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PAPER

The direct conversion of xylan to lactic acid by *Lactobacillus brevis* transformed with a xylanase gene

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A xylanase gene (*xyn*R8), obtained from the DNA of a pool of uncultured rumen microbes, was introduced *via* a plasmid into *Lactobacillus brevis*. The recombinant xylanase, with an estimated molecular weight of 32 KDa, was expressed in the transformants and showed obvious xylanase activity (0.412 U ml^{-1}) against oat-spelt xylan in broth when compared to the wild-type *Lactobacillus brevis* ATCC367. The transformants all shared a similar ability to utilize and metabolize xylooligosaccharides. When a selected transformant was inoculated into modified MRS medium containing xylan as the main carbon source, the cell density reached 2.20×10^9 CFU ml^{-1} on day 4, while the wild-type strain without the plasmid containing the recombinant xylanase did not grow at all under the same conditions. After fermentation, 1.70 g l^{-1} of lactic acid and 0.44 g l^{-1} of ethanol were present in the culture supernatant of the strain containing the recombinant xylanase. These results indicate that *Lactobacillus brevis* containing the xylanase gene is capable of directly saccharifying and fermenting xylan to produce lactic acid in one step. This strain will enable the development of a feasible and economical approach to the production of lactic acid directly from xylan.

Introduction

Lactic acid is a valuable and multi-functional organic acid, and it has been widely used in many industries, including cosmetic, food, pharmaceutical and chemical, due to its attractive properties.[1,2] It also has been shown to have potential promise when used to manufacture the biodegradable and biocompatible polymer polylactic acid (PLA), which is an environmentally friendly alternative to non-degradable plastics derived from the planet's limited petrochemical resources.[1,3] Intensive screening for various potential and economical feedstock substrates for the production of lactic acid is currently under way to meet future demand. Lactic acid can be produced by chemical synthesis or by biotechnological processes.[4] The chemical synthesis of lactic acid uses petroleum as the raw material and is less friendly to the environment.[1] The production of lactic acid by biotechnological processes is becoming more and more preferred due to issues of environmental pollution, the reducing reserves of petroleum and the increased requirement for lactic acid worldwide.

Direct fermentation to produce lactic acid from starch has been achieved using various *Lactobacillus* strains that possess extracellular amylase activity.[2,5] However, it is clear that the conversion of starchy feedstocks to lactic acid would compete with food for humans and feed for livestock, and that this is an important limitation. Excessive conversion of starchy feedstock like corn grain into fuel has already increased the prices of cereal and dairy products,[6,7] and the situation would be worse if extra starchy feedstock was used for the production of biodegradable plastic due to limited availability. In this context, the use of abundant but inedible material would be a better feedstock when using fermentation for lactic acid production.

Hemicellulose, which is mainly composed of xylan, is the second most abundant carbohydrate after cellulose in lignocellulosic biomass. It is also an important waste product from agriculture and food processing.[8] Lactic acid bacteria are generally regarded as potentially good candidates for the production of lactic acid, but most lactobacilli lack the ability to hydrolyze lignocelluloses.[9] Consequently, a variety of pretreatment methods have been developed that allow the fermentation of lignocellulose to produce lactic acid. Enzymatic pretreatment, and simultaneous saccharification and fermentation (SSF) are the two most prevalent procedures used to convert lignocellulosic materials into lactic acid.[3,10,11] The raw material is depolymerized into its constituent monosaccharides or simple

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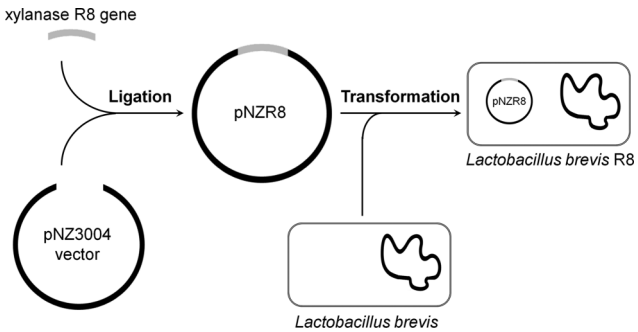
sugars, which can then be utilized by the lactic acid bacteria. This is done by adding the hydrolysate to media, which is then fermented by lactic acid-producing microorganisms. The two most common pre-treatment methods for the hydrolysis of lignocellulose to simple sugars are acid hydrolysis and enzymatic hydrolysis. Enzymatic treatment is more economic because it uses less energy, has relatively mild reaction conditions that avoid the use of toxic and corrosive chemicals, and does not produce inhibitors that affect the microbial fermentation.[10] Nonetheless, enzyme treatment is overall a more costly approach when used for lactic acid production. In these circumstances, the integration of enzyme production and microbial fermentation would be a feasible and cost-effective way to produce lactic acid from lignocellulose.

Lactobacillus brevis is a heterofermentative microorganism found in milk, cheese, sauerkraut, sourdough, silage and the gastrointestinal tracts of animals.[12] It can simultaneously consume a wide variety of carbon sources, including xylose and glucose, for growth.[13] It also has been shown to have β-xylosidase activity when wheat straw hydrolysate is used as the starting material for lactic acid production.[10] However, *Lactobacillus brevis* is unable to grow using xylan as its sole carbon source since no xylanase gene is present in its genome.[14] It seems highly probable that *Lactobacillus brevis* would be able to directly utilize xylan for growth if a xylanase gene was incorporated into its genome. In this study, a xylanase gene from a metagenomic sample of rumen fungi was introduced to *Lactobacillus brevis* (Scheme 1), and the expression of the xylanase and the ability of the transformed *Lactobacillus brevis* to produce lactic acid using xylan as the main carbon source (Scheme 2) were examined.



Scheme 1 Construction of a xylanase-producing transformant of *Lactobacillus brevis*.

Experimental

Microorganisms and media

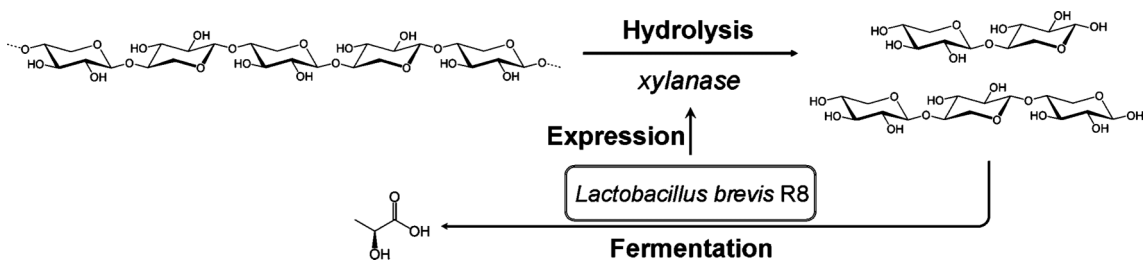
Escherichia coli DH5α (Invitrogen Corporation, Carlsbad, CA) was used as the host when carrying out recombinant DNA experiments and was grown at 37 °C on Luria-Bertani (LB) medium.[15] *Lactobacillus brevis* ATCC 367, which was purchased from the Bioresource Collection and Research Center (Hsingchu, Taiwan), was selected as the candidate for transformation with the xylanase gene. The *Lactobacillus* strain was maintained on de Man, Rogosa and Sharpe (MRS) medium.[10,16] MRS agar plates containing chloramphenicol (10 mg l^{-1}) was used to screen for transformants containing the xylanase gene plasmid. MRS-X medium, which was modified from MRS medium by replacing the glucose with oat-spelt xylan (20 g l^{-1}), was used for lactic acid production. MRS-XOS medium was used to test sugar utilization. The components of the MRS-XOS medium were the same as for the MRS medium, except that glucose was replaced with a 2% (w/v) xylooligosaccharide mixture that contained 1.41% xylose, 29.11% xylobiose, 31.31% xylotriose, 15.06% xylopentaose, 6.73% xylotetraose, 4.39% xylohexaose, 8.55 xyloheptaose, 0.73% glucose and 0.75% arabinose (Sweet Town Biotech. Crop, Taiwan). *Lactobacillus* strains were grown at 30 °C without shaking.

Plasmid construction and transformation

The xylanase R8 gene (accession no. AY941119) was obtained from the metagenomic DNA sample of a water buffalo by PCR (polymerase chain reaction) amplification, as published previously.[17] The PCR fragment encoding *xyn*R8 was digested with *Xho*I and *Pst*I, and ligated into similarly digested pNZ3004 to generate pNZR8. Next, pNZR8 was transformed into competent *Lactobacillus brevis* by electrophoration.[18] The electrophorated cells were spread onto MRS plates containing 10 μg ml^{-1} chloramphenicol and incubated at 30 °C until transformants appeared. The transformants were selected and transferred to another MRS plate containing 0.5% oat-spelt xylan. The plate was stained with Congo red solution after 2 d of incubation.[19]

Zymogram and enzyme activity assays

Culture supernatants were collected to determine xylanase activity, and zymogram analysis was also performed.[20] For image documentation, the stained gels were scanned at a resolution of 600 dpi using a V200 Photo Scanner (Epson Corporation, Nagano, Japan), and Adobe Photoshop software, version 6.0 (Adobe Systems Incorporated, San Jose, CA, USA).



Scheme 2 Direct conversion of xylan to lactic acid by a *Lactobacillus brevis* transformant bearing a xylanase gene.

The xylanase activity was detected by measuring the quantity of reducing sugar produced by enzymatic hydrolysis. The reducing sugar was determined by the DNS (dinitrosalicylic acid) method. One unit of xylanase activity was defined as 1 μmol of reducing sugar equivalents released from oat-spelt xylan per minute under assay conditions.[20]

Lactic acid fermentation from xylooligosaccharides and xylan

The wild-type *Lactobacillus brevis* and the *Lactobacillus brevis* strain containing the xylanase gene plasmid were cultured in MRS-X and MRS-XOS media to assess the fermentation of lactic acid. The lactic acid production experiments were carried out as batch fermentations under aerobic conditions. The strains were grown in a 250 ml Erlenmeyer flask containing 100 ml MRS-X broth, with the *Lactobacillus brevis* strains inoculated with 5% (v/v) of a bacterial suspension obtained after 15–20 h growth. The inoculated broth was then incubated at 30 °C for 5 d without shaking. The pH values of the broth were measured by a pH meter. The total reducing sugars in the fermentation broth were determined by the DNS colorimetric assay, as described by Cheng *et al*.[20] To determine viable cell counts (CFU (Colony Forming Units) ml^{-1}), serial dilutions of the samples were spread on MRS plates and incubated for 48 h at 30 °C. The *Lactobacillus brevis* CFU ml^{-1} in the broth were transformed to logarithmic values before statistical analysis. The reported bacterial count values are the mean values of triplicate determinations ± the standard deviations (SD).

Analysis of sugars and fermentation products

Samples taken during the fermentation were centrifuged (4 °C, 10 000 g, 30 min) on a Sigma 2K15 Centrifuge (Sigma Ltd., Osterode, Germany). The supernatant was filtered through a 0.45 μm filter (Pall Corporation, East Hills, NY, USA) and stored at 4 °C. The sugars and fatty acids of the filtrate were analyzed by high performance liquid chromatography (HPLC) using a Hitachi D7100 (Merck–Hitachi, Tokyo, Japan) with an ICSep COREGEL 87H3 column (Transgenomic Inc., Omaha, NE, USA) coupled to a UV detector and an RI detector; 0.01 M sulfuric acid (J. T. Baker Inc., Phillipsburg, NJ, USA) was used as the mobile phase at a flow rate of 1.0 ml min^{-1}. All chemicals in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

Results

Constructing the *Lactobacillus brevis* containing the xylanase gene

The transformants of *Lactobacillus brevis* harboring the xylanase gene plasmid were constructed and screened. The transformant shown in Fig. 1 shows xylanolytic ability on an MRS plate containing xylan. A yellow area around the colonies is visible after Congo red staining, and no such region of xylanolytic activity was found when a similar plate was inoculated with the wild-type untransformed strain. The transformant with xylanolytic ability was designated as *Lactobacillus brevis* R8. The plasmid present in *Lactobacillus brevis* R8 was isolated and checked for the presence of the xylanase gene by PCR. A single

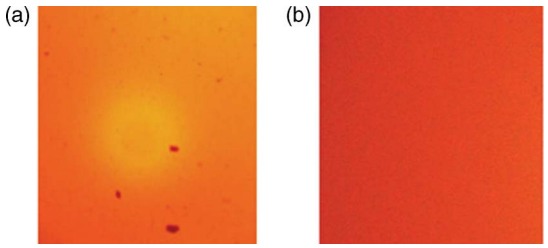


Fig. 1 Clear zones and orange-colored zones formed by (a) *Lactobacillus brevis* R8 and (b) *Lactobacillus brevis* ATCC 367. Both strains were inoculated on an MRS plate containing 0.5% (w/v) xylan. The plates were incubated at 30 °C for 3 d and stained with 0.5% (w/v) Congo red solution.

product of approximately 900 bp was amplified and was then detected by agarose gel electrophoresis. The sequence of the xylanase gene incorporated in the plasmid from *Lactobacillus brevis* R8 was confirmed by DNA sequencing, and the sequence was found to be identical to the sequence of the xylanase R8 gene, as previously described.

Analysis of the broth by zymogram after *Lactobacillus brevis* R8 had been grown in it revealed that xylanase activity was present and that the protein involved was a single clear band with an apparent molecular weight of 32 kDa (Fig. 2). The size of this expressed xylanase is close to the predicted molecular weight of xylanase R8. These results indicate that he xylanase R8 gene was successfully transformed, and is expressed and secreted by *Lactobacillus brevis* R8.

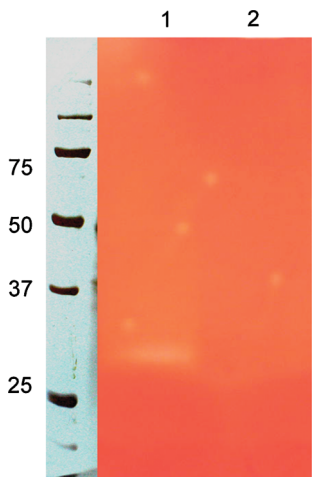


Fig. 2 Zymogram analysis of xylanase activity in crude extracts from *Lactobacillus brevis* R8 (lane 1) and *Lactobacillus brevis* ATCC 367 (lane 2). The crude extracts were separated on a 10% acrylamide gel supplemented with 1% oat-spelt xylan. Following electrophoresis, the gels were incubated in the presence of a citrate buffer (50 mM, pH 6) containing 50 mM dithiothreitol and stained with Congo red solution.

Lactic acid fermentation from xylooligosaccharides

To investigate the ability of *Lactobacillus brevis* R8 to utilize xylooligosaccharides, the glucose in the MRS medium was replaced with xylooligosaccharides. *Lactobacillus brevis* ATCC367, the wild-type strain, and *Lactobacillus brevis* R8 were inoculated individually into the modified medium, and the bacterial cell density, consumption of xylooligosaccharides

and accumulation of fermentation products were analysed. *Lactobacillus brevis* ATCC367 and the transformant both grew quickly and reached $10^{9.30}$ CFU ml^{-1} and $10^{9.18}$ CFU ml^{-1}, respectively, during which time the xylooligosaccharides were utilized (Fig. 3). The quantitative disappearance of xylotriose, xylobiose and xylose can be detected using HPLC, as shown in Fig. 3. Of these three sugars, which were present in the broth initially, xylobiose and xylotriose were quickly consumed, but xylose slightly increased after 1 d of incubation by both *Lactobacillus brevis* ATCC367 and *Lactobacillus brevis* R8.

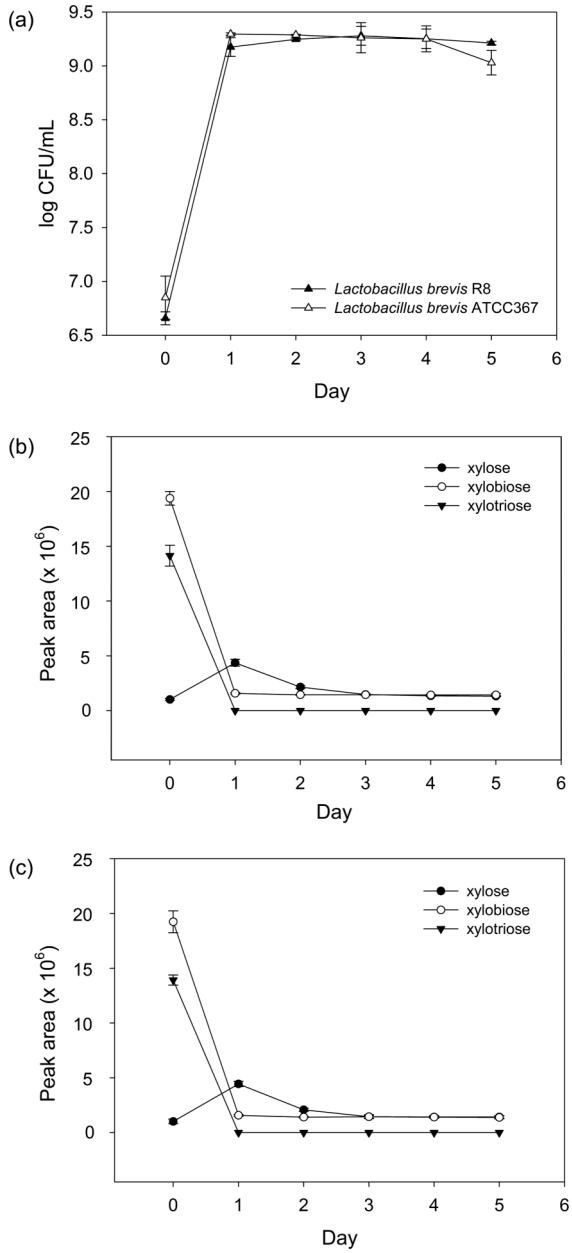


Fig. 3 The (a) growth and trend of xylooligosaccharide consumption in MRS-XOS broth inoculated with (b) *Lactobacillus brevis* R8 and (c) *Lactobacillus brevis* ATCC 367 during 5 d of fermentation. The peak areas of refractive index chromatograms were obtained from HPLC analysis using an 87H3 column. The samples were examined as described in the Experimental section. Data points represent the mean and standard deviations from triplicate experiments.

Xylotriose was totally exhausted after fermentation. In contrast, xylose and xylobiose were not completely depleted and a small amount remained in the broth after fermentation. The consumption of these substrates during fermentation indicates that both *Lactobacillus brevis* wild-type and R8 are able to utilize the various sugars present in the medium, including xylose, xylobiose and xylotriose. The concentration of lactic acid reached 6.81 ± 0.17 and 7.10± 0.53 g l^{-1} in the culture supernatant after 3 d of fermentation with *Lactobacillus brevis* ATCC367 and *Lactobacillus brevis* R8, respectively. The amount of acetic acid also increased to 0.76± 0.06 and 0.71± 0.04 g l^{-1}, respectively. Ethanol could not be found after fermentation when either strain was grown in MRS-XOS medium. These results show that *Lactobacillus brevis* ATCC367 and *Lactobacillus brevis* R8 are both capable of utilizing xylooligosaccharides as a carbon source for growth, and seem to possess a similar growth rate and similar metabolic pathways for xylooligosaccharide utilization.

The growth of *Lactobacillus brevis* R8 in xylan medium

Both the wild-type and *Lactobacillus brevis* R8 were subcultured in MRS-X broth to examine their growth and to determine whether there was heterologous xylanase expression. Xylanase activity increased, reaching a level of 0.412 U ml^{-1} after 5 d of incubation, when *Lactobacillus brevis* R8 was used for the fermentation, whereas no detectable xylanase activity was found when the wild-type strain was cultured under the same conditions (Fig. 4). The accumulation of reducing sugars in the fermentation depended on the production of xylanase by *Lactobacillus brevis* R8. As shown in Fig. 4, the level of reducing sugars (equivalent to xylose) in the broth continuously increased during the *Lactobacillus brevis* R8 fermentation on the MRS-X medium and reached 2.62 g l^{-1} after 5 d of incubation. However, there was no detectable increase in reducing sugar concentration when the wild strain was cultured under the same conditions. These results show that reducing sugars were released from the oat-spelt xylan, and that this was very likely to be due to the action of the expressed xylanase in *Lactobacillus brevis* R8.

The growth curves of the wild-type and *Lactobacillus brevis* R8 are presented in Fig. 4. *Lactobacillus brevis* R8 can be seen to grow continuously on MRS-X medium and reached a maximum cell density of 2.20×10^9 CFU ml^{-1} after 4 d of incubation; the bacterial counts then decreased slightly to 1.46×10^9 CFU ml^{-1} on day 5. Although *Lactobacillus brevis* ATCC367 without the xylanase gene showed a similar level of growth at the beginning of the incubation period, the cell density did not rise noticeably after the second day of fermentation. These results support the idea that the presence of the xylanase activity in *Lactobacillus brevis* R8 allows growth without pre-treatment on 0.5% oat-spelt xylan and that the xylan is utilized as its main carbon source. This does not occur with the wild-type *Lactobacillus brevis* because it lacks the plasmid encoding the xylanase gene.

Lactic acid fermentation from xylan

The ability of the *Lactobacillus brevis* strain containing the plasmid encoding the xylanase gene to directly ferment xylan to lactic acid was then assessed, together with an analysis of the intermediate products and the pH of the broth (Fig. 5). During fermentation, the pH was found to fall from an initial

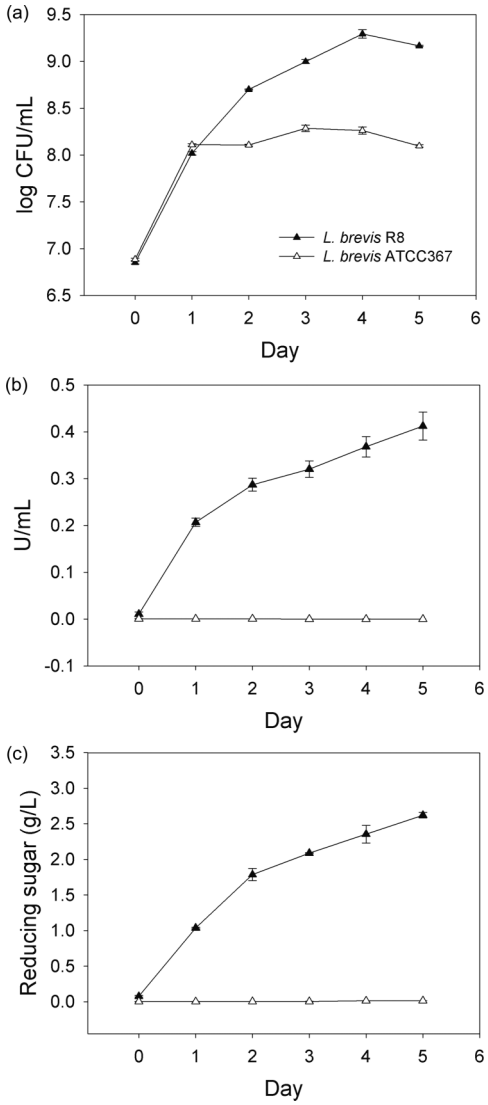


Fig. 4 Changes in (a) growth, (b) xylanase activity and (c) reducing sugar (equivalent to xylose) accumulation in the broth of MRS-X inoculated with *Lactobacillus brevis* R8 and *Lactobacillus brevis* ATCC367 during 5 d of fermentation. The number of *Lactobacillus brevis* was counted colony-forming units (CFU). The bacteria in the broth were spread on an MRS medium plate after dilution and incubated for 1 d at 37 °C. The xylanase activity was detected by measuring the quantity of reducing sugar produced by enzymatic hydrolysis from oat-spelt xylan for 30 min at 30 °C. The reducing sugar was determined by the DNS (dinitrosalicylic acid) method. One unit of xylanase activity is defined as 1 μmol of reducing sugar equivalents released from substrate per minute. Data points represent mean and standard deviations from triplicate experiments.

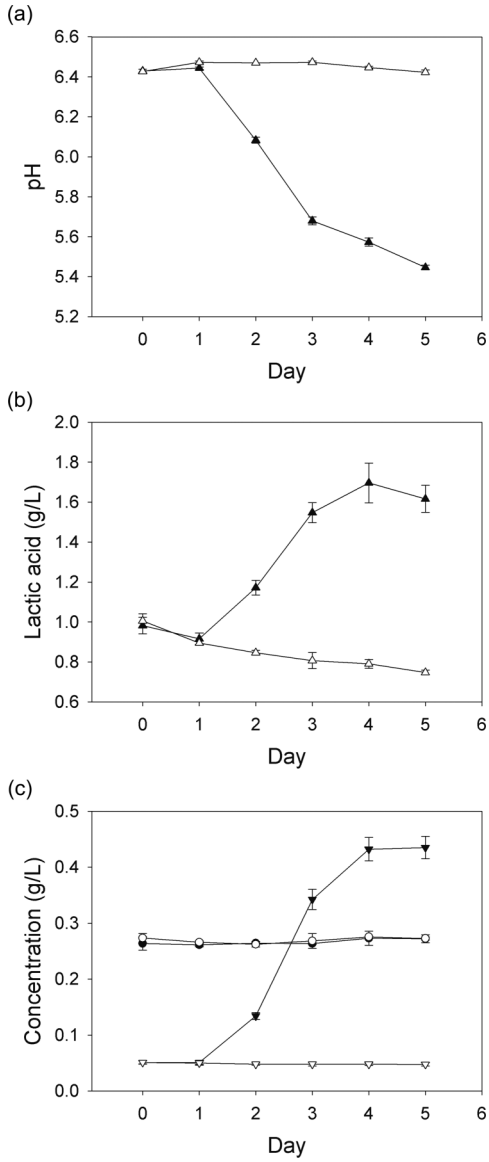


Fig. 5 Changes in (a) pH, (b) lactic acid production and (c) acetic acid (circle symbols), and the ethanol (triangle symbols) concentration in MRS-X broth inoculated with *Lactobacillus brevis* R8 (solid symbols) and *Lactobacillus brevis* ATCC367 (open symbols) during 5 d of fermentation. The samples were examined as described in the section of Experimental. Data points represent the mean and standard deviations from triplicate experiments.

value of 6.4 to 6.1 after 2 d, and then continue to fall, finally reaching 5.4 on day 5. Lactic acid was found to be the primary product of the fermentation by *Lactobacillus brevis* R8, and the concentration of lactic acid increased continuously during the 5 d of incubation, reaching a maximum of 1.70 g l^{-1} in the culture supernatant after 4 d of fermentation. The amount of lactic acid did not increase but decrease day-by-day when wilt-type *Lactobacillus brevis* was cultured in the same conditions. The levels of ethanol also increased during the fermentation of *Lactobacillus brevis* R8 and reached 0.46 g l^{-1}, whereas no significant change in either lactic acid or ethanol was detected with the wild-type strain. For both strains, the concentration of acetic acid changed only negligibly during the fermentation. These results show that *Lactobacillus brevis* R8 is able to directly ferment xylan and generate lactic acid and ethanol.

Discussion

It has been found that *Lactobacillus brevis* possesses a metabolic preference for pentose sugars such as xylose and arabinose over glucose, and xylose utilization in *Lactobacillus brevis* is not stringently controlled, as has been seen in other

xylose-utilizing lactobacilli such as *Lactobacillus pentosus*.[13,21] In the present study, we found that xylotriose was quickly exhausted, but that some xylobiose and xylose remained in the broth after fermentation with *Lactobacillus brevis* R8 (Fig. 3). In a previous study, it was also found that *Lactobacillus brevis* ATCC 8287 prefers a xylooligosaccharide mixture to pure xylose for growth.[22] These results suggest that *Lactobacillus brevis* might be better at utilizing xylotriose compared to xylose, supporting the idea that *Lactobacillus brevis* is an excellent candidate for the utilization and fermentation of xylan hydrolysate.

Obligately heterofermentative lactobacilli like *Lactobacillus brevis* are able to use the pentose phosphoketolase (PK) pathway for the metabolism of pentose, and the enzymes involved in the PK pathway, except for pyruvate-formate lyase and pyruvate decarboxylase, are also present in the genome of *Lactobacillus brevis* ATCC 367. In these circumstances, lactic acid and ethanol or acetate should be generated when the sugar is consumed,[13–14] and there should be a direct correlation between the sugar present and the production of ethanol or acetate. Acetate, rather than ethanol, is quickly utilized at the beginning of incubation, and increased amounts of acetate are only produced by *Lactobacillus brevis* when the amount of sugar present is high. The accumulation rate of acetate then slows down and trace amounts of ethanol are produced when sugars are almost depleted. This phenomenon has been found with several strains of *Lactobacillus brevis*.[13,23] The production of acetate rather than ethanol allows the synthesis of additional ATP *via* acetate kinase and leads to a higher specific growth rate. Protons are transferred from a coenzyme like NADH, which is generated by the PK pathway, to oxidative metabolites that allow faster growth. However, NADH will accumulate quickly when the sugar is metabolized on a large scale, and the accumulated NADH will then retard the production of lactate from glyceraldehyde-3 phosphate and will also have an influence on the generation of energy. Ethanol is made by recycling NADH, and this would allow the regeneration of the coenzyme.[13,23]

In the present study, acetic acid and ethanol can only be found in MRS-XOS and MRS-X, respectively (Fig. 5). The sugar concentration in MRS-XOS was sufficiently high (20 g l^{-1} xylooligosaccharides) to allow the fast growth of *Lactobacillus brevis* and the cell density reached a high level (Fig. 3). This was accompanied by the generation of ATP *via* acetate kinase, and as a result acetate accumulated significantly in the *Lactobacillus brevis* broth. In contrast to the above scenario, reducing sugars were released slowly from the xylan in MRS-X medium due to hydrolysis by the xylanase expressed by *Lactobacillus brevis* R8, and the available xylooligosaccharides were depleted as they were produced; as a result, the sugar concentration (Fig. 4) remained low (0.08~2.62 g l^{-1}). Under these circumstances, the production of ethanol was able to regenerate NADH, which allowed lactate to be scavenged for ATP generation due to the low levels of the available sugar.

Conclusion

In conclusion, our results clearly demonstrate that *Lactobacillus brevis* R8, a lactobacillus expressing a xylanase carried on a transformed plasmid, is able to directly ferment xylan to lactic acid in one step. This is an attractive approach to the production of lactic acid from a xylan-containing material and is worthy of further development in the future.

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