio 80/20 or 50/50) as carbon source was also tested (Fig. 2). The complementation with glucose did not improve the xylanase activity during serial transfers in stationary phase. The successive cultivations with only xylan as carbon source exhibited higher xylanase activity level. Thus, after 4 transfers, the xylanase activity was 2.48-fold higher on xylan alone compared to the activity on a mixture of xylan:glucose 80/20. The increase of glucose proportion (50/50) in the medium led to lower xylanase activity.

During the cultivations with the complementations, the growths did not change significatively, and the measured OD_{600nm} values were close to the ones obtained with only xylan. The mean values of OD_{600nm} were 2.78 ± 0.56 and 2.88 ± 0.71 for the successive cultivations performed with mixtures of xylan and glucose with ratio 80/20 or 50/50, respectively.

Study of strain stability for xylanase production

To evaluate the stability of the metabolic change related to the xylanase production after successive cultivations with xylan as carbon source, the strains obtained after six and ten successive cultivations (strains C6 and C10) and kept at 4 °C were further cultivated (Fig. 3). Six new successive cultivations were performed with the same cultivation parameters as the ones used for the optimization of the xylanase production with only xylan as a carbon source.

The strain C6, conditioned on xylan, exhibited originally a xylanase activity of 37.94 ± 0.54 IU/mL (or 1014.21 ± 137.48 IU/mg). When recultivated, the strain exhibited firstly a low xylanase activity of 4.55 ± 0.03 IU/mL (253.92 ± 90.58 IU/mg) before reaching a value of 38.11 ± 7.06 IU/mL (707.46 ± 233.42 IU/mg) after four successive cultivations (p value < 0.01). Then, the xylanase activity decreased to 16.82 ± 3.12 IU/mL (299.04 ± 95.91 IU/mg) even if this decrease was not statistically significant (Fig. 4). The results of xylanase activity after four successive cultivations were statistically similar to the original level of xylanase activities of the conditioned strain C6 (p value of 0.9829). It corresponded approximatively to the levels of xylanase activity from the wild strain after four successive cultivations on xylan. The maximum level of xylanase was obtained after a lower number of cultivations i.e., four successive cultivations instead of six during the first experiment even if the activities after four cultivations of the wild strain and C6 strain were not significantly different. However, at the opposite of the wild strain, the xylanase activity of the C6 strain remained stable after only two to three cultivations after the maximal activity production has been reached. The same trend was observed for the strain C10 obtained after ten cultivations, but with lower xylanase activity during all successive cultures (data not shown). These results indicated that *T. xylanilyticus* cells stored at 4 °C are still able to produce xylanase but required a minimal of four cultivations with transfers in stationary phase to recover the maximal level of xylanase activities. As for the main serial cultivation experiments, the growth of *T. xylanilyticus* did not change during the study of reversibility. The mean values of OD_{600nm} after 15 h of cultivation were 2.81 ± 0.81 for C6 and 2.18 ± 0.24 for C10.

To get more insights about the reversibility and stability of the obtained phenotypes, flow cytometry analyses were

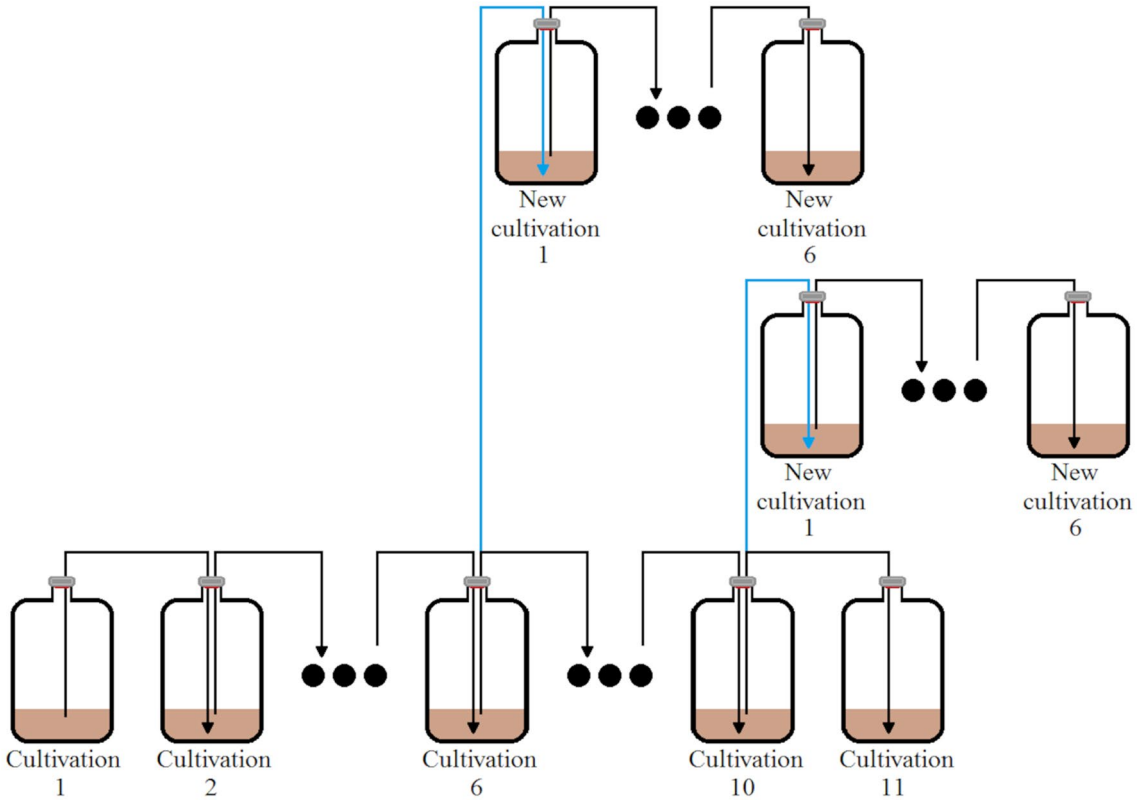

Fig. 3 Strategy for the study of *T. xylanilyticus* strain stability for xylanase production with xylan as carbone source. The blue lines correspond to the starting of new cultivations from the conditioned strains C6 and C10 conserved at 4 °C for 2 months

performed in stationary phase of each successive cultivations on xylan. The analysis of the forward scatter signal (FSC-A) defined during our previous works allowed to discriminate between two subpopulations of cells i.e., one exhibiting FSC-A signals higher than 30,000 and one exhibiting signals lower than 30,000. These subpopulations were labeled *high* subpopulation and *low* subpopulation, respectively. The *high* subpopulation contained cells in premature sporulation process (cells in the *high* population) [16]. In the present work, only one population with signals below 30,000 in FSC-A (*low* population) was observed during the successive cultivations with transfers in stationary phase. We then analyzed the metabolic activity based on the use of the RedoxSensor Green (RSG) molecular marker and flow cytometry. After the first cultivation, the cells exhibited a very high metabolic activity according to RSG staining (with an average green fluorescent level of 54,051 ± 3718, as recorded by the FL1-A channel of the flow cytometer). Interestingly, xylanase activity was very low after the first cultivation (Fig. 4). During the second and third cultivation rounds, the metabolic activities of the cultures decreased (around 9626 ± 2559 in FL1-A), by comparison with the first cultivation (p value < 0.001). After four cultivations, the metabolic activity increased (into 21,227 ± 1352 in FL1-A), together with the xylanase activity that reached the maximal peak of production (Fig. 4). The two last cultivations exhibited metabolic activities that tend to increase, but not significatively different from the fourth cultivation (29,794 ± 18,088 and 40,610 ± 27,136 in means FL1-A, respectively). For fifth and sixth cultivations, the standard deviations were very important. Several features can explain these high standard deviations. Some differences in cells metabolic activities (due to various physiological states of the cells) between each replicate of the fifth and sixth cultivations have probably high impact on the cytometric signals (FL1-A) and on the measurement of GFP positive cells. Moreover, the presence of some traces (insoluble) in the medium could have impacted the cytometric measurements. Xylanase activities decreased significantly during the last phase. Taken altogether, these data pointed out that, upon serial cultivations, low xylanase production levels are related to high metabolic activities.

To better highlight this relation, a gating procedure based on a FL1-A threshold of 50,000 was applied in flow cytometry. Based on this procedure, two subpopulations were observed in terms of metabolic activities (Fig. 5). These two subpopulations exhibited a high metabolic activity (with a mean FL1-A signal above 50,000, i.e. corresponding to the *high metabolism* subpopulation) or low metabolic activity

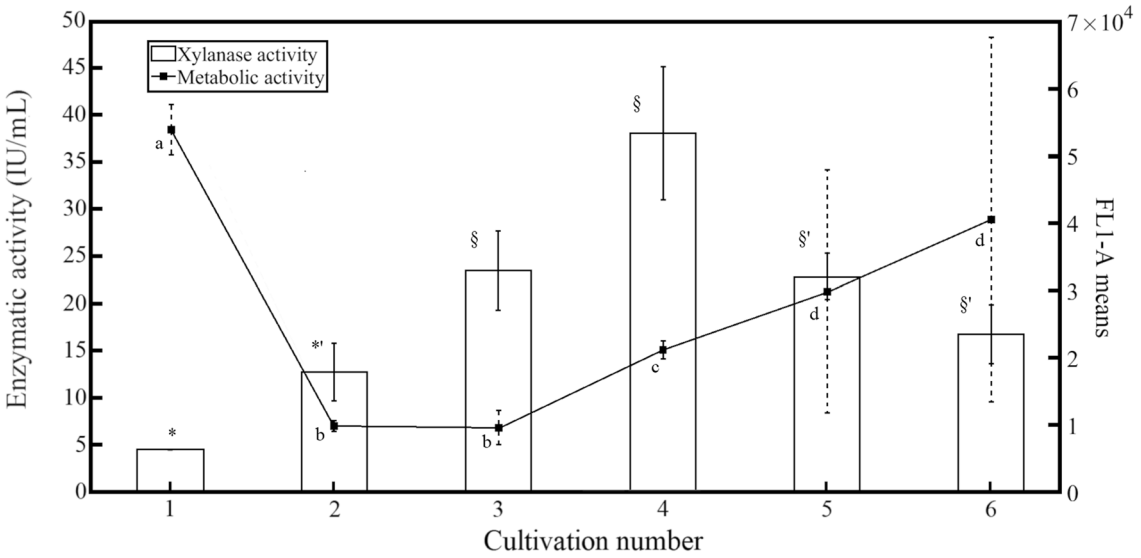

Fig. 4 Evolution of *T. xylanilyticus* metabolic activity and xylanase activity during successive cultivations in presence of xylans and with transfers in stationary phase. The cultivations were done with C6. The xylanase activity is expressed in IU/mL. The metabolic activities are represented by the green fluorescence collected at 525 nm ± 30 nm (FL1 detector). For each cultivation replicate, the mean fluorescence was collected and used to obtain the global mean fluorescence of the cultivation and the fluorescence standard deviations. The symbols * and § represent the significatively different group of xylanase activity between each cultivation (* = significantly lower of each other cultivation (p value < 0.05); *' = significantly different of each other cultivation excepted cultivations 5 and 6 (p value < 0.05); § = significantly higher from cultivations 1 and 2 (p value < 0.05) but not significantly different from cultivations 3, 4, 5 and 6; §' = significantly higher from cultivations 1 (p value < 0.05) but not significantly different from cultivations 2, 3, 4, 5 and 6). The alphabetical symbols represent the significatively different group of metabolic activity between each cultivation (a = significantly higher of each cultivations (p value < 0.001) excepted cultivations 5 and 6; b = significantly lower of cultivations 1 and 4 (p value < 0.01) but not significantly different from cultivations 2, 3, 5 and 6; c = significantly lower than cultivation 1 (p value < 0.001), higher than cultivations 2 and 3 (p value < 0.01) but not significantly different from cultivations 5 and 6; d = not significantly different of each cultivation)

(with a mean FL1-A signal below 50,000, considered as the *low metabolism* subpopulation). Upon the first cultivation, the *high metabolism* subpopulation was predominant (63.4 ± 4.4%). During the second, third and fourth cultivations, an abrupt shift in the metabolic activity profile was observed, the *high metabolism* population disappearing to the benefit of the *low metabolism* subpopulation with ratio of 99.9 ± 0.06%, 99.8 ± 0.06% and 98.2 ± 0.62%, respectively. The *low metabolism* subpopulation was also predominant after the fifth and sixth serial cultivation but with the presence of *high metabolism* subpopulation too (Fig. 5).

Evolution of enzymatic activities during serial cultivation on wheat bran and wheat straw

To study the evolution of hemicellulases production by *T. xylanilyticus*, the production levels of the extracellular xylanase activity as well as the intracellular β-xylosidase and acetyl esterase activities were measured during successive cultivations in presence of two different complex carbon sources i.e., wheat bran and wheat straw. Indeed, these complex substrates are known to induce the production of debranching enzymes by *T. xylanilyticus* (particularly esterases). Bacterial growth was lower on these two carbon sources. The mean OD_{600nm} was 1.10 ± 0.25 on wheat bran and 1.40 ± 0.27 on wheat straw after 22 h of cultivation.

At the beginning of the experiments, in presence of wheat bran, the extracellular xylanase activities quantified were similar to the activities obtained with xylan (Fig. 6a). The xylanase production firstly significatively decreased from 8.47 ± 1.89 IU/mL to 0.54 ± 0.38 IU/mL (p value < 0.05) after three successive transfers. Then, it increased to a level similar to the one obtained for the first cultivation round (i.e., around 7.73 ± 1.90 IU/mL), before stabilizing around a value of 5.72 ± 1.76 IU/mL. In presence of wheat straw, at the beginning of the experiments, the xylanase activity produced was 80.39 and 4.88-fold lower than activity obtained on xylan and wheat bran, respectively. Xylanase production significatively increased from 1.73 ± 0.37 IU/mL to 8.67 ± 0.42 IU/mL (p value < 0.001) after four successive cultivations. Then, the activity remained similar at this level until the end of the transfers. For both complex substrates, after stabilization at the maximal level, the xylanase activity did not decrease until the end of the experiments.

In presence of xylan as carbon source, the acetyl esterase activity level was around 187 ± 36 mIU/mL and did not change significatively over six successive cultivations. This activity was significatively increased from

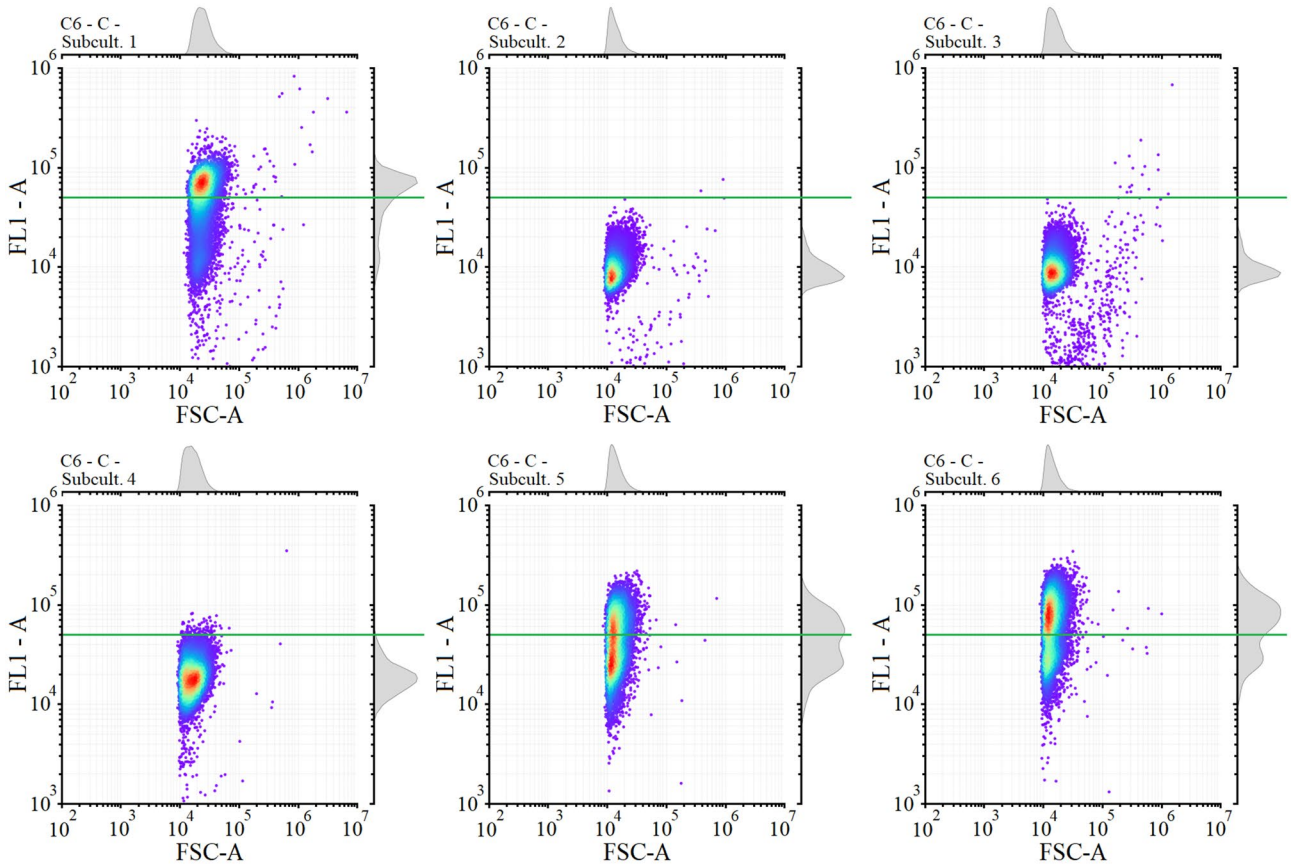

Fig. 5 Evolution of *T. xylanilyticus* metabolic activity and presence of subpopulations during cultivations in presence of xylans and with transfers in stationary phase. An example of cytograms corresponding to the six successive cultivations of one replicate from the "conditioned strain C6". The green line corresponds to the gate of 50,000 in FL1-A for the detection of green fluorescence from RedoxSensor Green (between 490 and 520 nm)

168.35 ± 12.71 mIU/mL to 466.43 ± 16.32 mIU/mL (increase of 2.77-fold, p value < 0.001) with wheat bran and increased from 71.30 ± 19.76 mIU/mL to 419.26 ± 26.59 mIU/mL (significant increase of 5.88-fold, p value < 0.001) in presence of wheat straw. These increased enzymatic activities were observed for both substrates after four successive cultivations. If the esterase production values stabilized for wheat straw until the end of the cultivation, for wheat bran, fluctuations in activity are still observed (Fig. 6b).

The mean values of β-xylosidase activity were around 167 ± 23 mIU/mL with xylan and did not change significantly over the six successive cultivations. During cultivation on wheat bran and wheat straw, the maximal level of this activity was lower (between 11.8-fold and 4.9-fold, respectively) than for xylan cultures. However, it significatively increased from 4.15 ± 3.56 mIU/mL to 13.87 ± 0.94 mIU/mL (3.34-fold increase, p value < 0.05) after four cultivations with wheat bran and increased to a maximal value of 31.23 ± 3.64 mIU/mL after four cultivations with wheat straw. Taken altogether, the data point out that serial cultivations led to a significant, 57.2-fold increase, of enzymatic activities by comparison with the first cultivation round (p value < 0.001) (Fig. 6c).

Discussion

In microbial processes, most of the strategies used for improving growth or metabolite productions by microorganisms rely on strain modifications, metabolic engineering or nutrient changes (or stress) [22, 23]. Strain optimization, also can be done by successive cultivations [24, 25] even if the phenotypes are not always stable [26]. For example, the lignocellulolytic enzymes production of *Aspergillus niger* and a thermophilic *Geobacillus* strain have already been enhanced by this methodology [27, 28]. The cultivation conditions, including the composition of the medium, the substrate diffusion rate, the presence of inductor, … can impact directly the enzymatic production of various bacterial species [12, 29–31]. The basic principle behind the optimization of enzyme production based on serial cultivation relies on the genotypic diversification of the strain of interest. However,

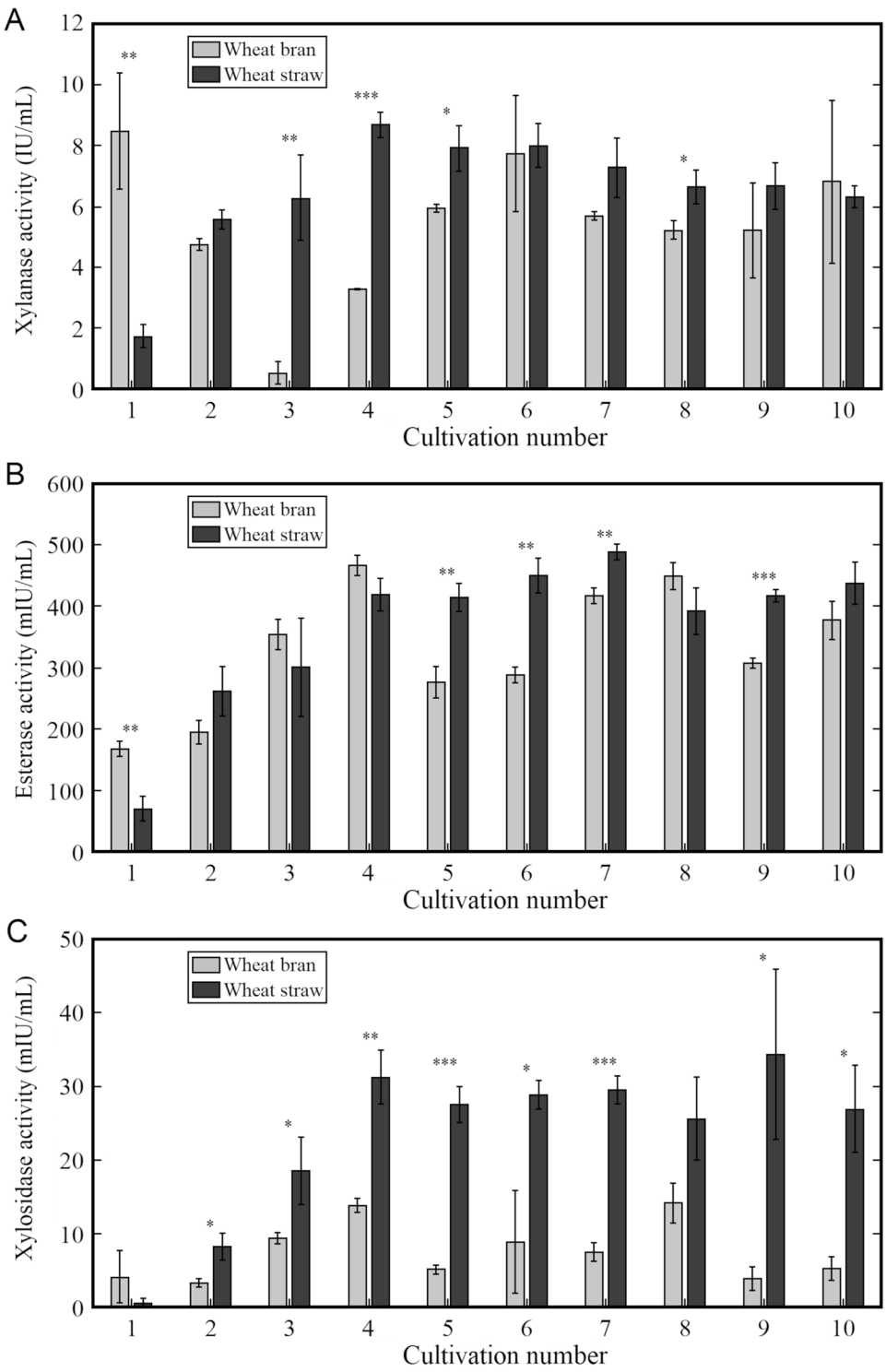

Fig. 6 Evolution of the extracellular xylanase activity (**a**) and intracellular enzymatic activities (**b** and **c**) changes during cultivations in presence of wheat bran and wheat straw. The intracellular activities correspond to acetyl esterase activity (**b**) and β-xylosidase activity (**c**). The asterisk represents the significatively different activity between wheat bran and wheat straw (* = (p value < 0.05), ** = (p value < 0.01) and ** = (p value < 0.001)

in our work, we point out that phenotypic diversification, involving epigenetic mechanisms without changes in DNA composition, are also involved. In our study, the application of successive cultivations resulted in the management of subpopulations and the improvement of hemicellulases production by *T. xylanilyticus*. The strategy used in this study is the transfer of cells after reaching the stationary phase, since transfer of exponentially growing cells has been shown to lead to a progressive reduction in extracellular xylanase activity due to cheaters' selection [16]. During our previous works, the xylanase activity was observed to be similar in stationary phase for the different subpopulations (i.e. cheaters and producing cells characterized upon cell sorting) [16]. Our hypothesis was that a transfer in stationary phase would

allow to increase the xylanase production without selection of cheaters over generations (Fig. 1). With all the cells in advanced sporulation, there would be no selection of vegetative cheater cells when a new cultivation is started and producing cells would be favored over the successive cultivations. The results obtained confirmed this hypothesis as the maximal level of enzyme production was reached more quickly under these conditions. After only four successive cultivations on xylan and complex substrates, the extracellular xylanase activity and intracellular enzymatic activities (esterase and xylosidase) reached their maximal levels. Additionally, the loss of xylanase activity typically observed during classical serial cultivation is delayed. The hemicellulases production by *Thermobacillus xylanilyticus* was stabilized and was maintained for a longer period of time. To our knowledge, few studies focusing on the improvement or stabilization of microbial populations rely on successive cultivations in stationary phase, except some studies about the influence of the inoculum age on the frequency of persistent subpopulation in *E. coli* [32, 33]. The impact of growth phases on phenotypic diversification and the presence of cheaters is already known for some bacteria, such as *Pseudomonas aeruginosa* or *Escherichia coli* [34–36]. For both bacteria, the cheater invasion (due to quorum sensing and cooperation for *Pseudomonas aeruginosa* and due to growth arrest of ancestral population from *E. coli*) seems to be a case of evolutionary cheating but affects the representation of the wild type in the following generations [34, 35].

The different diversification control strategies can also rely on adaptation to carbon limitation and switching to an alternative carbon source as previously studied [23, 34, 37, 38]. By the combinations of successive cultivations on cellobiose or by switching carbon sources (cellobiose and glucose) daily, two mutant strains (muC and muS) of *Thermobifida fusca* with higher cellulase activities compared to the wild-type strain were obtained [38]. In case of *T. xylanilyticus,* the switch between glucose and xylan as carbon source during successive cultivations allowed to improve (~ 1.5-fold to ~ 15-fold in function of generations) and stabilize the xylanase production compared to continuous cultivation on xylan [16].

In this study, we tried to go further by testing the effect of nutrient changes and combining glucose and xylan with various ratios (80/20 or 50/50). However, this strategy did not increase the xylanase activity upon serial transfers in stationary phase. An explanation could be that the strain efficiently used all the glucose before switching to the consumption of xylan due to the production of the xylanase. After this switch, the strain was not in the same growth phase (or production state) than during cultivation with only xylan. With both carbon sources in medium, the cultivation time required to reach the stationary phase (and the significant level of xylanase activity production) is increased. Indeed, xylanase production is known to be higher at the end of exponential phase and the beginning of the stationary phase [12]. To improve the production of other hemicellulases (mainly debranching enzymes), complex lignocellulosic substrates were used as carbon sources during successive cultivations. Previous studies indicated that *T. xylanilyticus* can adapt its enzymatic production, depending on the carbon sources used for the microbial growth [12]. The results from this study validated these observations, as the acetyl esterase activity was higher on WS than on WB, probably due to a higher content of acetyl ester into WS as seen in Table 1 [12]. As for the cultures performed on xylan, the higher β-xylosidase activity obtained during cultivation on WS could be related to an increased xylanase production with these carbon sources leading to a higher accumulation of xylo-oligosaccharides which are substrates for β-xylosidases. Xylan and wheat straw can then be considered as complementary for the improvement of hemicellulases production by *T. xylaniticus*. An efficient hydrolysis of hemicelluloses requires an optimal mixture of hemicellulolytic enzymes and is dependent on the composition of the lignocellulosic substrate and also on the physiological state of the producing cells [14]. Given an optimal production of xylanase (by successive cultivations with xylan as carbon source) and induction of β-xylosidase and acetyl esterase productions (by successive cultivations with complex lignocellulosic biomass), specific and adapted hemicellulasic cocktails containing xylanase and debranching enzymes can be obtained. For this purpose, the strategy of successive cultivations by playing on the age of inoculum is efficient. It is preferable to transfer *T. xylanilyticus* cells in stationary phase to manage the presence of cheater cells and to control phenotypic diversification.

Interestingly, our study pointed out that the phenotypic changes of *T. xylanilyticus* were not irreversible, as most of the changes in enzymatic activity were not stable over time particularly for xylanase activity (Figs. 2, 4, 6). These phenotypic changes could therefore not be considered as an evolutionarily stable configuration as described by Wagner and Schwenk, because the obtained changes were not stable over short period of time [26].

Another possible explanation about this effect is that enzyme production can be controlled by regulatory loops, particularly phosphorelays and two-component systems [39, 40]. It is indeed observed that transcriptional regulators and sensor proteins are present in the genome of *T. xylanilyticus* and some of these components are found within gene clusters coding for hemicellulolytic enzymes [11]. However, the functional dynamics of the network is not yet known. To verify the involvement of this type of regulation in population stability and reversibility and to better improve enzyme production, further studies are then needed. Our reversibility study pointed out that the strains obtained during the first successive cultivations (C6 and C10) exhibited higher level

of xylanase activities. Additionally, this production occurred more quickly when the isolates C6 and C10 were recultivated but less stable over successive cultivations. One mechanism possibly explaining this effect is the microbial memory. Indeed, microbes are able to display memory based on the accumulation of stable proteins to obtain fitness advantages [41, 42]. Memory is mainly due to epigenetic mechanisms, and can be diluted over successive generations if the proteins involved are very stable [41, 43]. The phenotypic memory is due to the transmission of stable cytoplasmic proteins between the mother and daughter cell during cell division as already proven for *lac* proteins transmitted during *E. coli* cultivations [43]. In the case of *T. xylanilyticus,* the epigenetic mechanisms responsible for the memory to recover higher levels of hemicellulases productions, as well as the mechanisms leading to the emergence of the cheating behavior and population heterogeneity, are not yet identified. The possible memory effect observed in this study seems to be closer to a phenotypic memory obtained by transmission of cytoplasmic proteins under fluctuating conditions.

Further studies implicating other approaches, like advanced single-cell technologies (microfluidics, automated flow cytometry with feedback control) have to be considered in order to refine the resolution about microbial population dynamics of *T. xylanilyticus* and stabilize the obtained phenotypes. Omic analyses, such as proteomics or genomics analyses, could also be used to better understand the diversification process occurring in *T. xylanilyticus*, as well as the organization of regulation network over successive cultivations [44].

Conclusions

This study pointed out that epigenetic mechanisms, like phenotypic switching and memory, are involved in the long-term production of enzymes by *T. xylanilyticus*. Accordingly, the use of successive transfers in stationary phase with xylan or complex lignocellulosic biomasses led to the effective control of enzyme production by avoiding the generation of cheaters and promoting the presence of actively producing cells. This strategy was successfully used and improved the extracellular activity (xylanase) and some debranching activities (acetyl esterase and β-xylosidase) from *T. xylanilyticus* in this study. However, this strategy was never adopted before for the control of population dynamics for the production of hemicellulases and open up new ways for the effective management of microbial population during long-term, serial, cultivations.

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Author contributions BR, CR and HR designed the experiments. BR performed the cultivations, enzymatic assays and flow cytometry experiments with the contribution of Coralie Pierrot. BR, FD, CR and HR wrote and revised the manuscript.

Declarations

Conflict of interest The authors declare that they have no competing interest.

References

1. Subramaniyan S, Prema P (2002) Biotechnology of microbial xylanases: enzymology, molecular biology, and application. Crit Rev Biotechnol 22:33–64
2. Bhardwaj N, Kumar B, Verma P (2019) A detailed overview of xylanases: an emerging biomolecule for current and future prospective. Bioresour Bioprocess. https://doi.org/10.1186/s40643-019-0276-2
3. Takkellapati S, Li T, Gonzalez MA (2018) An overview of biorefinery-derived platform chemicals from a cellulose and hemicellulose biorefinery. Clean Technol Environ Policy 20:1615–1630. https://doi.org/10.1007/s10098-018-1568-5
4. Gírio FM, Fonseca C, Carvalheiro F, Duarte LC, Marques S, Bogel-Łukasik R (2010) Hemicelluloses for fuel ethanol: a review. Bioresour Technol 101:4775–4800. https://doi.org/10.1016/j.biortech.2010.01.088
5. Dodd D, Cann IKO (2009) Enzymatic deconstruction of xylan for biofuel production. GCB Bioenergy 1:2–17
6. Samain E, Touzel JP, Brodel B, Debeire P (1992) Isolation of a thermophilic bacterium producing high levels of xylanase. Xylans Xylanases, Prog Biotechnol 7:467–470
7. Touzel JP, O'Donohue M, Debeire P, Samain E, Breton C (2000) *Thermobacillus xylanilyticus* gen. nov., sp. nov., a new aerobic thermophilic xylan-degrading bacterium isolated from farm soil. Int J Syst Evol Microbiol 50:315–320
8. Samain E, Debeire P, Touzel JP (1997) High level production of a cellulase-free xylanase in glucose-limited fed batch cultures of a thermophilic Bacillus strain. J Biotechnol 58:71–78
9. Debeche T, Cummings N, Connerton I, Debeire P, O'Donohue MJ (2000) Genetic and biochemical characterization of a highly thermostable α-L- arabinofuranosidase from *Thermobacillus xylanilyticus*. Appl Environ Microbiol 66:1734–1736
10. Rakotoarivonina H, Hermant B, Chabbert B, Touzel JP, Remond C (2011) A thermostable feruloyl-esterase from the hemicellulolytic bacterium *Thermobacillus xylanilyticus* releases phenolic acids from non-pretreated plant cell walls. Appl Microbiol Biotechnol 90:541–552
11. Rakotoarivonina H, Loux V, Doliwa C, Martin V, Rémond C (2022) Draft genome sequence of the lignocellulolytic and thermophilic bacterium *Thermobacillus xylanilyticus* XE. Microbiol Resour Announc 11:e00934-21
12. Rakotoarivonina H, Hermant B, Monthe N, Rémond C (2012) The hemicellulolytic enzyme arsenal of *Thermobacillus xylanilyticus*

depends on the composition of biomass used for growth. Microb Cell Fact 11:1–12
13. Rakotoarivonina H, Revol PV, Aubry N, Rémond C (2016) The use of thermostable bacterial hemicellulases improves the conversion of lignocellulosic biomass to valuable molecules. Appl Microbiol Biotechnol 100:7577–7590
14. Rakotoarivonina H, Hermant B, Aubry N, Rabenoelina F, Baillieul F, Rémond C (2014) Dynamic study of how the bacterial breakdown of plant cell walls allows the reconstitution of efficient hemicellulasic cocktails. Bioresour Technol 170:331–341. https://doi.org/10.1016/j.biortech.2014.07.097
15. Debeire-Gosselin M, Loonis M, Samain E, Debeire P (1991) Purification and properties of a 22kDa endoxylanase excreted by a new strain of thermophilic Bacillus. Prog Biotechnol 7:463–466
16. Bouchat R, Vélard F, Audonnet S, Damien R, Delvigne F, Rémond C et al (2022) Xylanase production by *Thermobacillus xylanilyticus* is impaired by population diversification but can be mitigated based on the management of cheating behavior. Microb Cell Fact. https://doi.org/10.1186/s12934-022-01762-z
17. Velicer GJ (2003) Social strife in the microbial world. Trends Microbiol 11:330–337
18. Allison SD (2005) Cheaters, diffusion and nutrients constrain decomposition by microbial enzymes in spatially structured environments. Ecol Lett 8:626–635
19. Kidby DK, Davidson DJ (1973) Ferricyanide estimation of sugars in the nanomole range. Anal Biochem 55:321–325
20. Konopka MC, Strovas TJ, Ojala DS, Chistoserdova L, Lidstrom ME, Kalyuzhnaya MG (2011) Respiration response imaging for real-time detection of microbial function at the single-cell level. Appl Environ Microbiol 77:67–72
21. Davey HM, Hexley P (2011) Red but not dead? Membranes of stressed Saccharomyces cerevisiae are permeable to propidium iodide. Environ Microbiol 13:163–171
22. Binder D, Drepper T, Jaeger KE, Delvigne F, Wiechert W, Kohlheyer D et al (2017) Homogenizing bacterial cell factories: analysis and engineering of phenotypic heterogeneity. Metab Eng 42:145–156
23. Sassi H, Nguyen TM, Telek S, Gosset G, Grünberger A, Delvigne F (2019) Segregostat: a novel concept to control phenotypic diversification dynamics on the example of Gram-negative bacteria. Microb Biotechnol 12:1064–1075
24. Portnoy VA, Bezdan D, Zengler K (2011) Adaptive laboratory evolution-harnessing the power of biology for metabolic engineering. Curr Opin Biotechnol 22:590–594. https://doi.org/10.1016/j.copbio.2011.03.007
25. Dragosits M, Mattanovich D (2013) Adaptive laboratory evolution - principles and applications for biotechnology. Microb Cell Fact 12:1–18
26. Schwenk K, Wagner GP (2001) Function and the evolution of phenotypic stability: connecting pattern to process. Am Zool 41:552–563
27. Govil T, Saxena P, Samanta D, Singh SS, Kumar S, Salem DR et al (2020) Adaptive enrichment of a thermophilic bacterial isolate for enhanced enzymatic activity. Microorganisms 8:1–16
28. Patyshakuliyeva A, Arentshorst M, Allijn IE, Ram AFJ, de Vries RP, Gelber IB (2016) Improving cellulase production by Aspergillus niger using adaptive evolution. Biotechnol Lett 38:969–974
29. Dalmau E, Montesinos JL, Lotti M, Casas C (2000) Effect of different carbon sources on lipase production by Candida rugosa. Enzyme Microb Technol 26:657–663
30. da Silva RN, Melo LFdA, Luna Finkler CL (2021) Optimization of the cultivation conditions of *Bacillus licheniformis* BCLLNF-01 for cellulase production. Biotechnol Reports 29:e00599
31. Allison SD, Lu L, Kent AG, Martiny AC (2014) Extracellular enzyme production and cheating in *Pseudomonas fluorescens* depend on diffusion rates. Front Microbiol 5:1–8
32. Jõers A, Kaldalu N, Tenson T (2010) The frequency of persisters in *Escherichia coli* reflects the kinetics of awakening from dormancy. J Bacteriol 192:3379–3384
33. Luidalepp H, Jõers A, Kaldalu N, Tenson T (2011) Age of inoculum strongly influences persister frequency and can mask effects of mutations implicated in altered persistence. J Bacteriol 193:3598–3605
34. Feng X, Kostylev M, Dandekar AA, Greenberg EP (2019) Dynamics of cheater invasion in a cooperating population of *Pseudomonas aeruginosa*. Sci Rep 9:1–9. https://doi.org/10.1038/s41598-019-46651-5
35. Vulić M, Kolter R (2001) Evolutionary cheating in *Escherichia coli* stationary phase cultures. Genetics 158:519–526
36. Heins AL, Weuster-Botz D (2018) Population heterogeneity in microbial bioprocesses: origin, analysis, mechanisms, and future perspectives. Bioprocess Biosyst Eng 41:889–916. https://doi.org/10.1007/s00449-018-1922-3
37. Nguyen TM, Telek S, Zicler A, Martinez JA, Zacchetti B, Kopp J et al (2021) Reducing phenotypic instabilities of a microbial population during continuous cultivation based on cell switching dynamics. Biotechnol Bioeng 118:3847–3859
38. Deng Y, Fong SS (2011) Laboratory evolution and multi-platform genome re-sequencing of the cellulolytic actinobacterium *Thermobifida fusca*. J Biol Chem 286:39958–39966
39. Smits WK, Kuipers OP, Veening JW (2006) Phenotypic variation in bacteria: the role of feedback regulation. Nat Rev Microbiol 4:259–271
40. Burbulys D, Trach KA, Hoch JA (1991) Initiation of sporulation in B. subtilis is controlled by a multicomponent phosphorelay. Cell 64:545–552
41. Casadesús J, D'Ari R (2002) Memory in bacteria and phage. BioEssays 24:512–518
42. Wolf DM, Fontaine-Bodin L, Bischofs I, Price G, Keasling J, Arkin AP (2008) Memory in microbes: quantifying history-dependent behavior in a bacterium. PLoS ONE 3:e1700
43. Lambert G, Kussel E (2014) Memory and fitness optimization of bacteria under fluctuating environments. PLoS Genet 10:e1004556
44. Delvigne F, Goffin P (2014) Microbial heterogeneity affects bioprocess robustness: dynamic single-cell analysis contributes to understanding of microbial populations. Biotechnol J 9:61–72