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A direct and sensitive method for screening fructooligosaccharides-digesting microorganisms useful in food and health science

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Short-chain fructooligosaccharides (FOS) stimulate selectively the growth and activity of microorganisms in the colon providing positive health effects and well-being in humans and animals. The lack of accurate isolation methods, however, hampers the possibility of getting new potential fructo-oligosaccharides (FOS)-fermenting yeast or bacterial strains. A valuable screening procedure to visually detect bacterial and yeast strains able to ferment FOS in liquid or solid rich media supplemented with an innocuous pH indicator is described. Using this assay, 15 FOS-consuming strains isolated from different sources were successfully evaluated to prove the utility of the method. This screening procedure is a new and valuable tool in rapid large-scale detection of potential FOS fermenting-strains useful in food and health science.

Key words: FOS-fermenting microorganisms, prebiotics, probiotics, symbiotics.

INTRODUCTION

The selection of bacterial or yeast strains able to efficiently ferment short chains-carbohydrates with prebiotic properties for example, fructooligosaccharides (FOS), is a subject of permanent interest. However, the lack of direct screening methods hampers the rapid identification of such important microorganisms. The ability to ferment short chain oligosaccharides is a key property for any bacterial or yeast strain to provide desirable clinical effects (Kaplan and Hutkins, 2000). Main targets for FOS consumption in the colon are Bifidobacteria and Lactobacilli as these intestinal bacteria have shown several positive effects upon human or animals' well-being (Kaplan and Hutkins, 2003).

According to that explained above, if certain carbo-hydrates, such as fructooligosaccharides, are fermented by only specific beneficial microbial strains, then a liquid or solid growth medium containing these "prebiotic" substrates as unique carbon sources could efficiently and directly select for those useful FOS-fermenters microbial strains.

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Abbreviations: FOS, Short-chain fructooligosaccharides; **TLC,** thin layer chromatography; **HPLC,** high pressure liquid chromatography; **YNB,** yeast nitrogen base.

In previous research the ability to ferment FOS of some Bifido and Lactic acid bacterial strains was assayed (Kaplan and Hutkins, 2000). However, two main drawbacks still to date make it difficult to establish an accurate screening procedure based on FOS consumption. Firstly, FOS commercial preparations may have different polymerization degrees and/or contain contaminants like glucose, fructose, sucrose, or other fermentable sugars being rather difficult to establish if microbial growth in FOS-containing medium is really due to FOS metabolism.

This fact makes necessary the use of additional confirmatory analytical techniques such as thin layer chromatography (TLC) or High Pressure Liquid Chromatography (HPLC). Secondly, the selection of FOS-fermenter strains by using continuous culture (Sghir et al., 1998) or rich media such as De Man, Rogosa and Sharpe (MRS), to evaluate FOS consumption by bacteria or yeast undoubtedly could mask the final results. To overcome this problem, instead of a rich media like MRS or any similar, the use of minimal medium might be the best choice to evaluate FOS fermentation, but another problem arises.

Contrary to yeasts*, Lactobacillus* and *Bifidobacterium* strains for example, need elevated nutritional requirements in the media, and their growth in minimal medium is almost null. Following this line we developed a new feasible and simple pH shift-based procedure for rapid screening of pure cultures of FOS-fermenting yeast and bacterial strains growing in rich media. As the principle, FOS fermentation by bacteria and yeast provokes acidification of the culture medium (pH below 6) due to production of organic acids. By contrast, microbes unable to metabolize FOS, for example enteric bacteria like *Escherichia coli* or *Salmonella* sp., use the nitrogen-containing components in yeast extract or peptone causing alkalinization (pH above 7) due to ammonia release. The slightly pH switches from acidic to basic conditions can be detected by the addition of the innocuous pH indicator Bromthymol blue (transition interval pH 6.0 to 7.6; yellow-blue, Sigma Co., USA). At this stage, a simple visual inspection is enough to detect color change in liquid or solid media allowing a rapid and accurate screening and detection for those microorganisms able to metabolize FOS.

MATERIALS AND METHODS

Microorganisms

Bifidobacterium bifidum 15696, *B. dentium* 27678 were obtained from ATCC collection and *E. coli* TOP10F*, Pichia pastoris* GS115*, P. pastoris* X33 were purchased from Invitrogen SA. Other bacterial and yeast strains such as: *Saccharomyces boulardii* L/25/4/96*, Saccharomyces cerevisiae* L/25-7-82*, S. cerevisiae* L/25-7-76*, Kluyveromyces fragilis* L/12-8-1 *K. fragilis* L/12-8-6, *Lactobacillus acidophilus* B/103-5, *Lactobacillus rhamnosus* B/103-1-5, *Lactobacillus reuteri B/108-1*, *Lactobacillus fermentum* B/103-11-3, *Lactobacillus casei* B/103-11-6, *Lactobacillus paracasei* B/103-11-7, *Lactobacillus bulgaricus* B/103-12-6, *Streptococcus thermophilus* B/103-12-7*,* were isolated from different sources and belongs to the microorganism collection of the Cuban Research Institute for Sugarcane Derivates (ICIDCA) Havana, Cuba.

Culture media

The commonly used minimal Yeast Nitrogen base (YNB) and rich media YP (Yeast extract and Peptone) and LB (Luria Bertani) for yeast and bacterial grow, respectively, were prepared according to the *Pichia* expression vectors for constitutive expression and purification of recombinant proteins Catalog nos., V200-20 and V205-20, Invitrogen SA (USA). Minimal M9 media was purchased from Sigma Co., (USA) catalog number: M6030 and prepared according to the manufactures instructions. Solid media was prepared similar that liquid media but including 20 g of agar per liter. For the pH shift assays using any of the solid or liquid media mentioned above, 0.0025% of Bromothymol blue (transition interval pH 6.0 to 7.6: yellow-blue, Sigma Co., USA Catalog number: 34656), filter-sterilized solutions of glucose, fructose and sucrose (BDH) added at 2% final concentration or FOS solution obtained in this work 2 and 3% final concentration were incorporated to the adequate culture media according to the different experiments requirements. Final pH in the different media was adjusted to 6.5.

Microbial growth under anaerobic conditions

COY Chambers (COY Laboratory Products Inc.) were used to create and maintain anaerobic conditions as needed during growth of some of the microorganisms mentioned above in the different experiments.

FOS mixture composition

The initial FOS mixture was composed by: 4.6% nystose (GF3), 56% 1-kestose (GF2), 21% sucrose (GF), 17% glucose (G), and 1.4% fructose (F). This syrup was obtained as described in the European patent application EP 2 899 282 A1 (29-07-2015) with some modifications. Then, the mixture was further submitted to HPLC separation as described below to get an enriched FOS solution composed mainly by 1-kestose and nystose.

Sugars separation by high performance liquid chromatogrphy (HPLC)

Sugar composition in the enriched FOS solution were separated in an Aminex HPX-42C column (0.78 by 30 cm; Bio-Rad Laboratories, Hercules, Calif.) and detected with a RI-410 (Waters) detector. The column temperature during the analysis was kept constant at 85°C, and water was used as the mobile phase at 0.6 ml/min.

RESULTS

Unlike yeasts, some lactobacilli and bifido bacterial strains were not able to grow in minimal media supplemented with FOS. Previously, syrup composed of 4.6% nystose (GF3), 56% 1-kestose (GF2), 21% sucrose (GF), 17% glucose (G), and 1.4% fructose (F) was produced during sucrose transformation by recombinant *P. pastoris* cells entrapped in Ca-alginate beads. After sugars separation, an enriched FOS solution containing

1-kestose (96%), nystose (3%) and sucrose (1%) was obtained, as judged by HPLC analysis (Figure 1A). To evaluate microbial FOS fermentation, the use of minimal media appears to be ideal because of the lack of carbohydrates or proteins as alternative energy sources for cell growth. On this basis, the enriched filter-sterilized FOS solution was incorporated to a final concentration of 2 and 3% into solid or liquid minimal Yeast Nitrogen Base (YNB, Invitrogen Co., USA) or minimal M9 (Sigma Co., USA) medium supplemented with 0.05% L-cysteine. Two yeasts (*S. boulardii* and *K. fragilis*) and 2 bacterial strains (*B. bifidum* and *B. dentium*) were streaked on YNB-FOS or M9-FOS plates and incubated 48 h at 30 or 37°C, respectively, under anaerobic conditions. The assayed yeasts were able to grow in solid or liquid YNB-FOS demonstrating that FOS was equally as good substrate as glucose (BDH), fructose (BDH) and sucrose (BDH) in supporting growth (Figure 1B). Additionally, HPLC analysis confirmed the complete depletion of the FOS fraction after microbial growth (Figure 1C). Unlike yeasts, the two *Bifidobacteria* and two additional FOS-consumers *Lactobacillus* strains used as controls, failed to grow in solid or liquid M9-FOS (results not shown). Likely, their limited growth on minimal media may not guarantee the initial production of enzymes for FOS transport inside the cell and further FOS hydrolysis.

Lactobacilli and Bifidobacteria, organisms that are generally considered to be desirable members of the colonic microbiota, release mainly lactic and acetic acids during FOS catabolism under anaerobic conditions (Collins and Gibson, 1999; Kaplan and Hutkins, 2000; 2003) so, provoking acidification of the culture medium reaching pH values below 5. As shown above, these bacteria are unable to grow in minimal media due to their high nutritional requirements. These characteristics prompted us to test whether the addition of an adequate pH indicator as Bromothymol blue (transition interval pH 6.0 to 7.6: yellow-blue) to the LB media supplemented with 2% FOS could produce visible changes in the medium depending on the bacterium ability to FOS consumption. As shown in Table 1 or Figures 2A and B, the growth of 10 of the screened bacterial strains, in solid and liquid LB media turned the medium color from initial green (pH 6.5) to yellow indicating acidification, pH below 6, due to FOS consumption (Table 1). On the other hand, when FOS was not added, the medium color turned to blue revealing alkalinization due to ammonia release from utilization of the nitrogen-containing carbon sources in yeast extract and peptone so, pH values raised up from initial 6.5 to 8 (Table 1). The same results were obtained when five yeast strains were assayed (Figures 3A and B). Additionally, the use of YNB-FOS minimal medium supplemented with Bromothimol blue revealed also, by simple visual inspection, the capacity to consume these short chain carbohydrates by these yeasts (results not shown). As expected, the enteric bacterium *E. coli* and the methylotrophic yeast *P. pastoris,* which are unable to use FOS, turned the medium color to blue raising the pH values over 8 (Table 1) due to also utilization of the nitrogen-containing carbon sources in yeast extract and peptone.

FOS depletion was further verified by HPLC analysis demonstrating that the assayed bacteria and yeasts consumed totally the GF2 (1-kestose) and GF3 (nystose) fractions (results not shown). In addition, no accumulation of other monosaccharide was seen which suggest that they were also degraded by the microorganisms. These results agree with previous reports dealing with efficient consumption of GF2, GF3 together with mono and disaccharide fractions (Saulnier et al., 2007).

DISCUSSION

The intestinal flora is part of a complex ecosystem and many of its beneficial constituents remain unidentified despite there is strong evidence about their influences over the immune system of both, human and animals (Kohler et al., 2003; Patterson, 2005). FOS stimulates selectively the growth and activity of this beneficial ecosystem in the colon providing positive health effects and well-being in humans and animals. However, there are not reports regarding to rapid and accurate qualitative detection methods to get new potential fructooligosaccharides-fermenting yeast or bacterial strains based on consumption of these short chain carbohydrates widely used as prebiotics (Roberfroid, 1998; Tokunaga, 2004). During evaluation of microbial growth in FOS containing media, the use of FOS commercial preparations with different polymerization degrees, together with the presence of background levels of other carbohydrates and the use of rich media have been two of the major drawbacks that undoubtedly mask final results. So, it is not possible that, the implementation of an accurate screening procedure based on FOS consumption under these circumstances. Several bacterial strains, previously identified as FOS non-fermenters, grew in MRS-FOS broth (Kaplan and Hutkins, 2000), likely due to the catabolic oxidation of the yeast extract and peptone media constituents instead of FOS as carbon source.

In this research a direct qualitative screening method that overcomes these two problems is proposed. Firstly, the enriched 96% FOS preparation composed mainly by GF2 (1-kestose) and GF3 (nystose) is useful as a carbon source since guarantee that microbial growth is mainly due to FOS consumption as unique carbon source. Additionally, this FOS preparation has further commercial application as a food or feed additive to stimulate several potential probiotics strains in the gut or in new symbiotic designs.

Secondly, the addition of an innocuous pH indicator like Bromothymol blue (transition interval pH 6.0 to 7.6: yellow-blue) to any of the used media supplemented with2% FOS produced rapid and visible changes in the

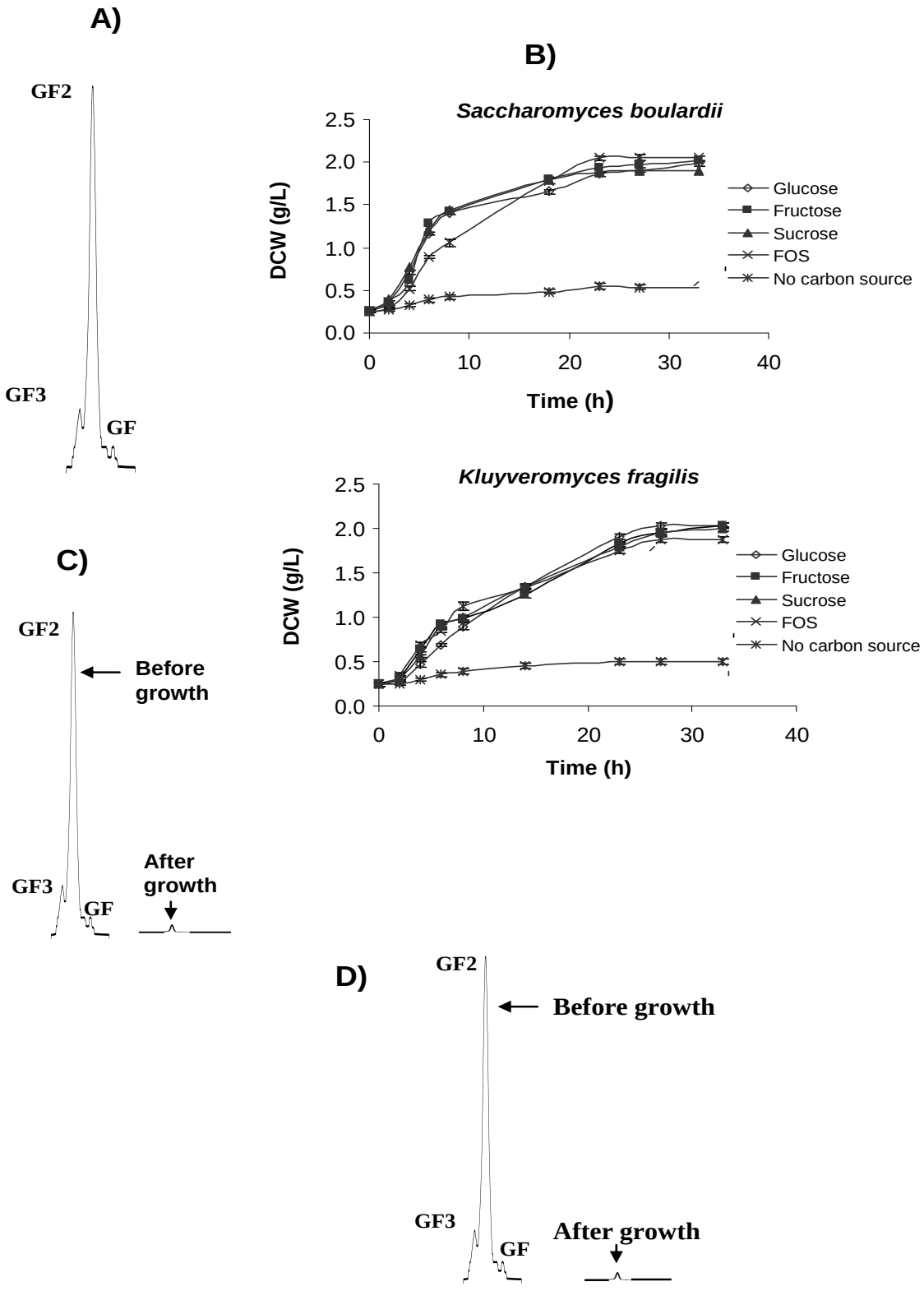

Figure 1. FOS utilization by two yeasts currently used as probiotic. **A)** HPLC profile of FOS solution (96% purity) used in this and subsequent experiments. Letters represent nystose (GF3), 1-kestose (GF2), and remaining sucrose (GF). **B)** Growth in liquid minimal YNB medium supplemented with 2% sucrose, 2% fructose, 2% glucose, or 2% FOS. Culture growth was followed by measuring dry cell weight (DCW) at different time points during 35 h. **C)** HPLC profile of culture media samples containing 2% FOS before and after microbial growth. Letters represent: GF3: nystose, GF2: 1-kestose, GF: sucrose.

Table 1. pH values reached after growth in FOS-LB or YP rich media of bacterial and yeast strains screened with this method able or not to metabolize FOS as unique carbon source.

Microorganisms	Source	pH FOS(+)	FOS(-)
Saccharomyces boulardii L/25/4/96	Commercial preparation	3.44 ±0.02	8.02 ±0.03
Saccharomyces cerevisiae L/25-7-82	Honey	3.35 ±0.06	8.22 ±0.02
Saccharomyces cerevisiae L/25-7-76	Honey	3.30 ±0.04	8.01 ±0.02
Kluyveromyces fragilis L/12-8-1	Sugarcane industrial process	3.42 ±0.06	8.06 ±0.05
Kluyveromyces fragilis L/12-8-6	Sugarcane industrial process	3.40 ±0.03	8.03 ±0.04
Lactobacillus acidophilus B/103-5	yogurt	4.14 ±0.06	8.31 ±0.02
Lactobacillus rhamnosus B/103-1-5	pigs faecal blend	4.45 ±0.04	8.33 ±0.01
Lactobacillus reuteri B/108-1	human faecal blend	4.09 ±0.02	8.03 ±0.04
Lactobacillus fermentum B/103-11-3	human faecal blend	4.01 ±0.05	7.98 ±0.03
Lactobacillus casei B/103-11-6	human faecal blend	4.51 ±0.02	8.13 ±0.02
Lactobacillus paracasei B/103-11-7	Cheese process	4.48 ±0.04	8.32 ±0.06
Lactobacillus bulgaricus B/103-12-6	Cheese process	5.19 ±0.01	7.94 ±0.05
Streptococcus thermophilus B/103-12-7	Cheese process	5.31 ±0.03	8.03 ±0.04
Bifidobacterium bifidum 15696	ATCC	4.35 ±0.04	8.06 ±0.06
Bifidobacterium dentium 27678	ATCC	4.32 ±0.02	8.04 ±0.03
Escherichia coli TOP10F	Invitrogen SA	8.52 ±0.02	8.31 ±0.04
Pichia pastoris GS115	Invitrogen SA	8.44 ±0.01	8.43 ±0.03
Pichia pastoris X33	Invitrogen SA	8.46 ±0.04	8.42 ±0.05

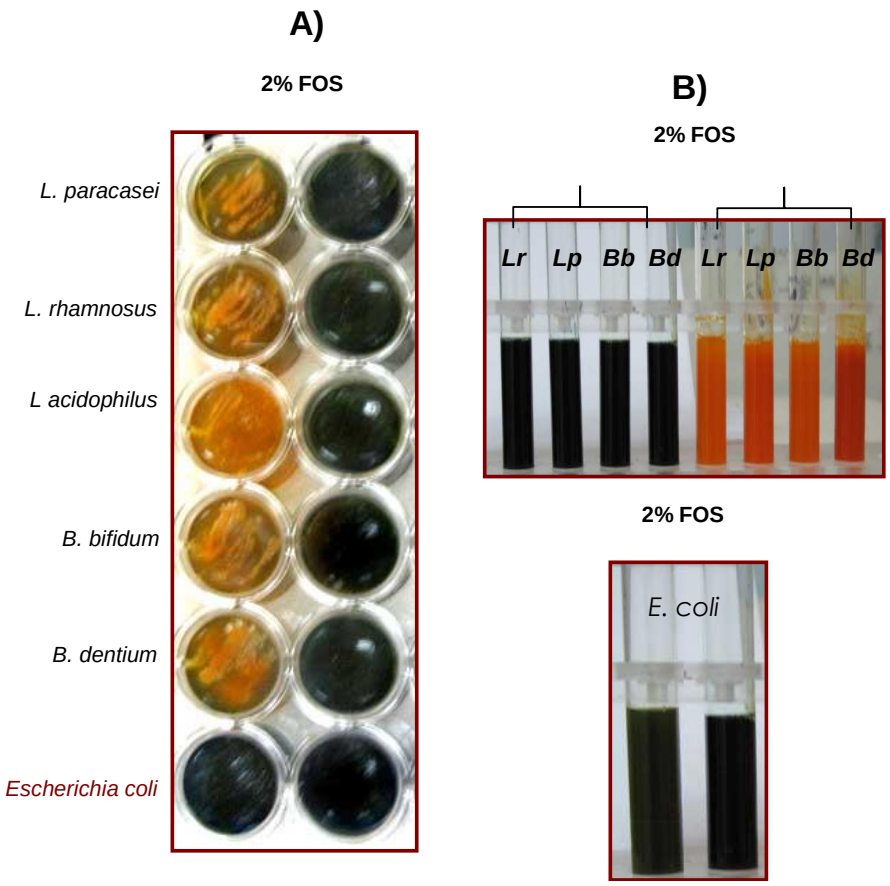

Figure 2. Growth of five of the assayed bacteria: *L. paracasei (Lp), rhamnosus (Lr), acidophilus (La), B. bifidum (Bb) and dentium* (*Bd*) under anaerobic conditions at 37°C in a covered 12 wells plates with solid (A) and liquid (B) LB medium supplemented or not with 2% FOS and 0.025% (final concentration) of the pH indicator Bromothymol blue and initial pH 6.5. The FOS non-consumer enteric bacterium *Escherichia coli* were used as negative control.

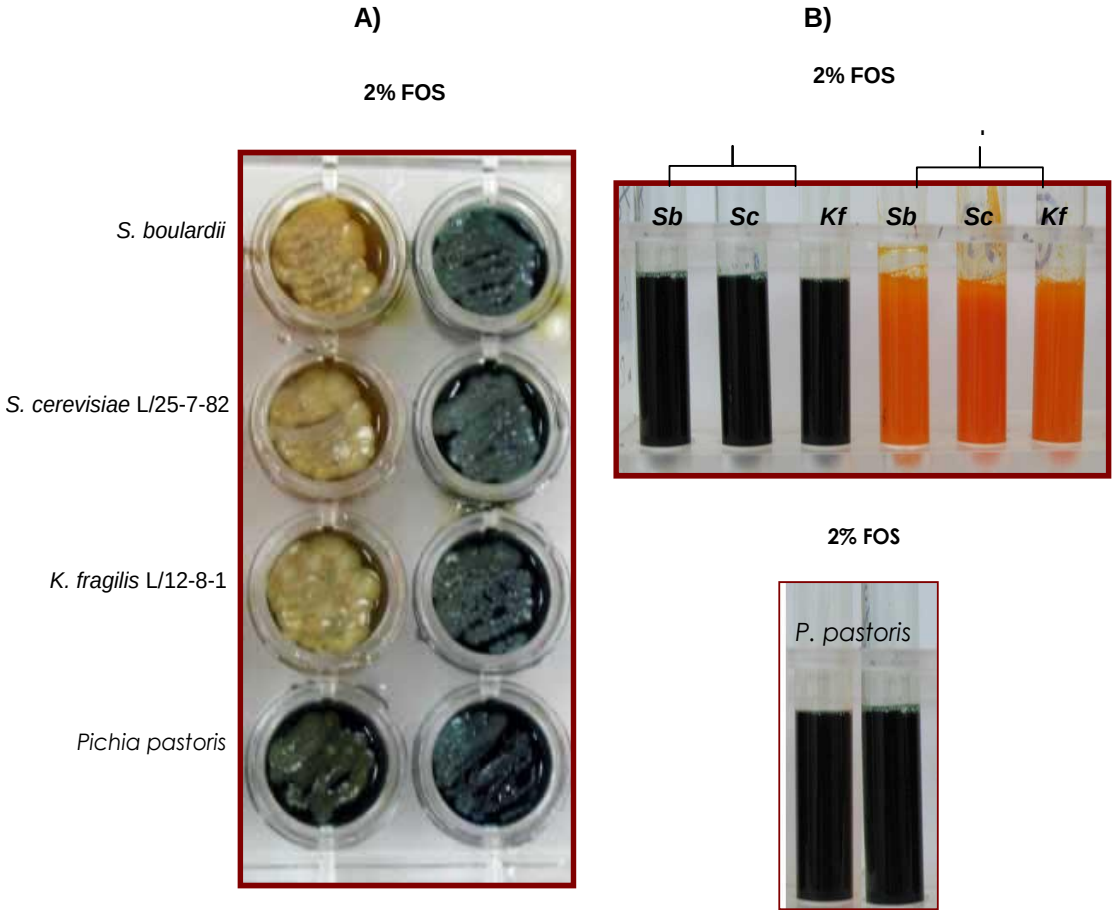

Figure 3. Growth of three of the assayed yeasts: *Saccharomyces cerevisiae* (*Sc*), *S. boulardi*i (*Sb*) and *Kluyveromyces fragilis* (*Kf*) at 30°C under anaerobic conditions in a 12 wells plates with solid (A) and liquid (B) YP medium supplemented or not with 2% FOS and 0.025% (final concentration) of the pH indicator Bromothymol blue. Initial pH medium 6.5. The FOS non-consumer yeast *Pichia pastoris* was used as negative control.

medium color depending on the bacterium or yeast ability to consume or not FOS as unique carbon source. Fifteen of the assayed strains were able to switch to yellow the media color due to FOS consumption. According to the results described above, the proposed screening method is a valuable tool in large-scale detection of pure cultures of potential FOS fermenters-yeast or bacterial strains.

Conflict of interests

The author(s) did not declare any conflict of interest.

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