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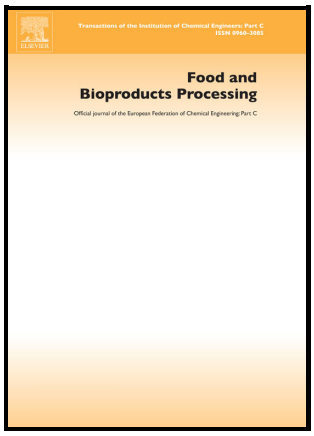

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Upcycling of carrot discards into prebiotics (fructooligosaccharides) as high value food ingredients

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Abstract

A great amount of carrots is discarded daily in the word because they do not meet the quality standards imposed by consumer market. The aim of this work was to evaluate the feasibility of using carrot discards as substrate of solid-state fermentation to produce β-fructofuranosidase by *Aspergillus niger* and its subsequent use to catalyze the synthesis of

fructooligosaccharides (prebiotics) using carrot juice. Results indicated that carrot bagasse has adequate nutrients for the growth of *A. niger*, but supplementation with a source of nitrogen is required to obtain a high production of β-fructofuranosidase (90.82 U/mL). Maximum specific activity (251 U/mg) was obtained after 3 days of fermentation at 30 ºC, using an inoculum of 10^6 conidia/ mL. β-fructofuranosidase was successfully used to synthesize fructooligosaccharides using carrot juice as substrate, raising the possibility for future development of a natural functional beverage.

Keywords: carrot discards, β -fructofuranosidase, prebiotics, fructooligosaccharides

1. Introduction

β-fructofuranosidase or invertase (FFase, EC 3.2.1.26) is an enzyme that catalyzes the hydrolysis of β-1,4-glycosidic bonds into simpler carbohydrates as glucose and fructose, (Ire et al., 2018). This enzyme is very important in the production of invert sugar (equimolar mixture of glucose and fructose), a popular sweetener used in jams and candies because it does not crystallize easily (Ohara et al., 2015). β-fructofuranosidase can also be used for production of cattle feed, honeybees food or in bakery products because it provides fructose or glucose for CO_2 production in fermentation processes (Mase et al., 2009) FFase can be found in plants, animal, bacteria, yeasts, and filamentous fungi. Among filamentous fungi, *Aspergillus genus* has been extensively reported as producer of FFase under submerged and solid-state fermentation (Carvalho et al., 2019). *Aspergillus niger* can produce β-fructofuranosidase in the presence of β-fructofuranoside sugars, demonstrating that the synthesis of FFase is inducible (Rubio and Navarro, 2006).

Industrially FFase is produced by submerged fermentation (SmF), however solid state fermentation (SSF) is advantageous for filamentous fungi which are more difficult to operate in SmF than bacteria and yeasts (Wang and Yang, 2007). SSF also provides an environment similar to the natural habitat where fungi are better adapted to grow, since mycelium can grow and expand better in a solid matrix (Ocano and Postigo et al., 2021). Additionally, it requires minimal pretreatment of the raw material, generates less wastewater and the products are obtained at higher concentration (Meini et al., 2021). The solid phase used in SSF systems can be natural or inert material and both can act as a physical structure for the growth of microorganisms. However natural ones also provide a carbon and nitrogen source, and growth factors for the microorganisms (Krishna, 2005). The components of the culture medium used in SSF for enzyme production, represent around 30–40% of the production cost (De la Rosa et al., 2019). Therefore, the use of agricultural waste as substrate of fermentation is an interesting alternative since they are mainly composed of complex carbohydrates and crude proteins. The bibliography reports different agro–industrials wastes for FFase production by fungi in SSF such as banana peels, orange peels, sugarcane bagasse, molasses, wheat bran, apple pomace, rye flour (Manoochehri et al., 2020; Ire et al., 2018; Veana and Aguilar, 2014; Raju et al., 2016). The transformation of these agro-industrial into high-value products will contribute to their circular economy.

In Argentina a big number of carrots are discarded every year due they do not meet the quality standards and the requirements of size and shape imposed by the consumer market. The estimated discard volume is 80–100 t/d during the harvest time (June to December) (Aimaretti et al., 2013). This problem is repeated in other production areas of America and Europe. Carrot discards have the same optimal maturity, freshness level and nutritional

characteristics than packaged carrots. These properties allow its use as a raw material in several process to obtain bioethanol, pectins, fibers, carotenes, lactic acid (Clementz et al., 2019; Salvañal et al., 2021; Clementz et al., 2015).

At high concentration of sucrose, FFase can transfer a fructosyl group to a sucrose molecule acceptor, allowing its use in the synthesis of fructooligosaccharides (FOS), prebiotics that affect many human physiological functions, promoting positive impact to health (Huang et al., 2016; Mohanti, 2018; Alvarez et al., 2020). FOS are composed by units of fructose linked by β-(2-1) bonds with a degree of polymerization between 2 and 9 and a D-glucosyl unit at the non-reducing end (Borromei et al., 2011). These compounds are present naturally in low concentration in many fruits and vegetables, however commercially FOS are synthesized by transfructosylation process from sucrose using enzymes with high fructosyltransferase activity such as FFase (Flores-maltos et al., 2014). The aim of the present work was to evaluate the production of β- fructofuranosidase from carrot bagasse by solid state fermentation using *A. niger* and its subsequent use as a FOS-producing enzyme in carrot juice. These results will allow evaluating the future production of a natural beverage with FOS from carrot discards.

2. Materials and methods

2.1.Raw material

Carrots discards (*Daucus carota*) were provided by a packing shed located in Santa Fe, Argentina. Carrots were processed raw in a domestic juicer to obtain juice and bagasse separately. Both by-products were stored frozen at -4 °C until their use.

2.2.Microorganism

FFase production was carried out by solid state fermentation (SSF) by *Aspergillus niger* NRRL3, provided by The National Center for Agricultural Utilization Research (ARS), USDA, USA. It was propagated on potato dextrose agar (Britania, CABA, Argentina) at 30 °C for 96 h. Fungal spores were collected with sterile 20% glycerol and kept at -4 °C. The concentration of spores in the inoculum was determined by Neubauer counting chamber.

2.3.Carrot bagasse and juice composition

Proximal composition of bagasse and carrot juice was determined using standard methods of Association of Official Analytical Chemistry (AOAC, 2000). The nitrogen content was determined by the Kjeldahl method (AOAC 920.87). Protein content was estimated as the nitrogen content multiplied by 6.25. Total fat content and dietary fiber were determined employing AOAC 920.85 and AOAC 973.18, respectively. Moisture and ashes were carried out according to AOAC 925.10 and 923.03 standards, respectively. Carbohydrates were determined by subtraction of the sums of the protein content, total fat, moisture, and ash presents in the sample.

2.3.1. Sugar content in carrot juice

Determination of sugar content in carrots was performed by high performance liquid chromatography coupled with refractive index detector (HPLC-IR) using an Aminex HPX-87H column, 300mm x 7.8mm (packed with a matrix based on co-polymers, poly-(styrene-divinylbenzene sulfonated) at 8% cross-linking, ionically hydrogen, 9 µm particle size and pH range1-3). The chromatographic run was performed at 0.6 mL/min with a temperature of 45°C in the column compartment and 25°C in the autosampler. The isocratic mobile phase consisted of 5 mM H_2SO_4 in MiliQ H_2O (pH 2). The pressure was maintained at 50

bar for the duration of the test. Sugar standards (glucose, fructose, sucrose) were dissolved in MiliQ water and diluted to perform the calibration curve on HPLC. Each standard was injected separately, to register the retention time. For the analysis of the sugar content (glucose, sucrose and fructose) in samples, they were centrifuged (spin down for 5 min at 14,000 rpm), diluted 1/10 with MiliQ water and filtered through 0.22 μm Millipore Durapore membranes (Billerica, MA, USA), they were injected into the HPLC-IR system. Quantification was obtained by peak integration in comparison with standards.

2.4.Fermentations conditions

Solid state fermentation was carried out in 250 mL flask containing 13 g of carrot bagasse. This amount allows to obtain a homogeneous fermentation system and completely covered by substrate. After sterilization at 120 ºC during 20 min, systems were inoculated with 0.1 ml of variable concentration (conidia/ mL) of *A. niger* and incubated at 30 ºC. A control performed using 90 g/L sucrose solution and polypropylene sponge as solid support was incubated under the same conditions. After fermentation, the content was filtered through cloth fabric filter to get the crude enzyme extract that was then centrifuged at 10,000 rpm for 10 min and filtered with a 0.45 μm nylon syringe filter.

2.4.1. Supplementation of carrot bagasse

Two media for supplementation of carrot bagasse were used: i) media enriched in nitrogen (MN) (Ashokkumar et al., 2001) and ii) MN with traces solution (MNtz). MN consist of: $(NH_4)_2SO_4$ 10 g/L; KH_2PO_4 40 g/L; $FeSO_4$ 0,05 g/L; $MgSO_4.7H_2O$ 2 g/L; yeast extract 10 g/L; urea 10 g/L. MNtz consist of MN with 1 μL/mL of trace solution ($FeSO_4 \cdot 7H_2O$, 1,0 g/L, EDTA 10,0 g/L, $ZnSO_4 \cdot 7H_2O$ 4,4 g/L, H_3BO_3 2,2 g/L, $MnCl_2 \cdot 4H_2O$ 1,0 g/L, $CoCl_2$

·$6H_2O$ 0,32 g/L, $CuSO_4$ ·$5H_2O$ 0,32 g/L, $(NH_4)6Mo_7O_2$ ·4 H2O 0,22 g/L) (Hill and Kafer, 2001). Carrot bagasse with the addition of 5 mL of sterile water was used as a control. After fermentation, the crude enzyme extract was obtained following the steps descripted in Section 2.4, and FFase activity was determined as explained in Section 2.11.

2.5.Optimization of fermentation parameters

2.5.1. Effect of added volume of MNtz

The effect of adding different volumes of MNtz (1, 3, 5, 7 and 9 mL), was evaluated in systems containing 13 g of carrot bagasse. Systems were inoculated with 0.1 ml of $1x10^8$ conidia/mL of *A. niger* and incubated at 30 ºC for 4 days. The crude enzyme extract was obtained following the steps descripted in Section 2.4 and FFase activity was determined as explained in Section 2.11.

2.5.2. Effect of inoculum size

The effect of inoculum size was evaluated in systems containing 13 g of carrot bagasse supplemented with 5 mL of MNtz and inoculated with 0.1ml of different inoculum size (10^5 – 10^8 conidia/mL of *A. niger*). Systems were incubated at 30 °C for 4 days. After fermentation the content was filtered and centrifuged to obtain crude enzyme extract (Section 2.4). FFase activity was determined as described in section 2.11.

2.5.3. Effect of incubation time

The effect of incubation time on FFase activity was evaluated at 24-hour intervals for 7 days. Systems containing 13 g of bagasse were supplemented with 5 mL of MNtz and inoculated with 0.1 ml of *A. niger* $1x10^6$ conidia/mL. After incubation at 30 °C, the content

was filtered and centrifuged to obtain crude enzyme extract (Section 2.4). FFase activity was determined as described in section 2.11.

2.6.Determination of total proteins

Total proteins were determined using the Bradford method (Bradford, 1976) adapted for microassay, which is based on the reaction of aromatic amino acids with the Coomasie G-250 dye, generating a bluish complex that can be measured at 595 nm. Assays were performed in duplicate in microplates, using bovine serum albumin as a standard. For the colorimetric reaction, 200 µL of Bradford's reagent (Sigma) and 20 µL of sample were placed in each well, and the reaction was carried out for 5 minutes shaking gently. Absorbance was determined at 595 nm.

2.7.Analysis by SDS-PAGE and nLC-MS

The secretome of *A. niger* was analyzed by mass spectrometry technique. In order to concentrate all the proteins in a single band for further analysis, the enzyme extract was run on an SDS-PAGE electrophoresis gel up to 1 cm in the resolving gel, using 10% stacking gel and 13% resolving gel. The gel was stained with colloidal Coomassie Brillant Blue according to the procedure by Neuhoff et al. (1988). The band containing all the proteins of the optimized enzymatic extract was cut off the SDS-PAGE gel and analyzed by nano liquid chromatography-mass spectrometry (Nano LC-MS/MS). The analysis was performed at Centro de Estudios Químicos y Biológicos por Espectrometría de Masa (CEQUIBIEM), Universidad de Buenos Aires - CONICET, Buenos Aires, Argentina.

Samples were reduced with 20 mM DTT for 45 min and alkylated with 50 mM Iodoacetamide for 45 min in the dark. Then, the samples were digested with trypsin at 37

°C overnight. Extraction of peptides was performed with a gradient of two solutions: Water with 0.1% formic acid and acetonitrile with 0.1 mL formic acid. The sample was lyophilized by Speed Vac and resuspended with 10 µL of 0.1% Formic Acid. A desalting was carried out passing through zip Tip C18 (Merck). The samples were then analyzed by nano-LC–MS/MS using an EASY-nLC 1000 system (Thermo Scientific) coupled to an LTQ Orbitrap XL mass spectrometer (Thermo Scientific, Bremen, Germany). The ionization of the samples was carried out by electrospray (ThermoScientific, model EASY-SPRAY). The LC-MS spectra was analyzed by Proteome Discoverer 1.4 software, using genome of *A. niger* UP000006706 as database to identify the proteins in the sample. A high confidence filter for peptide identification was used.

2.8.SDS-PAGE – Zymography

Zymogram and SDS- PAGE under non-denaturing conditions (without β-mercaptoethanol) were performed as complementary analysis to evaluate the presence of FFase in the crude enzyme extract. Zymogram was achieved following polyacrylamide gel electrophoresis using 4.5% stacking gel and 10% resolving gel. The samples were seeded in duplicate and, at the end of the run, the gel was cut in half, obtaining two identical gels that were revealed by different methods. Zymogram was performed following the methodology described by Gabriel and Wang (1969), replacing the 2,3,5-triphenyltetrazolium chloride reagent with dinitrosalicylic acid (DNS). For this, one of the gels was washed in water three times and incubated in 1% X-100 triton solution for 10 min. Then, it was incubated in a 68 g/L sucrose solution in sodium acetate buffer pH=4.5 at 37 ºC for 10 min and washed with distilled water twice. Development was completed by incubation of the gel with DNS solution at 100 °C for 5 min. As a result of the reaction of DNS, bands with FFase activity

turned dark red. The remaining gel was stained with coomasie blue R-250 to reveal all protein bands by shaking the gel in the solution for 30 min. Then, it was destained with distilled water at 100 ºC. To determine molecular weight of the bands, the digitized images of the gels were analyzed using Gel analyzer 19.1 software. A calibration curve of molecular weights (MW) was made using molecular weight markers (GE Healthcare Bio-Sciences Amersham Low Molecular Weight Calibration Kit for SDS Electrophoresis), and then it was extrapolated to calculate the MW of bands of interest.

2.9.FOS production using carrot juice as substrates

For transfructosylation reaction the crude enzymatic extract was concentrated by ultrafiltration with 10 KDa cut-off membranes (Milipore) at 7000 rpm for 30 min at 15 ºC. Carrot juice was concentrated by controlled heating until final concentration of 35 °brix. Transfructosylation reaction was carried out following the conditions reported by Vega-Paulino and Zúniga-hansen (2012). Systems containing 8 mL of carrot juice and 2% of concentrated crude enzyme extract were incubated at 50 °C, 150 rpm for 3 h. Samples were taken every 1 h, stopping the reaction by heat shock at 100 °C.

2.10. High performance thin layer chromatography

FOS and sugars obtained after transfructosylation were qualitative determinate by high performance thin layer chromatography (HPTLC) using the CAMAG system (Cheong et al., 2014; Kaur et al., 2019). 2 μL of each sample were seeded in 8 mm bands using a semi-automatic applicator (Automatic TLC sampler 4, CAMAG). For the calibration curves, standard solutions of glucose, fructose, sucrose, 1-kestose and nystose 0.1 mg/mL were seeded in a range of 0.5 to 10 μL.

Silica gel G-60 F254 plates on aluminum support (1.05554.0001 Merck) 20 x 10 cm were used as stationary phase and as the mobile phase a mixture of 1-Butanol / 2-Propanol / Boric acid (3:5:1 v/v/v) was used (Islam et al., 2020). All solvents were HPLC grade. Chromatographic separation was performed in an automatic development chamber (ADC2, CAMAG), previously saturated with the mobile phase for 5 min, at room temperature. The run was performed vertically for approximately 30 min, until the front reached 5 mm before the upper limit of the plate. Excess mobile phase was then evaporated from the plate by air current for 15 min and derivatization proceeded. A reagent prepared as follows was used as developer: 2 g of diphenylamine and 2 mL of aniline were dissolved in 80 mL of methanol. After the addition of 10 mL of phosphoric acid (85%) it was made up to 100 mL using methanol (Islam et al., 2020). The development was performed manually, immersing the obtained chromatogram in the developer and heating with a Stanley heat gun at 120 °C until sugars revealing. The chromatogram was observed under UV 254 nm, UV 366 nm and visible radiation. Images were digitized using TLC Visualizer (CAMAG) and data was processed using VisionCATS v2.0 (CAMAG) software, which was also used to control individual instrumentation modules.

2.11. FFase activity determination

FFase activity was determined by measuring the reducing sugars released by the hydrolysis of a sucrose solution. The reaction mixture consisted of 700 µL of 68 g/L sucrose as substrate in 0.2 M sodium acetate buffer pH 4.5 and 50 µL of crude enzymatic extract. The reaction was carried out at 37 °C for 10 min and terminated by thermal shock at 100 °C for 10 min. Released reducing sugars were determined by the DNS method at 560 nm (Miller,

1959). One unit of FFase activity was determined as the amount of enzyme required to release 1 µMol of reducing sugars per minute.

2.12. Data analysis

All experiments were performed in triplicate. Statistical analysis was performed using Minitab 17.0 software. For means comparisons, the experiments were performed in triplicates, and the mean values were analyzed statistically by ANOVA followed by the Tuckey Test. The significance of the results was established at p-value < 0.05. All graphics were carried out using the software SigmaPlot 12.0 for Windows.

3. Results and discussion

3.1. Chemical composition of carrot bagasse and carrot juice

The composition of the fermentation substrate can significantly affect the production of enzymes and the growth of microorganisms due it provides nutrients and allows anchorage of the microbial cells. Thus, substrate selection is one of the most important parameters to evaluate (Dinarvand et al., 2013; Martínez-Avila et al., 2021).

As a result of the preprocessing of carrots discards, juice and bagasse were obtained separately. Carrot bagasse was used as substrate in SSF to obtain FFase that was then added to the carrot juice to produce FOS. Chemical composition of carrot bagasse and juice are shown in Table 1. The results indicate that the main components in both bagasse and juice are carbohydrates (59.26 % and 83.96%, respectively), which consist in sucrose, glucose and fructose (3.90 %, 4.43 % and 1.3 % w/w, respectively, sugar composition was measured as described in Section 2.3.1).When agro-industrial wastes are used as substrate of fermentation is important to consider that the presence of some carbohydrates can

influence microorganism growth and enzyme production due to induction or repression mechanisms. Sucrose was reported as the best carbon source for FFase production while concentrations above 5 g/L of glucose could act as a repressor (Rubio and Navarro, 2006; Oyedeji et al., 2017). For FFase production, a wide range of fungi use only sucrose as carbon source and inducer. This fact is due to its ability to use sucrose hydrolysates for both growth and FFase production (Ire et al., 2018).

Previous reports indicated that carrot discards are a suitable substrate for microbial cultivation and production of molecules with biotechnological importance (Aimaretti et al., 2012). Salvañal et al.(2021) reported the production of lactic acid (22.18 g/L) using carrot syrup (obtained after β-carotene extraction from carrot discards) as substrate of fermentation by *Rhizopus arrhizus*. Aimaretti and Ybalo(2012), evaluated the ethanol production from carrot discards using *Saccharomyces cerevisiae.* The authors reported that carrot discards provide the necessary ions and the appropriate C:N balance for the fermentation.

3.2. Carrot bagasse as substrate of FFase production

Two types of support can be used in SSF: natural materials or inert supports (Krishna, 2005). Natural materials play two roles in SSF i) as physical structure for the growth of microorganisms, ii) as source of carbon, nitrogen, and growth factors for the microorganisms. In order to evaluate the carrot bagasse as natural substrate and support of SSF for FFase production, four types of fermentation systems were evaluated: I) control with polypropylene sponge as inert support embedded with 13 ml of sucrose solution (9.6 g/L) as sole carbon and 5 ml of water, II) systems containing 13g of carrot bagasse and 5 ml of water, III) systems with 13 g of carrot bagasse and 5 ml of synthetic media enriched

in nitrogen (MN), which contains urea, yeast extract and $(NH_4)_2SO_4$ (See Section 2.4.1) and IV) systems with 13 g of carrot bagasse and 5 ml of MN with traces (MNtz) (see Section 2.4.1.). All systems were inoculated with 0.1 mL of *A. niger* of 10^8 conidias/ml and incubated at 30°C for 4 days. Figure 1 shows the FFase production in different fermentation systems. When system I was used, the FFase production was the lowest. This fact indicates that solution of sucrose is not sufficient for fungal growth, while carrot bagasse may provide other nutrients like lipids, proteins, minerals that promote microorganism growth and enzyme production. Ohara et al.(2015) evaluated four agro-industrial wastes as substrate of SSF for FFase production using *A. niger* as microorganism: wheat bran, soybean meal, cottonseed meal and orange peel. Soybean meal and cottonseed meal had a higher protein content (55.91% and 27.69 %, respectively) while wheat bran and orange peel showed the greatest carbohydrates content. The higher FFase production was obtained when soybean meal and cottonseed meal were used as substrate (154.27 and 25.91 $\mu\ g^{-1}$, respectively) showing that the protein content positively influences FFase production. In accordance with Figure 1, carrot bagasse must be supplemented to obtain high FFase production. The protein content in carrot bagasse, 4.94%,(Table 1) is not sufficient for the grown of *A. niger* thus it is necessary the supplementation with MN. Mashetty and Biradar (2019) tested the supplementation of orange peel with three different sources of nitrogen: yeast extract, peptone, and urea, being the yeast extract the one that allowed the maximum FFase production.

Figure 1 also illustrates the effect of the supplementation of carrot bagasse with traces, showing that supplementation with MNtz allows to reach the highest value of FFase production. This may be due to trace elements have significant effects on the growth, metabolism and enzyme synthesis of many microorganisms (Dinarvand et al., 2013). The

main sources of nitrogen in MNtz are organic. These type of sources allow supply of amino acids and many growth factors that the microorganism need for cell metabolism and enzyme production (Maiorano et al., 2008).

3.3. Optimization of fermentation conditions

3.3.1. Effect of added volume of MNtz

SSF are fermentation process that occurs under similar condition to the natural environment where microorganisms are adapted, in the absence or near-absence of free water (Sánchez-martínez et al., 2020; Gowthaman et al., 2001). The use of large amount of water in fermentation systems has some disadvantages as well as the dilution of the molecule of interest and the risk of bacterial contamination (Melnichuk et al., 2020).

In order to evaluate the optimum amount of MNtz and initial moisture, systems containing 13 g of carrot bagasse were supplemented with 1 to 9 mL of MNtz. Figure 2 shows the effect of varying the volume of MNtz and the final moistures in the systems in FFase production. The results show that low FFase activity was obtained using volumes of MNtz lower than 5 ml and moisture values lower than 91 %. This fact could be due to the low amount of nitrogen present in the system when 1 or 3 ml are used. Additionally, low moisture in the system decreases nutrient diffusion and microorganism growth which results in a low FFase production (Singhania et al., 2010). Volumes greater than 5 ml did not improve FFase production. Higher levels of moisture in system produce particle agglomeration, transfer limits of gas and bacterial competition (Singhania et al., 2010). Thus, 5 ml of MNtz and 91 % of moisture were the optimal values that allowed to reach a maximum FFase production.

3.3.2. Effect of the inoculum size

Four inoculum sizes were studied to optimize FFase production. System containing 13 gr of carrot bagasse and 5 ml of MNtz were inoculated with 0.1 ml of 10^5 to 10^8 conidia/mL of *A. niger* and incubated for 96 h at 30°C. Results are shown in Figure 3. In accordance with the results, inoculum size influences significantly the production of FFase (p-value < 0.05). The highest FFase production was achieved with $1x10^6$ conidia/mL. High inoculum size (10^7-10^8 conidia/mL) causes intraspecific competition between fungal cells. This leads to rapid nutrient depletion and low enzyme production (Ganaie et al., 2017). On the contrary a small inoculum size (10^5 conidia/mL) is not enough to produce an adequate concentration of FFase. Thus, an inoculum size of 10^6 conidia/ mL was selected as optimum for FFase production.

3.3.3. Effect of incubation time

To evaluate the effect of incubation time on FFase production, systems containing 13 gr of carrot bagasse and 5 ml of MNtz were inoculated with 0.1 ml of 10^6 conidia/mL of *A. niger* and incubated at 30 ºC for 1 to 7 days. Figure 4 shows enzymatic activity at different incubation times. Results indicated that there are significant differences in the production of FFase in the range of time evaluated. Maximum FFase value (117 U/mL) was achieved at day 4, with a tendency to reach a plateau at later tested days (6 and 7 days). This could be a consequence of nutrient depletion or the accumulation of toxic products of metabolism in the fermentation medium, which could affect enzyme stability (Mamma et al., 2008). Total protein, specific activity and productivity of the crude enzyme extract was determined from 2 to 4 days of fermentation process (Table 2). In accordance with Figure 4, the maximum FFase activity was reached after 4 days of fermentation, however, when considering the

specific activity and productivity, it is observed that there are no significant differences between 3 and 4 days of fermentation. Therefore 3 days are enough to reach the maximum values of FFase activity.

The bibliography reported the evaluation of various agro-industrial wastes to produce FFase using *A. niger* as producer. Raju et al.(2016) analyzed three wastes as substrates: orange peel, pomegranate peel and pineapple peel. After optimization of fermentation conditions, the maximum enzyme production was 43 U/ml when orange peel was used as substrate. Veana and Aguilar(2014) evaluated molasses and sugarcane bagasse as substrates for FFase production using *A. niger* GH1 as producer. The SSF were performed using virgin and hydrolyzate bagasse (treatment with concentrated sulfuric acid). The maximum enzyme production achieved was 5.23 U/ml when hydrolyzated bagasse was used. The reported values are significantly lower than the results obtained in the present study, showing that carrot bagasse is a good substrate with adequate nutrients for FFase production.

3.4. FFase in enzymatic extract

To evaluate futures strategies of FFase purification, the presence of this enzyme in the extract was verified. Enzymatic extract was analyzed by mass spectrometry. The analysis of secretome of *A. niger* grown under optimized conditions, showed the presence of FFase with a molecular weight of 63 kDa (see Supplementary material, Table 3). Similar weights were reported in the bibliography. Oyedeji et al., 2017 produced FFase from *A. niger* with native molecular weight of 67.7 ± 0.21 kDa using pineapple peel as substrate.

Zymogram of enzymatic extract in native conditions, SDS-PAGE of *A. niger* enzymatic extract under optimal conditions and molecular weight markers are illustrated in Figure 5

A, B and C, respectively. As can be observed, the zymogram shows two bands with FFase activity whose molecular weight was estimated using Gel Analyzer 19.1 software. The first band corresponds to a molecular weight of 117 kDa and the second one is estimated to have a molecular weight of 84 kDa. In accordance with the results, FFase should be present in the second band. SDS-PAGE) shows two bands that correlate with the wide red band of the zymogram indicating that these bands may contain other enzymes with FFase activity of similar molecular weight.

A proof-of-concept study was carried out using crude enzyme extract and carrot juice. The enzymatic extract was concentrated by ultrafiltration until final FFase activity of 212 U/mL before reaction. Carrot juice was concentrated until a sugar concentration of 270 g/L. Transfructosylation reaction was carried out following the conditions descripted in Section 2.9. for 3h. Figure 6 shows sugars and FOS present in reaction mixture at different times (1-3 h), the standards samples (ST) and their retention factor (RF). In this proof-of-concept study the determination of FOS and sugars were qualitative. As can be seen in Figure 6, the band corresponding to fructose (RF 0.19) in ST is hardly detected, this effect may be due to the reagent developer used. Transfructosylation reaction is a complex process with simultaneously reactions of synthesis and hydrolysis (Vega and Zuniga-hansen, 2014). In presence of sucrose, enzymes with transfructosilase activity cleave the β–2,1-glycosidic bond of sucrose and transfers the fructose moiety onto any acceptor such as sucrose or another FOS present in the solution, releasing glucose as a by-product (Ganaie et al., 2014). Figure 6 showed that crude enzyme extract has transfructosylase activity and produces mainly 1-kestose in the reaction conditions and glucose as by-product. This shows that is possible to obtain a natural prebiotic juice with FOS from carrot discards. However, the

purification and characterization of enzymes with FFase activity present in the crude extract enzyme is essential to obtain high yields of FOS. This is the aim of future investigations.

4. Conclusion

A biorefinery process for the upcycling of carrot discards was outlined. It consisted in the production of β-fructofuranosidase in SSF by *A. niger* using carrot bagasse, and its subsecuent application to catalyze the synthesis of FOS in carrot juice. This process allowed obtaining a high yield of enzyme, which was successfully used to catalyze FOS (1-kestose) synthesis using sugars present in carrot juice. These compounds are well known prebiotics with high nutritional value, since they contribute to improve human health. Results are promising, since they open the door for the future development of a functional prebiotic beverage. However, purification of enzyme and optimization of reaction conditions should be studied in order to improve FOS yield.

Solid-state fermentation is a low-cost and efficient process for the upcycling of agro-industrial wastes to generate enzymes of industrial interest, and the process shown in this work could be applied to other discards with similar characteristics.

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Tweetable abstract

A great amount of carrots is discarded daily. This paper evaluates the use of carrot bagasse to produce β-fructofuranosidase and its subsequent application in carrot juice to produce a natural beverage with prebiotics.

Conflict of interest:

The authors declare no conflict of interest

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Tables

Table 1: Chemical composition of carrot bagasse and carrot juice. Results were expressed as % dry matter

	Carrot bagasse (%)	Carrot juice (%)
Fat Matter	2.47 ±0.15	2.83±0.10
Proteins	4.94 ±0.44	6.60±0.35
Ashes	4.94 ±0.22	5.66±0.25
Fiber	28.40±0.50	0.94±0.55
Carbohydrates	59.26±1.20	83.96±1.57

Values are means ± standard deviations (n = 3).

Table 2: Effect of incubation time on FFase specific activity and productivity by *A. niger* in SSF from carrot bagasse.

Days	FFase activity (U/mL)	Proteins (mg/mL)	Specific Activity (U/mg prot.)	FFase productivity (U/h)
2	35.1 ± 0.30	0.18 ± 0.30	197±22.00	0.73
3	93 ± 1.00	0.37 ± 0.01	251 ± 7.00	1.29
4	117 ± 4.00	0.48 ± 0.01	244 ± 5.00	1.22

Values are means ± standard deviations (n = 3).

Figures

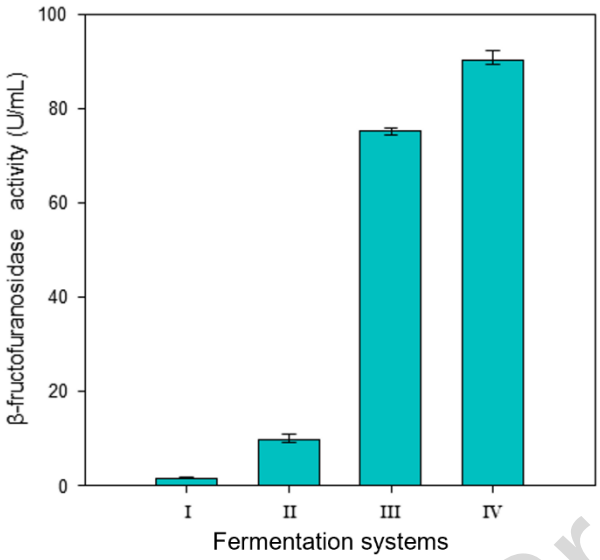


Figure 1: FFase production by SSF with *A. niger* from carrot bagasse. SSF system: (I) Control, (II) carrot bagasse with water, (III) carrot bagasse supplemented with nitrogen source (MN) and (IV) carrot bagasse supplemented with nitrogen source and traces (MNtz). Values are means ± standard deviations (n = 3)

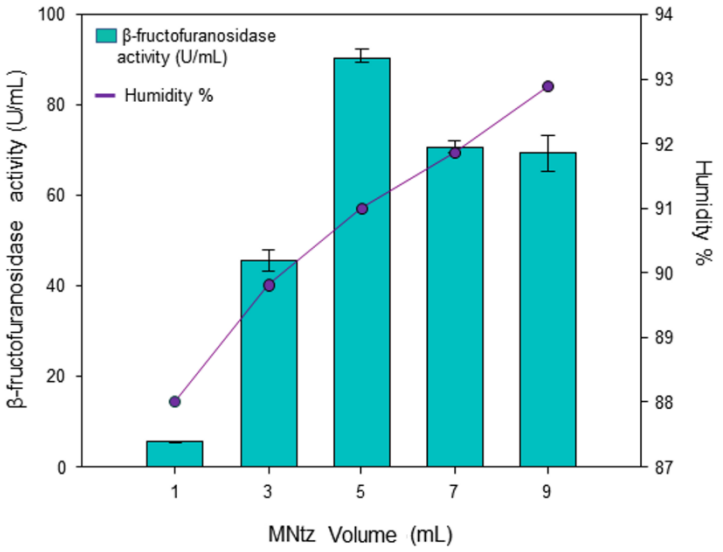


Figure 2: Effect of MNtz volume and moisture on FFase production by SSF with *A. niger* from carrot bagasse. Values are means ± standard deviations (n = 3)

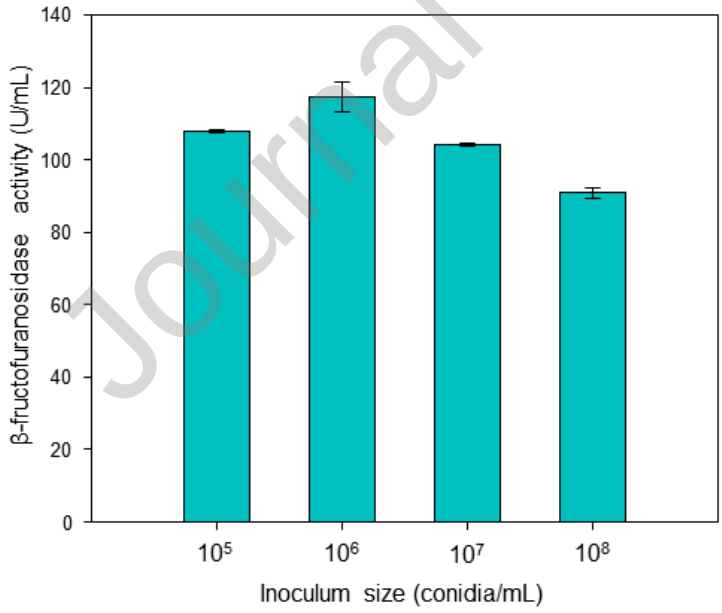


Figure 3: Effect of inoculum size on FFase production by SSF with *A. niger* using carrot bagasse as substrate. Values are means ± standard deviations (n = 3)

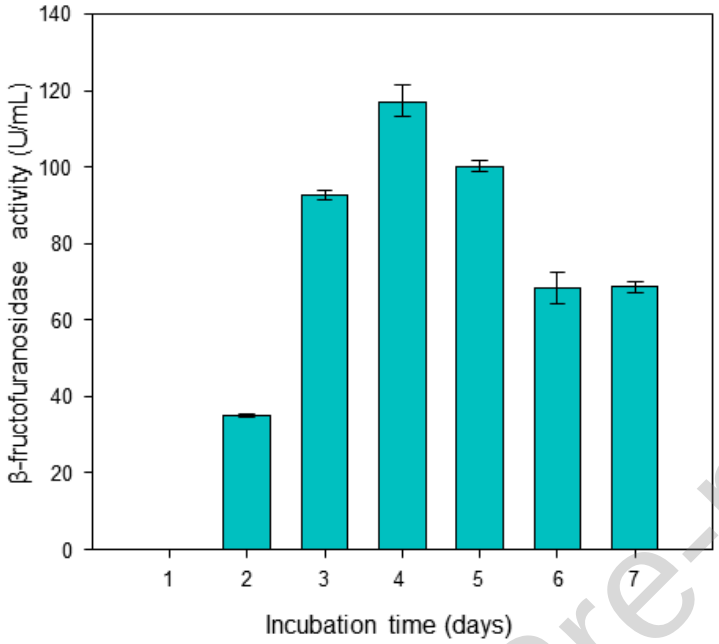


Figure 4: Effect of incubation time on FFase production by SSF with *A. niger* from carrot bagasse. Values are means ± standard deviations (n = 3)

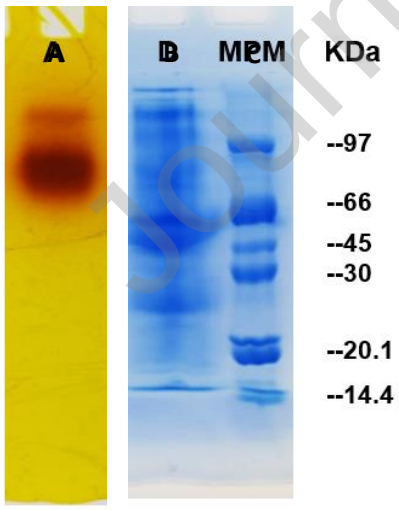


Figure 5: FFase in enzymatic extract: A) Zymogram showing bands with FFase activity stained red as product of the reaction of reducing sugars with DNS after hydrolysis. B)

SDS-PAGE of *A. niger* enzymatic extract obtained under optimal conditions and revealed by Coomasie Blue R-250. C) Molecular weight markers.

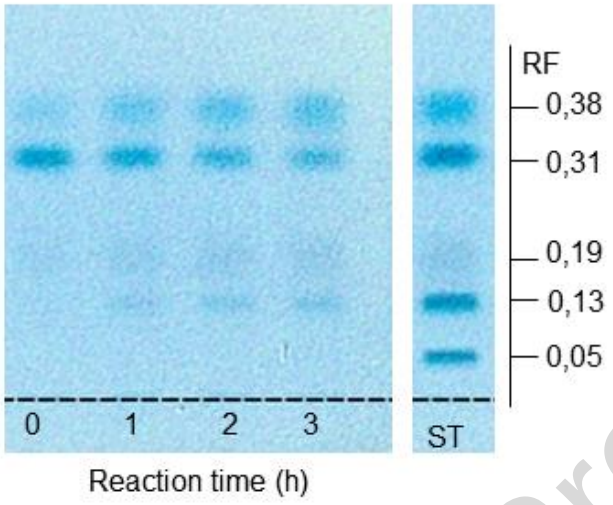
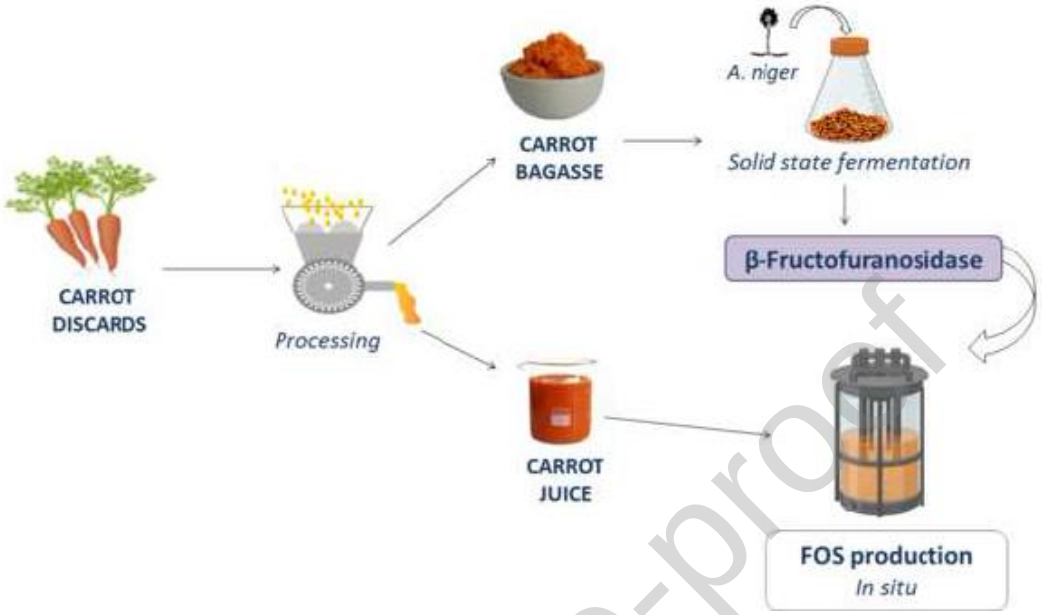


Figure 6: TLC on G-60 silica gel plates, using 1-butanol/ 2-propanol/ boric acid 3:5:1 (v/v/v) as mobile phase and aniline/diphenylamine/phosphoric acid reagent as developer. RF: retention factor, ST: standards sample. Bands corresponding to nystose (RF 0.05), 1-kestose (RF 0.13), fructose (RF 0.19), sucrose (RF 0.31) and glucose (RF 0.38) can be observed at 0, 1, 2 and 3 h of reaction.

Graphical abstract



Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Highlights

- Upcycling of carrot discards into high value food ingredients is proposed.
- Carrot bagasse is an adequate substrate for β-fructofuranosidase production.
- β-fructofuranosidase from carrot bagasse has transfructosylase activity.
- HPTLC analysis showed the production of fructooligosaccharides in carrot juice.