Engineering *Zymomonas mobilis* to produce tailor-made fructooligosaccharides

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Background

Fructooligosaccharides (FOS) are promising prebiotics in the increasing market of functional food. Industrially, these compounds are produced from sucrose using fructosyltransferase (Ftase) or β-fructofuranosidase enzymes (Ffase) [1]. Although it is known that these compounds may have a great impact in health, it has also been described that the beneficial effects may depend on the relative FOS composition. Therefore, to obtain a product with high market value it is necessary to optimize not only the FOS yield, but also the composition of the mixture. The search for novel microbial strains that have high transfructosylation and/or produce FOS with different patterns is currently being investigated [2]. The generally regarded as safe (GRAS) bacterium *Zymomonas mobilis* represents an excellent recombinant expression system to produce FOS, since it contains native enzymes able to convert the non-prebiotic sugars (glucose and fructose) into added-value products, such as levan, sorbitol and ethanol [3,4]. This microorganism contains an extracellular sucrase/invertase (SacC) and an extracellular levansucrase enzyme (SacB) that converts sucrose into levan and FOS [5,6]. Although the heterologous production of FOS by different organisms has proven to be a successful strategy to optimize and change the FOS composition [7,8], there are no reports regarding the heterologous production of FOS by *Z. mobilis*.

Methods

The strong and constitutive pyruvate decarboxylase (*pdc*) (ZMO1360) promoter (P*pdc,* 500 bp) and *pdc* terminator (T*pdc*, 200 bp), were amplified from the *Z. mobilis* ZM4 genome by PCR. P*pdc* and T*pdc* were cloned into pBBR1MCS plasmid, creating the pB1 plasmid. The s*acB* gene was amplified from *Z. mobilis* ZM4 genome and cloned in pB1 (pB1-*sacB*). A mutated version of the Ffase gene from *Schwanniomyces occidentalis* (Ffase-Leu196) [9] was amplified from a plasmid previously constructed in our research group (p-L196). Ffase-Leu196 present in plasmid pL196 (originally amplified from Ffase-pYES-L196 kindly provided by Prof. María Fernández-Lobato, CSIC Madrid, Spain) has an upstream native signal sequence of 29 aa from *Z. mobilis* acid phosphatase gene (*phoC*), to allow the engineered strains to export the heterologous enzymes to the media and ensure the FOS synthesis extracellularly. After amplification, the signal sequence and the Ffase-Leu196 were cloned into pB1, leading to pB1-L196.

FOS production experiments were performed in 250 mL flasks containing 100 mL of production medium previously optimized (350 g L^{-1} sucrose, 20 g L^{-1} YE, 2.5 g L^{-1}

NaCl, 2 g L^{-1} potassium phosphate monobasic, 1 g L^{-1} ammonium sulphate and 2.04 g L^{-1} magnesium sulphate), supplemented with 100 mg L^{-1} of chloramphenicol. Flasks were incubated for 72 h at 30 ºC, without agitation.

Results

As an attempt to modulate the composition of FOS mixture, two different *Z. mobilis* strains were constructed. The first one was constructed by overexpressing the native *sacB* gene. The second one was constructed by heterologous expression of a mutated Ffase variant (Ffase-Leu196) from *S. occidentalis*.

The mutant strains, including the control strain *Z. mobilis* ZM4 carrying the empty plasmid pB1, were cultivated in shake-flasks with production medium containing 350 g L^{-1} of sucrose (**Figure 1**).

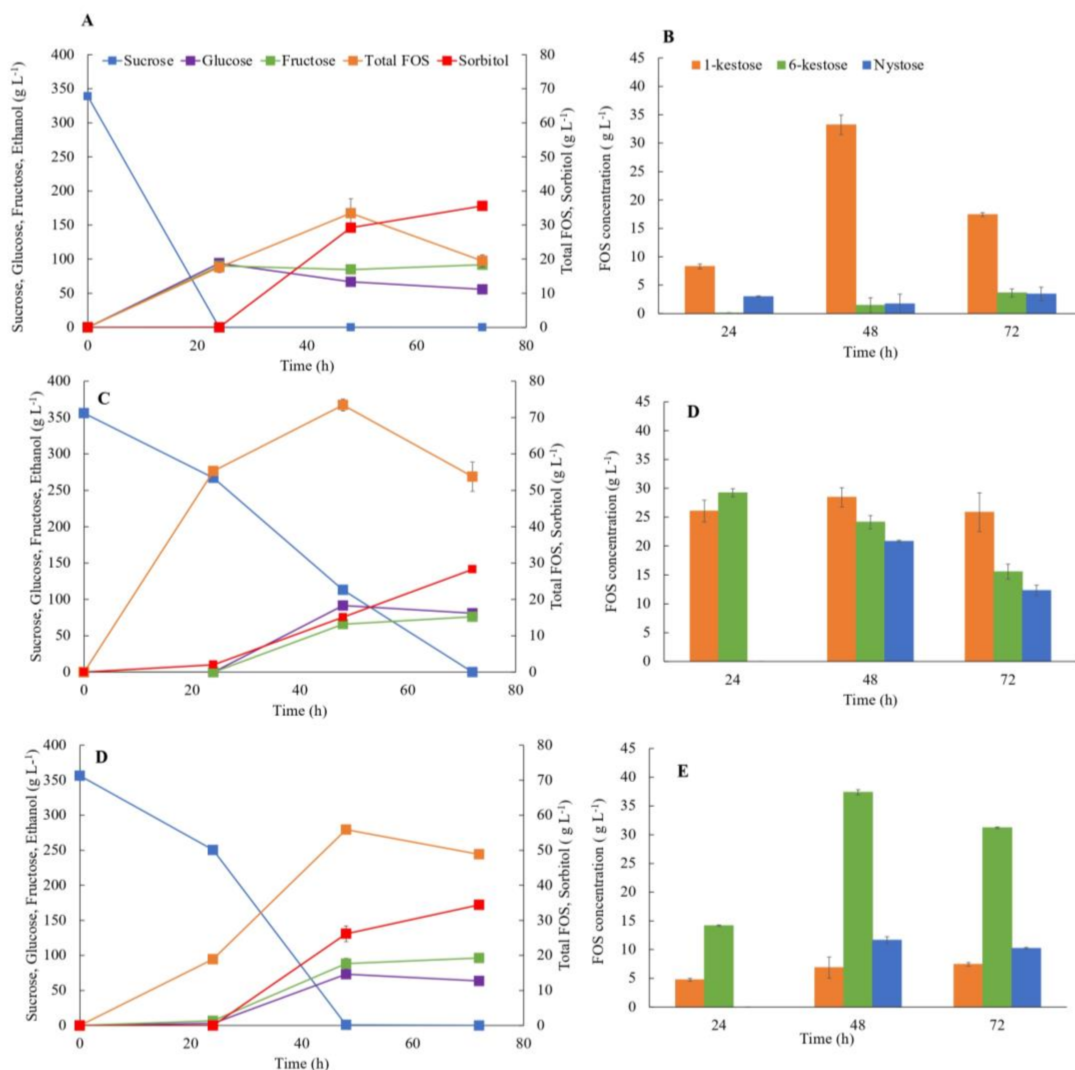

Figure 1-Time course of fructooligosaccharides (FOS), sorbitol, sucrose, glucose and fructose by *Zymomonas mobilis* ZM4 pB1 (A), *Z. mobilis* ZM4 pB1-*sacB* (C) and *Z. mobilis* ZM4 pB1-L196 (E) in shake flask using 350 g L^{-1} of sucrose as substrate. FOS production profile of *Z. mobilis* ZM4 pB1 (B), *Z. mobilis* ZM4 pB1-*sacB* (D) and *Z. mobilis* ZM4 pB1-L196 (E). The

values presented correspond to the average of three independent tests ± standard deviation.

It was possible to observe that, for all the strains, the maximum FOS concentration was obtained after 48 h of fermentation. In addition, it was shown that all strains were able to produce FOS. These results confirm the ability of *Z. mobilis* ZM4 to natively produce FOS using the one-step approach (33.5 ± 0.5 g L^{-1}) (**Figure 1A**). Comparing the performance of all the strains under study, the overexpression of *sacB* gene (*Z. mobilis* ZM4 pB1-*sacB* strain, **Figure 1C**) allowed a higher FOS titer (73.4 ± 1.6 g L^{-1}), with a productivity of 1.53 ± 0.03 g L^{-1} h^{-1} and a yield of 0.31 ± 0.03 g_{FOS} $g_{sucrose}^{-1}$. On the other hand, the heterologous expression of Ffase-Leu196 in *Z. mobilis* ZM4 (*Z. mobilis* ZM4 pB1-L196, **Figure 1D**) lead to a FOS titer of 55.9 ± 0.9 g L^{-1}, with a productivity of 1.16 ± 0.08 g L^{-1} h^{-1} and a yield of 0.16 ± 0.02 g_{FOS} $g_{sucrose}^{-1}$. The two mutant strains overexpressing the native *sacB* gene or the heterologous Ffase-Leu196 constructed allowed a significant increase ($p>0.05$), 2.2-fold and 1.7-fold increase, respectively, in the production of FOS when compared with the results obtained with the control strain. Regarding the composition of the FOS mixture obtained at 48 h, the main produced FOS are 1-kestose, 6-kestose and nystose. However, the mixtures produced by the different strains contained different ratios of FOS. For example, the control strain produced a FOS mixture with 93 ± 4 % of 1-kestose, 4 ± 0.5 % of 6-kestose and 5 ± 1 % of nystose (Figure 1B). On the other hand, *Z. mobilis* ZM4 pB1-*sacB* produced a mixture with 38 ± 2 % of 1-kestose, 32 ± 1 % of 6-kestose and 28 ± 0.1 % of nystose (Figure 1D). In this case, large amounts of 6-kestose and nystose were accumulated in addition to 1-kestose, confirming the effectiveness of overexpressing the *sacB* gene. However, a different FOS production profile was observed in the experiments performed with *Z. mobilis* ZM4 pB1-L196 strain. The FOS mixture contained 69 ± 2 % of 6-kestose, 13 ± 1 % of 1-kestose and 21 ± 1 % of nystose (Figure 1F). Herein it was also possible to observe that, the total FOS concentration started to decrease for all strains (Figure 1A, C, D) after 48 h of fermentation, showing that the FOS composition can be tailored by ending the process at specific time points.
In addition, *Z. mobilis* is also able to produce sorbitol and levan when grown on sucrose or on a mixture of fructose and glucose. Figures 1A, 1D and 1C shown that sorbitol was produced during the fermentation, attaining a maximum concentration of 35.62 ± 1.04 g L^{-1}, 34.44 ± 1.41 g L^{-1} and 28.25 ± 0.32 g L^{-1}, for the experiments with *Z. mobilis* ZM4 pB1, *Z. mobilis* ZM4 pB1-L196 and *Z. mobilis* ZM4 pB1-*sacB*, respectively, after 72 h of fermentation. At the end of the fermentation, 5.09 ± 0.69 g L^{-1}, 6.59 ± 0.56 g L^{-1} and 6.93 ± 0.16 g L^{-1} of levan were produced in the experiments with the strains *Z. mobilis* ZM4 pB1, *Z. mobilis* ZM4 pB1-L196 and *Z. mobilis* ZM4 pB1-*sacB*, respectively.

Conclusion

This study demonstrated an efficient system to produce FOS by one-step approach from sucrose using two recombinant *Z. mobilis* ZM4 strains. It was demonstrated that the mutant strains constructed in this work allowed to control the relative FOS composition, originating FOS mixtures with high concentrations of 6-kestose. The best results were

obtained with *Z. mobilis* ZM4 pB1-sacB (73.4 ± 1.6 g L^{-1} of FOS). In addition, the prebiotic potential of the mixtures here produced was enriched due to the presence of the non-digestible sugar alcohol sorbitol and levan. For this reason, this work opens a new route to produce tailor-made FOS mixtures with potentially different bioactivities.

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