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Development of new biocatalytic processes for fructooligosaccharides (FOS) preparation

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Development of new biocatalytic processes for fructooligosaccharides (FOS) preparation

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ABSTRACT

This PhD research project was aimed at the development of new biocatalytic processes to produce natural sugars by selection and characterisation of new enzymes able to produce fructooligosaccharides. Biochemical studies were performed to obtain information on the mechanism of action and understand the structural elements that define the activity. After the development of the biotransformation conditions, a continuous production of FOS was studied and a cheap separation method of the transformation products was also assessed, in order to obtain FOS in purified form. Mass spectrometry studies were performed on the purified enzymes after purification from wild-type strains.

After a screening for FOS production from sucrose two microorganisms were chosen for their activity up to 30 % (w/w) conversion and differences in FOS mixture production:

$CF_2$15 = *Cladosporium cladosporioides*

CK1 = *Penicilium sizovae*

$CF_2$15 produce a mixture similar to the commercial product Actilight®, while CK1 produce for almost kestose (GF3). Under the optimised biotransformation conditions the maximum accumulation of FOS was 56 % (w/w) and 31 % (w/w) for $CF_2$15 and CK1 respectively. We were able to isolate and characterise seven different carbohydrates such as 1-kestose, 1-nystose, 1-fructofuranosylnystose, 6-kestose, neo-kestose and neo-nystose for $CF_2$15 while CK1 produce only 1-kestose, 1-nystose, 1-fructofuranosylnystose and 6-kestose. Another oligosaccharides was isolated and fully characterised from $CF_2$15 mixture, named blastose (Fru-β(2→6)-Glc). An immobilization study was carried using the DALGEEs (Dried Alginate Entrapped Enzymes) method on the mycelium of $CF_2$15 strain. The maximum accumulation of FOS using DALGEEs mycelium was 51 % (w/w), reached in common buffer and seawater. With this cheap technique we develop a continuous FOS production using the facilities of Flow chemistry. The reactor, filled with DALGEEs and celite, was stable for months and the maximum accumulation of FOS was 52% (w/w). At this flow stream of FOS mixture we added a batch step to purify the FOS from glucose that represent the 26 % (w/w) of entire mixture. Glucose Oxidase from Novozymes® named Glyzyme® MONO 10.000 BG was employed and the result was the reduction of glucose from 26 % (w/w) to 3% (w/w). This purification step was added for two reasons: to obtain a cheap and fast method for FOS purification from glucose and to simplify the blastose purification by preparative HPLC. 56 mg of purified blastose were obtained and utilised to perform a preliminary study of blastose prebiotic action. The growth of 5 different lactobacillus strains (*Lactobacillus paracasei* DG, *Lactobacillus rhamnosus* GG, *Lactobacillus paracasei* SHIROTA, *Lactobacillus johnsonii* LC1 and *Lactobacillus reuteri* ATCC55730) were followed with the addiction of different carbohydrates as only carbon source (glucose, Actilight®, inulin, blastose). The best results were reached with *Lactobacillus johnsonii* LC1 where the Vmax using 0.5 % (w/v) of blastose was higher than glucose 0.5 % (w/v) (1.125 ± 0.023 1/h and 0.521 ± 0.054 1/h respectively).

In the second part of this PhD project the purification of the enzymes involved in FOS formation was achieved after several chromatographic steps. The molecular weight (MW) of the two proteins was ≈50 kDA for the enzyme from *Cladosporium cladosporioides* (monomeric) and ≈75 kDa for the one from *Penicilium sizovae* (monomeric). The enzyme from *C. cladosporioides* was biochemically characterised and shown a Km of 129 ± 6 mM, Vmax of 2.83 ± 0.04 U/mL, Kcat of 2.88 ± 0.04 1/s and a Kcat/Km of 22.3 ± 1.4 1/M*s with sucrose and a Km of 268 ± 6 mM, Vmax of 0.0328 ± 0.003 U/mL, Kcat of 0.0334 ± 0,003 1/s and Kcat/Km of 0.124 ± 0.014 1/M*s with 1-kestose. A mass spectrometry MALDI-TOF analysis study was performed on the protein, showing a MW of 61178 Da. A trypsin digestion was performed and the fragments analysed showed no homology with known proteins.

RIASSUNTO

Questo progetto ha avuto lo scopo di sviluppare nuovi metodi biocatalitici per la produzione di dolcificanti alternativi mediante la selezione, l'isolamento e la caratterizzazione di nuovi enzimi (fruttofuranosidasi) capaci di produrre fruttooligosaccaridi (FOS) da saccarosio. Sono stati fatti studi per ottenere informazioni sul processo produttivo dei FOS e migliorare la produzione anche mediante la progettazione di un processo in continuo. Gli enzimi, inoltre, sono stati isolati e caratterizzati per permettere l'elucidazione della sequenza e un futuro studio di ingegneria proteica per migliorare la capacità dei catalizzatori.

Al termine di uno screening volto alla scoperta di nuove fruttofuranosidasi, 2 ceppi di eumiceti filamentosi sono stati isolati ed identificati in quanto risultati migliori catalizzatori in termini di quantità di FOS prodotti e varietà nella miscela di fruttooligosaccaridi prodotti: $CF_2$15 indentificato come *Cladosporium cladosporioides* e CK1 identificato come *Penicilium sizovae.*

Nella prima parte del progetto di dottorato le condizioni di biotrasformazione sono state ottimizzate portando ad un massimo accumulo di FOS del 56 % (w/w) per $CF_2$15 e 31 % (w/w) per CK1. I FOS identificati nelle miscele sono stati: 1-chestosio, 1-nistosio, 1-fruttofuranosilnistosio, 6-chestosio, neo-chestosio e neo-nistosio, per $CF_2$15 e 1-chestosio, 1-nistosio e 1-fruttofuranosilnistosio per CK1. Un altro oligosaccaride non convenzionale è stato isolato, dalla miscela prodotta dal ceppo $CF_2$15, ed identificato come blastosio (Fru-β(2→6)-Glc). Evidenziate queste differenze tra i ceppi si è deciso di concentrare l'attenzione sul ceppo $CF_2$15. Uno studio di immobilizzazione del micelio è stato sviluppato usando l'innovativa tecnologia DALGEEs (Dried Alginate Entrapped Enzymes). L'utilizzo del micelio immobilizzato ha portato ad un buon riciclo del catalizzatore, utilizzando tamponi convenzionali o acqua di mare, e ad una massima produzione di FOS del 51 % (w/w). Per questo motivo i DALGEEs sono stati utilizzati per sviluppare una metodologia di produzione in continuo di FOS sfruttando i vantaggi offerti dalla tecnica di Flow chemistry. Questo approccio ha portato ad una produzione in continuo di FOS del 52 % (w/w) con una composizione della miscela di reazione simile a questa osservata con cellule intere o DALGEEs. La suddetta miscela è stata utilizzata come base di partenza per sviluppare un metodo economico di purificazione dal glucosio, il quale rappresenta un prodotto della reazione indesiderato e costituisce il 26 % (w/w) della miscela di reazione. Una preparazione di glucosio ossidasi, prodotta da Novozymes® chiamata Gluzyme® 10000 MONO BG, è stata usata per ossidare il glucosio ed ottenere una miscela purificata di FOS. Questo processo ha anche avvantaggiato la purificazione di 56 mg di blastosio mediante HPLC. Con la quantità di blastosio ottenuta è stato intrapreso uno studio pionieristico circa l'attività prebiotica del disaccaride. Mediante l'impiego di 5 diversi ceppi di lattobacilli (*Lactobacillus paracasei* DG, *Lactobacillus rhamnosus* GG, *Lactobacillus paracasei* SHIROTA, *Lactobacillus johnsonii* LC1 e *Lactobacillus reuteri* ATCC55730), la crescita è stata valutata in presenza di un solo carboidrato come unica fonte di carbonio (glucosio, Actilight®, inulina, blastosio). I risultati più interessanti si sono avuti con il ceppo *Lactobacillus johnsonii* LC1, il quale ha mostrato una Vmax superiore con blastosio 0.5 % (w/v) (1.125 ± 0.023 1/h) che con glucosio 0.5 % (w/v) (0.521 ± 0.054 1/h).

Nella seconda parte del progetto di dottorato si è deciso di purificare gli enzimi coinvolti nella biotrasformazione di saccarosio in FOS. Dopo alcuni passaggi cromatografici siamo riuscititi ad ottenere una frazione purificata contenete una solo proteina, controllata mediante elettroforesi su gel di poliacrilammide. Una singola banda è stata rilevata a circa 50 KDa per $CF_2$15 e 75 KDa per CK1. L'attenzione quindi, è stata focalizzata sulla proteina proveniente dal ceppo $CF_2$15, il quale si è rivelato il miglior catalizzatore. Utilizzando l'enzima proveniente da $CF_2$15 (*C. cladosporioides*) è stato effettuato uno studio biochimico. I valori cinetici sono stati calcolati con saccarosio e 1-chestosio. I risultati hanno mostrato: Km di 129 ± 6 mM, Vmax di 2.83 ± 0.04 U/mL, Kcat di 2.88 ± 0.04 1/s, Kcat/Km di 22.3 ± 1.4 1/M*s con saccarosio e Km di 268 ± 6 mM, Vmax di 0.0328 ± 0.003 U/mL, Kcat di 0.0334 ± 0,003 1/s, Kcat/Km di 0.124 ± 0.014 1/M*s con 1-chestosio. Un conseguente studio di spettrometria di massa ha portato all'identificazione del peso molecolare della proteina pari a 61178 Da. L'analisi dei frammenti, ottenuti dopo digestione tripsinica, non ha però portato ad una identificazione della sequenza primaria della proteina, la quale rimane ancora da elucidare.

PREFACE

A vast number of microorganisms inhabit the mammalian gut, and their symbiotic and mutualistic relationship with the host (host−bacteria and bacteria−bacteria interactions) determines a complex and dynamic ecosystem.

The human intestinal microbiota is composed of 10^{13} to 10^{14} microorganisms whose collective genome (‘‘microbiome’’) contains at least 100 times as many genes as our own genome.

Molecular analysis of microbial composition of fecal and mucosal samples using 16s ribosomal DNA and RNA have increased previous culture-based valuations to as high as 36000 individual species (Frank D. N. et al 2007).

There is a clear variability of specific composition in terms of species considering different zone of the gut, different ages and even different hosts. Greater than 99% of the gut microbiota is composed of species within four bacterial divisions: *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, and *Actinobacteria*

It is widely acknowledged that the commensal gut microbiota plays vital roles, although not yet fully understood, in the normal digestive function of the host, maturation of human immunity, brain development, and natural defence mechanism against pathogenic bacteria.

A healthy gut microbiota (i.e. properly balanced bacterial groups) is normally required for human/animal health. This guarantees host immune homeostasis and nutrient intake. Accordingly, a balanced gut microbiota composition confers benefits to the host, whereas gut microbiota imbalance may disturb the physiological homeostasis, leading to various diseases such as inflammatory bowel diseases, obesity, colon cancer, neonatal necrotizing enterocolitis, irritable bowel syndrome, and cardiovascular diseases (Li T., Lu X., and Yang X. 2013).

Therefore, the modulation of the microflora through dietary ingredients offers promises for reducing disorders that may be mediated by deviations from gut microbiota equilibrium. As the Nobel Prize recipient, Eli Metchnikoff suggested in 1907 “the dependence of the intestinal microbes on the food makes it possible to adopt measures to modify the flora in our bodies and to replace the harmful microbes by useful microbes”. This theory results nowadays in the development of two kinds of functional food: prebiotics and probiotics.

The World Health Organization's 2001 definition of probiotics is “live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host”. Many commercial preparations are available on the market. Now the two species of microorganisms widely use are Lactobacillus (e.g. *L. johnsonii* in the LC1® preparation from Nestle) and *Bifidobacterium.*

The term prebiotic means a “non-viable food component that confers a health benefit on the host associated with modulation of the microbiota”. “Component” means not an organism or drug, but a

substance that can be characterised chemically. In most cases, this will be a food grade component. “Health benefit” refers to measurable and not due to absorption of the component into the bloodstream or due to the component acting alone. “Modulation” means that the presence of the component in the formulation in which it is being delivered changes the composition or activities of the microbiota in target host. Mechanisms of modulations might include fermentation, receptor blockage or others (Fernandez R. C. et al 2007).

On the other hand, the term prebiotic refers to compounds able to improve the quality and the quantity of the human microbiota.

In this contest, the term “synbiotic” has been created, referring to nutritional supplements obtained by the combination of probiotics and prebiotics. The Food & Agriculture Organization (FAO) recommends that the term “synbiotic” is used only if the net health benefit is synergistic (Guida B. 2004; Asp N. 2007).

A significant expansion of the potential market for prebiotics and probiotics has led to deeper investigations by scientific groups that are dealing with new methods for the synthesis of prebiotic compounds, the preparation of better probiotic products, and the isolation of new beneficial strains of microorganisms

The most important prebiotics belong to a large variety of dietary carbohydrates such as starch, dietary fibres, and some non-digestible oligosaccharides (NDOs). Inulin-type fructans or fructooligosaccharides (FOS), galactooligosaccharides, and lactulose are some of the most important NDOs.

FOS are linear oligosaccharides of fructose containing a single glucose (G) moiety in which fructosyl units (F) are bound at the β(2→1) position of a sucrose molecule (GF). Among 1-FOS, 1-kestose (GF2), 1-nystose (GF3), and 1-β-fructofuranosylnystose (GF4) represent the most studied and used compounds. Other FOS families founded in commercial functional foods are 6-FOS and neo-FOS with the linkage between the fructose and glucose moiety in position β(6→2) β(2→6) respectively (Fernandez R. C. et al 2007).

References

Frank D. N., St. Amand A. L, Feldman R. A., Boedeker E. C., Harpaz N., and Pace N. R., 2007, Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. Proc. Natl. Acad. Sci. USA34:13780-13785.

Li T., Lu X., and Yang X., 2013, Stachyose-enriched alpha-galacto-oligosaccharides regulate gut microbiota and relieve constipation in miceJ. Agric.Food. Chem. 61:11825-11831.

Fernandez R. C., Ottoni C. A., Sabino da Silva E., Mitiko Saito Matsubara R., Carter J. M., Magossi L. R., Alves Wada M. A., de Andrade Rodrigues M. F., Maresma B. G., and Maiorano A. E., 2007, Screening of β-fructofuranosidase-producing microorganisms and effect of pH and temperature on enzymatic rate. Appl. Microbiol. Biotechnol.75:87-93.

Guida B., Germanò R., Trio R., Russo D., Memoli B., Grumetto L., Barbato F., and Cataldi M., 2014, Effect of the Synbiotic Probinul-Neutro® on Gastrointestinal Symptoms and Plasma p-Cresol Level in Chronic Renal Failure. Nutr. Metab. Cardiovasc. Dis. 24:1043-1049

Asp N., Brunser O., Macfarlane S., Morelli L., Reid G., Tuohy K., and Piñeiro M., 2007, FAO technical meeting on prebiotics.

STATE OF THE ART

Inulin-type fructooligosaccharides (1F-FOS) are fructose oligomers with a terminal glucose unit in which 2-4 fructofuranosyl moieties are linked by β(2→1) bonds (Antosova M., Polakovic M. 2001; Kelly G. 2008). 1F-FOS are used as food ingredients due to their properties, such as prebiotic action favouring the development of bifidobacteria and lactobacillus, low caloric intake (2 kcal/g), low glycemic index, improved gut absorption of Ca^{2+} and Mg^{2+}, lowering of blood lipid levels, prevention of urogenital infections and reduced risk of colon cancer (Huebner J. et al 2007;Scholz-Ahrens K.E. et al 2007). 1F-FOS are commonly obtained by controlled hydrolysis of inulin or other fructans (typically using inulinases, EC 3.2.1.7) (Nguyen Q.D. et al 2011). or by enzymatic transfructosylation of sucrose catalysed by β-fructofuranosidases (EC 3.2.1.26) or fructosyltransferases (EC 2.4.1.9) (Kurakake M. et al 2010). Short-chain 1F-FOS (1-kestose, nystose, 1F fructosylnystose, and so on) are currently produced at multi-ton scale from concentrated sucrose solutions using fungal transfructosylating enzymes from *Aspergillus niger*, *Aspergillus oryzae* or *Aureobasidium pullulans* (Sangeetha P.T. et al 2005; Plou F.J. et al 2002). Other FOS containing β(2→6) linkages between two fructose units (6F-FOS, also called levan-type FOS, such as 6-kestose or 6-nystose (Alvaro-Benito M. et al 2007; 2012; de Abreu M. et al 2011; 2013) or between a fructose and a glucose (6G-FOS, also called neoFOS, such as neokestose or neonystose) have also been described and are commonly produced by enzymes from yeasts (Linde D. et al 2009; 2013; Lafraya A. et al 2011). Levan-type FOS and neoFOS are reported to exhibit improved prebiotic properties and chemical stability compared to inulin-type FOS (Hayashi S. et al 2000; Kilian S. et al 2002; Lim J.S. et al 2007), although more studies are required to elucidate 1 the bioactivity of the different FOS series. Industrial scale production of FOS is commonly performed by either soluble enzymes in batch reactions (Hidaka H. et al 1987) or by entrapped cells in alginate gel beads using continuous fixed-bed reactors (Yun J.W. et al 1995; Park M.C. et al 2005;). Several immobilised enzymes for FOS synthesis have been also developed (Alvaro-Benito M. et al 2007; Smaali I. et al 2011; Fernandez-Arrojo L. et al 2013; Alvarado-Huallanco M.B. et al 2010). The use of biomass (e.g. mycelia) or immobilised biocatalysts minimizes the loss of activity during operation and allows establishing a continuous process.

Two industrials processes are involved for FOS production, based on sucrose modification:

- Beghin-meiji process (French-Japanese): FOS production with a fructofuranosidase from *Aspergillus niger*, commercial product named Actilight® (GF2 37% / GF3 53% / GF4 10%) (Hidaka H., et al 1988).
- Cheil Foods process (Korean): FOS production with immobilised cells from *Aureobasidium pullulans*, (mixture composition GF2 56% / GF3 44%). (Shin H.T. et al 2004).

Enzymatic process is characterised by the following critical points:

1. The reaction catalysed by FF is an equilibrium reaction in which the enzyme can catalyse both the formation and hydrolysis of FOS.
2. The accumulation of glucose in the reaction medium determines phenomena of enzyme inhibition.
3. The final purification step. Separation of FOS from glucose and other molecules is archived by:
    a. Chromatography, using SMB (simulating moving bed) with cationic resins (Gomes, P. S. et al 2006).
    b. Nanofiltration (Li W. et al 2004).

The identification of novel microbial strains with high transfructosylation activity and/or producing a distinctive FOS pattern is currently being investigated (Ganaie M.A. et al 2013).

References

Antosova M, Polakovic M., 2001, Fructosyltransferases: The enzymes catalysing production of fructooligosaccharides. Chem Pap-Chem Zvesti; 55:350-358.

Kelly G., 2008, Inulin-type prebiotics - A review: Part 1. Altern Med Rev; 13:315-329.

Huebner J., Wehling R.L., Hutkins R.W., 2007, Functional activity of commercial prebiotics. Int. Dairy J.; 17:770-775.

Scholz-Ahrens K.E., Ade P., Marten 1. B., Weber P., Timm W., Asil Y., Gluer C.C., Schrezenmeir J., 2007, Prebiotics, probiotics, and synbiotics affect mineral absorption, bone mineral content, and bone structure. J. Nutr; 137:838S-846S.

Nguyen Q.D., Rezessy-Szabo J.M., Czukor B., Hoschke A., 2011, Continuous production of oligofructose syrup from Jerusalem artichoke juice by immobilised endo-inulinase. Process Biochem; 46:298-303.

Kurakake M., Masumoto R.Y.O., Maguma K., Kamata A., Saito E., Ukita N., Komaki T., 2010, Production of fructooligosaccharides by β-fructofuranosidases from *Aspergillus oryzae* KB. J Agric Food Chem; 58:488-492.

Sangeetha P.T., Ramesh M.N., Prapulla S.G., 2005, Fructooligosaccharide production using fructosyl transferase obtained from recycling culture of *Aspergillus oryzae* CFR 202. Process Biochem; 40:1085-1088.

Plou F.J., Martin M.T., Gomez de Segura A., Alcalde M., Ballesteros A., 2002, Glucosyltransferases acting on starch or sucrose for the synthesis of oligosaccharides. Can J Chem; 80:743-752.

Alvaro-Benito M., de Abreu M., Fernandez-Arrojo L., Plou F.J., Jimenez-Barbero J., Ballesteros A., Polaina J., Fernandez-Lobato M., 2007, Characterization of a β- fructofuranosidase from *Schwanniomyces occidentalis* with transfructosylating activity yielding the prebiotic 6-kestose. J Biotechnol; 132:75-81.

Alvaro-Benito M., Sainz-Polo M.A., Gonzalez-Perez D., Gonzalez B., Plou F.J., Fernandez-Lobato M., Sanz-Aparicio J., 2012, Structural and kinetic insights reveal that the amino acid pair Gln-228/Asn-254 modulates the transfructosylating specificity of *Schwanniomyces occidentalis* β-fructofuranosidase, an enzyme that produces prebiotics. J Biol Chem; 287:19674-19686.

De Abreu M., Alvaro-Benito M., Plou F.J., Fernandez-Lobato M., Alcalde M., 2011 Screening β-fructofuranosidases mutant libraries to enhance the transglycosylation yield of β-(2-6) fructooligosacharides. 1 Comb Chem High T Scr; 14:730-738.

De Abreu M., Alvaro-Benito M., Sanz-Aparicio J., Plou F.J., Fernandez-Lobato M., Alcalde M., 2013 Synthesis of 6-kestose using an efficient beta-fructofuranosidase engineered by directed evolution. Adv Synth Catal; 355:1698-1702.

Linde D., Macias I., Fernandez-Arrojo L., Plou F.J., Fernandez-Lobato M., 2009 Molecular and biochemical characterization of a β-fructofuranosidase from *Xanthophyllomyces dendrorhous*. Appl Environ Microbiol; 75:1065-1073.

Linde D., Rodriguez-Colinas B., Estevez M, Poveda A., Plou F.J., Fernandez-Lobato M., 2012, Analysis of neofructooligosaccharides production mediated by the extracellular β-fructofuranosidase from *Xanthophyllomyces dendrorhous*. Bioresour Technol; 109:123-130.

Lafraya A., Sanz-Aparicio J., Polaina J., Marin-Navarro J., 2011, Fructo-oligosaccharide synthesis by mutant versions of *Saccharomyces cerevisiae* invertase. Appl Environ Microbiol; 77:6148-6157.

Hayashi S., Yoshiyama T., Fujii N., Shinohara S., 2000, Production of a novel syrup containing neofructo-oligosaccharides by the cells of *Penicillium citrinum*. Biotechnol Lett; 22:1465-1469.

Kilian S., Kritzinger S., Rycroft C., Gibson G., du Preez J., 2002, The effects of the novel bifidogenic trisaccharide, neokestose, on the human colonic microbiota. World J Microb Biot; 18:637-644.

Lim J.S., Lee J.H., Kang S.W., Park S.W., Kim S.W., 2007, Studies on production and physical properties of neo-FOS produced by co-immobilised *Penicillium citrinum* and neo-fructosyltransferase. Eur Food Res Technol; 225:457-462.

Hidaka H., Eida T., Adachi T., Saitoh Y., 1987, Industrial-production of fructooligosaccharides and its application for human and animals. J Jpn Soc Food Sci; 61:915-923.

Yun J.W., Kang S.C., Song S.K., 1995, Continuous 1 production of fructooligosaccharides from sucrose by immobilised fructosyltransferase. Biotechnol Tech; 9:805- 808.

Park M.C., Lim J.S., Kim J.C., Park S.W., Kim S.W., 2005, Continuous production of neo fructooligosaccharides by immobilization of whole cells of *Penicillium citrinum*. Biotechnol Lett; 27:127-130.

Smaali I., Soussi A., Bouallagui H., Chaira N., Hamdi M., Marzouki M.N., 2011, Production of high-fructose syrup from date by-products in a packed bed bioreactor using a novel thermostable invertase from *Aspergillus awamori.* Biocatal Biotransfor; 29:253-261.

Fernandez-Arrojo L., Rodriguez-Colinas B., Gutierrez-Alonso P., Fernandez-Lobato M., Alcalde M., Ballesteros A.O., Plou F.J., 2013, Dried alginate-entrapped enzymes (DALGEEs) and their application to the production of fructooligosaccharides. Process Biochem; 48:677-682.

Alvarado-Huallanco M.B., Maugeri-Filho F., 2010, Kinetics and modeling of fructo oligosaccharide synthesis by immobilised fructosyltransferase from *Rhodotorula* sp. J Chem Technol Biotechnol; 85:1654-1662.

Hidaka H., Hirayama M., Kaisha M. S., Horikawa-cho, Saiwai-ku, Kawasaki-shi, 1988, Fructooligosaccharide-producing Enzyme from *Aspergillus niger* ATCC 2061. Agricultural and Biological Chemistry 52 (5): 1181-1187

Shin H. T., Baig S. Y., Lee S. W., Suh D. S., Kwon S. T., Lim Y. B., Lee J. H., 2004, Production of fructo-oligosaccharides from molasses by *Aureobasidium pullulans* cell. Bioresource Technology 93: 59-62

Gomes, P. S., Minceva M., Rodrigues A., 2006, Simulated moving bed technology: old and new adsorption. Journal of the International Adsorption Society 12: 375-392

Li W., Chen T., Cuixian, 2004, Nanofiltration for purifying fructooligosaccharides. Journal of Membrane Science 245 (1-2): 123-129

Ganaie M.A., Gupta U.S., Kango N., 2013, Screening of biocatalysts for transformation of sucrose to fructooligosaccharides. J Mol Catal B-Enzym; 97:12-17.

AIMS OF THE STUDY

In this work, we will screen different microorganisms able to grow on sucrose-rich substrates, such as molasses or jams, with the aim of identifying new β-fructofuranosidases able to produce FOS with different composition compared with already known biocatalysts.

The proteins of the best microorganisms will be isolated and characterised to elucidate the sequences and the elements that define the fructofuranosidase activity.

The mains topics of this PhD project will be:

- Study of biotransformation
  - Study of thermodynamic/kinetic of reaction
  - Characterisation of products: HPLC, MS and NMR analysis.
- Enzymatic activity study
  - Activity/stability
- Enzymatic immobilization
  - Best technology from immobilisation
  - Ease of use and recycling
- Continuous fructooligosaccharides production
  - Development of parameter for continuous production of FOS
- Extraction methods
  - Cheapest method for FOS in high-purity yields.
- Study of prebiotic action of the most interesting FOS isolated
  - *In-vitro* studies with different lactobacillus strains
- Isolation and characterisation of the best proteins from wild-type strains
  - Mass spectrometry studies

RESULTS AND DISCUSSION

Production of fructooligosaccharides by mycelium-bound transfructosylation activity present in *Cladosporium cladosporioides* and *Penicilium sizovae*.

Materials and methods

Materials

Sucrose, glucose, fructose and p-anisaldehyde were from Sigma-Aldrich. Nystose and 1-kestose were from Fluka. 1F-fructosylnystose was from Megazyme. 6- Kestose, neokestose and neonystose were synthesised as previously described (Alvaro-Benito M. et al 2007; Linde D. et al 2009; 2012). Yeast extract was from Difco and barley malt flour from Diagermal. All other reagents and solvents were of the highest available purity and used as purchased.

Isolation and screening of microorganisms with transfructosylating activity

The fungal cultures employed in the study were isolated from molasses or from commercial and home-made kiwi and fig jams. The contents of each sample were uniformly mixed, and a sample (1.0 mL) was aseptically withdrawn, mixed with 9.0 mL of sterile water, and then diluted for isolation purposes. The inoculum was transferred to Potato Dextrose Agar (PDA) plates containing chloramphenicol (0.05 g/L) to inhibit bacterial growth. Plates were repeatedly incubated at 28°C until obtaining homogenous morphological colonies. Identification of the best performing strains was carried out at CBS (Centraal Bureau voor Schimmelcultures, Baarn, Holland) using standard molecular techniques. The microorganisms were routinely maintained on MYA slants (Barley malt flour 100 g/L, yeast extract 5 g/L, agar 15 g/L, pH 5.5) at 4°C. The screening for FOS production was performed by inoculating the strains into 1 L flasks containing 100 mL of liquid MY medium (Barley malt flour 100 g/L, yeast extract 5 g/L, pH 5.5) containing 200 g/L of sucrose. The growth was carried out on a reciprocal shaker (150 rpm) at 28°C. Sugar composition was analysed by HPLC during the growth after filtration of the mycelium.

Biotransformations with *Cladosporium cladosporioides* and *Penicillium sizovae*

Studies were carried out with mycelium obtained after growth on MY medium for different times (with and without 200 g/L sucrose) under the conditions described above. Mycelia with the highest activity were found when cultures were grown on MY medium in absence of sucrose after 96 h on a reciprocal shaker (150 rpm) at 28°C. After centrifugation, cells were washed with sodium acetate buffer (20 mM, pH 6.0), lyophilised and used for biotransformations.

The activity of mycelium-bound and extracellular enzymes was independently assayed (Gandolfi R. et al 2000). Experiments with mycelium free supernatant were accomplished using the liquid fraction obtained after centrifugation of the whole culture and ultrafiltration using a stirred ultrafiltration cell (Model 8050 Amicon, Millipore, capacity 50 mL) with a 10 kDa cut-off membrane. The extracellular fraction containing 0.41 g/L of total protein (Bradford assay) was used for biotransformation, started by incubating the mixture at 50ºC in an orbital shaker at 90 rpm after addition of 200 g/L of sucrose. Freshly suspended (40 g dry weight/L) and lyophilised mycelium (40 g/L) were added to 200-600 g/L sucrose solutions in 20 mM sodium acetate (pH 6.0) in a total reaction volume of 2 mL. The mixtures were incubated at 50ºC in an orbital shaker at 90

rpm. At different times, aliquots (50 µL) were withdrawn, diluted 1 with 200 µL of water, incubated for 10 min at 90ºC to inactivate the enzymes, and analysed by HPLC to determine the total FOS yield and by HPAEC-PAD to identify the synthesised FOS.

HPLC analysis

The screening of transfructosylation activity and the measurement of the FOS production were carried out analyzing the corresponding reaction mixtures using hydrophilic interaction chromatography (HPLC-HILIC) with a Delta 600 quaternary pump (Waters). The chromatographic column used was a 5-µm Luna-NH_2 100A (4.6 × 250 mm) from Phenomenex and the HPLC detector was a refraction index 2410 from Waters. The mobile phase was acetonitrile/water 78/22 (v/v) at 1 mL/min. The temperature of the column was set at 30°C. Under these conditions the following retention times were observed: Fructose 7.3 min, Glucose 8.8 min, Saccharose 11.7 min, Blastose 13.4 min, Neo-kestose 16.5 min, 1-kestose 19.1 min, 6-kestose 23.2 min, Neo-nistose 25.2 min, 1-nistose 27.5 min, 1-Fructofuranosylnistose 39.3 min

Analysis of FOS by HPAEC-PAD

Analysis of FOS composition was carried out by high-performance anion exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) on a Dionex ICS3000 system consisting of an SP gradient pump, an electrochemical detector with a gold working electrode and Ag/AgCl as reference electrode, and an autosampler (model AS-HV). All eluents were degassed by flushing with helium. A pellicular anion-exchange 4 × 250 mm Carbo-Pack PA-1 column (Dionex) connected to a 4 × 50 mm CarboPac PA-1 guard column was used at 30°C. Eluent preparation was performed with Milli-Q water and NaOH. The initial mobile phase (at 0.5 mL/min) was 100 mM NaOH. A gradient from 0 to 200 mM sodium acetate was performed in 50 min at 0.5 mL/min, and 200 mM sodium acetate was maintained 1 for 25 min. The chromatograms were analysed using Chromeleon software. The identification of the different carbohydrates was done on the basis of standards commercially available or purified in our laboratory.

Isolation of an unknown carbohydrate

The biocatalytic reaction with the *C. cladosporioides* $CF_2$15 enzymatic preparation was scaled up to 10 mL. At the point of maximum concentration of the unknown oligosaccharide, the reaction was stopped by inactivation at 100ºC (10 min) followed by filtration. The mixture was purified by semi-preparative HPLC using a system equipped with a Waters Delta 600 pump coupled to a 5 µm Kromasil-NH_2 column (10 x 250 mm; Analisis Vinicos). A three-way flow splitter (model Accurate, Dionex) and a refraction index detector (Waters, model 2410) equilibrated at 30ºC were used. Acetonitrile/water 70:30 (v/v), degassed with helium, was used as mobile phase at 4.7 mL/min for 40 min. The column temperature was kept constant at 30ºC. After collecting the different oligosaccharides, the mobile phase was eliminated by rotary evaporation using a R-210 rotavapor (Buchi).

Mass Spectrometry

The unknown carbohydrate was analysed by MALDI-TOF mass spectrometry (Bruker, model Ultraflex III TOF-TOF) using 2,5-dihydroxybenzoic acid doped with NaI as matrix, in positive reflector mode.

Nuclear Magnetic Resonance (NMR)

The structure of the unknown carbohydrate was elucidated using a combination of 1H, 13C and 2D-NMR (COSY, TOCSY, NOESY, HSQC, HMBC) techniques. The spectra of the sample (ca. 10 mM), dissolved in deuterated water, was recorded on a Bruker AVANCE DRX500 spectrometer equipped with a tuneable broadband 1H/X probe with a gradient in the Z axis, at a temperature of 298 K. Chemical shifts were expressed in ppm with respect to the 0 ppm point of DSS, used as internal standard. COSY, NOESY, HSQC, HSQC-TOCSY, DEPT-HSQC and HMBC sequences were provided by Bruker. COSY, TOCSY (80 ms mixing time), and NOESY (500 ms mixing time) experiments were performed with 8, 32, and 64 scans, respectively, with 256 increments in the indirect dimension and with 1024 points in the acquisition dimension. The spectral widths were 5 ppm in both dimensions. The HSQC and related experiments (16 scans) also used 256 increments in the indirect dimension and 1024 points in the acquisition dimension. The HMBC (64 scans) used 384 increments in the indirect dimension and 1024 points in the acquisition dimension. The spectral width for the heteronuclear correlations was 120 ppm in the indirect dimension and 5 ppm in the acquisition one.

Result and discussion

Screening of transfructosylation activity

Filamentous fungi (45 strains), isolated from sucrose-rich environments (jams and molasses), were firstly grown on a MY medium (see Experimental Section) containing 200 g/L sucrose, and FOS production was followed during the growth. The carbohydrate composition of the supernatant was analysed by HPLC-HILIC, showing that five 14 strains (Table 1) were able to produce FOS in different concentration (referred to the total amount of sugars in the sample) composition (indicated in the table by the kestose/nystose ratio, K/N). Two strains ($CF_2$15 and CK1, identified as *Cladosporium cladosporioides* and *Penicillium sizovae*, respectively) gave maximum FOS production in shorter times (48 h) and were selected for their ability to synthesize FOS with different K/N ratios (0.8 and 6.0, respectively), at the point of maximum FOS concentration. Notably, *Cladosporium cladosporioides* gave the highest production of total FOS, whereas *Penicillium sizovae* was very selective, furnishing kestose as the main product.

Table 1. Initial screening of FOS-synthesizing microorganisms in fruit jams and molasses. Experimental conditions: biotransformations with growing cells in MY medium (including 200 g/L sucrose), 28ºC, 150 rpm. Carbohydrates: F, fructose; G, glucose; S, sucrose; K, total kestoses; N, total nystoses; FN fructosylnystose; K/N, kestose/nystose ratio.

Strain	**Time (h)**[a]	**Composition (%)**[b]						**K/N**
		F (%)	**G (%)**	**S (%)**	**K (%)**	**N (%)**	**FN (%)**	**ratio**
$CF_2$3V	96	2	33	9	32	24	-	1.3
$CF_2$4V	96	8	43	9	12	18	9	0.7
$CF_2$15	48	5	34	6	23	27	5	0.8
CK1	48	12	25	35	24	4	-	6.0
M1A	96	4	30	13	36	17	-	2.1
$CF_1$1	72	2	18	50	21	9	-	4,4
$CF_1$2	48	11	14	69	3	-	-	-
$CF_2$7	96	3	12	67	13	7	-	-
$CF_2$9V	48	48	47	3	3	-	-	-
$CF_2$11	72	3	5	87	4	-	-	-
$CF_2$12	96	8	20	57	10	5	-	2.0
$CF_2$14	96	6	31	43	18	4	-	4.0
$CF_2$16	48	2	24	47	12	5	-	2.4
SD4	96	5	26	58	16	5	-	3.2

[a] Time of maximum FOS production

[b] Weight percentage referred to the total amount of sugars in the mixture

Total FOS production with lyophilised mycelium of *C. cladosporioides* and *P. sizovae*

The extracellular and cell-bound transfructosylating activity of *P. sizovae* and *C. cladosporioides* to produce FOS was evaluated using cultures grown on liquid MY medium. Cell-free supernatant and washed mycelium were independently assayed to locate the transfructosylating activity. The supernatant gave conversions into total FOS lower than 5% for both strains, showing that the activity was mostly mycelium-bound. No significant differences were observed in the activity of mycelium grown in the medium with or without sucrose. Therefore, mycelium of cultures grown in liquid MY medium without sucrose was used for further experiments aimed at optimization and product characterization. Mycelia did not lose any significant activity upon lyophilisation (data not shown). It is well reported that lyophilised mycelia of fungi are easy-to-handle biocatalysts often showing remarkable long-term stability (Gandolfi R. et al 2000; Converti A. et al 2002). Lyophilised mycelia of the two strains were used for FOS production using various initial sucrose concentrations (200-600 g/L), and the highest yields of total FOS were obtained with 600 g/L of sucrose (data not show).

The time course of total FOS formation with *P. sizovae* and *C. cladosporioides* using 600 g/L sucrose was followed by HPLC-HILIC and is represented in Figs. 1A and 1B, respectively. *P. sizovae* mycelium produced 184 g/L of total FOS (31% w/w of total sugars, after 24 h), which was obtained at 53% sucrose conversion, whereas *C. cladosporioides* mycelium synthesised 339 g/L of FOS (56% w/w, after 72 h) at 93% sucrose conversion. The fact that maximum FOS concentration with *C. cladosporioides* enzyme was obtained when only 7% of initial sucrose remains in the mixture indicates that the transglycosylation to hydrolysis ratio of this enzyme is notable (Plou F.J. et al 2009). The FOS yield obtained with *C. cladosporioides* is close to the maximum values reported (around 60%) for the industrial processes with *Aspergillus* or *Aureobasidium* sp. enzymes (Plou F.J. et al 2009; Fernandez R.C. et al 2004; Sangeetha P.T. et al 2005).

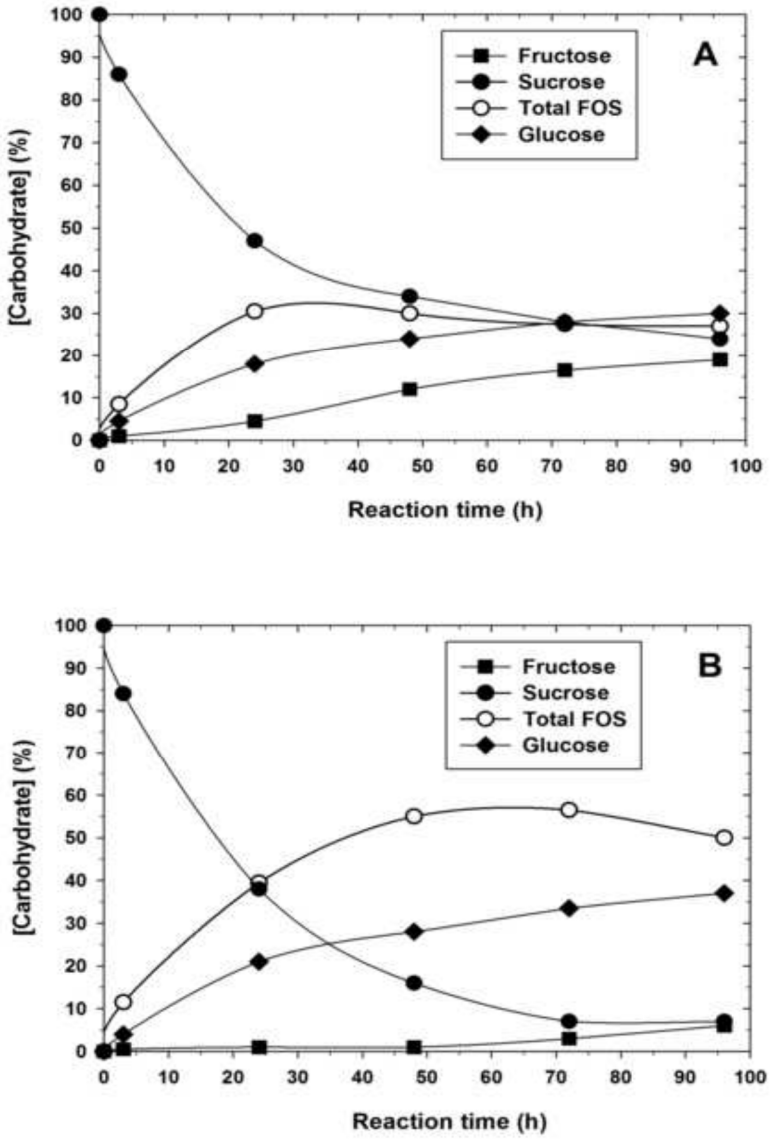


Figure 1. Time course of the reaction of sucrose with P. sizovae (A) and C. cladosporioides (B) mycelia. Reaction conditions: 600 g/L sucrose, 40 g/L lyophilised mycelium, 20 mM sodium acetate buffer (pH 6.0), 50ºC. Standard deviations were lower than 5%.

Characterization of synthesised FOS

HPAEC-PAD was employed for the characterization of the FOS synthesised in the reactions with *P. sizovae* and *C. cladosporioides* using 600 g/L sucrose. According to the chromatograms presented in Figure 2, we detected at least 13 different carbohydrates in the reactions mediated by *P. sizovae* (Fig. 2A) and *C. cladosporioides* (Fig. 2B) mycelia. Peaks 1, 2, and 3 corresponded to glucose, fructose and sucrose, respectively. As illustrated in the chromatogram 2B, the main products present in the reaction mixture with *C. cladosporioides* were peaks 4 (1-kestose) and 9 (nystose). Peaks 7, 8 and 10 were identified as 6-kestose, neokestose and neonystose, respectively, using standards previously purified in our laboratory as described (Alvaro-Benito M. et al 2007; Linde D. et al 2009). Peak 12 was the pentasaccharide 1F-fructosylnystose. The oligosaccharides corresponding to peaks 6, 11 and 13 could not be identified so far. Figure 3 illustrates the structures of the different carbohydrates obtained in these reactions. The compound corresponding to peak 5 was purified by semi-preparative HPLC. Its mass spectrum showed that it was a disaccharide. The 1D and 2D 1H NMR spectra displayed two anomeric signals, arising from the typical α/β equilibrium and a signal pattern recognizable as fructose and glucose residues. From the combination of the signals from COSY, TOCSY, NOESY, HSQC and HMBC spectra, full assignment of the 1H and 13C resonance signals belonging to the different residues was achieved. The glycosylation position 1 was determined from the existence of a crosspeak between the H6 from glucose and the quaternary carbon C2 from fructose in the HMBC spectrum. The NMR data unequivocally permitted to identify the compound as blastose [Fru-β(2→6)-Glc] (Figure 4), a sucrose isomer member of the neoFOS series. Despite it is a non-conventional disaccharide, the isolation and chemical characterization of blastose was first described in submerged cultures and honeydew of Claviceps africana and Clavicesshorgi (Flieger M. et al. 2012). Besides forming polyfructans, the levansucrase from Bacillus megaterium also synthesised five different oligosaccharides including blastose (Homann A et al 2007).

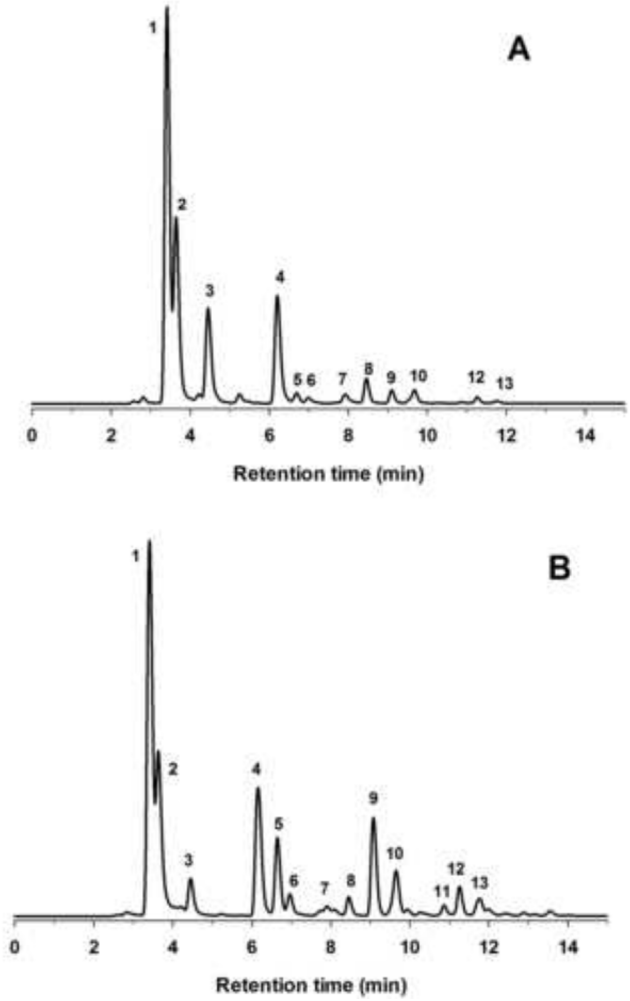


Figure 2. HPAEC-PAD analysis of the reaction of sucrose with P. sizovae (A) and C. cladosporioides (B) mycelia at the point of maximum FOS concentration. Peaks: 1: glucose; 2: fructose; 3: sucrose; 4: 1-kestose; 5: blastose; 7: 6-Kestose; 8: neokestose; 9:1- nystose; 10: neo-nystose; 12: 1F-fructosylnystose; 6, 11, 13: unknown.

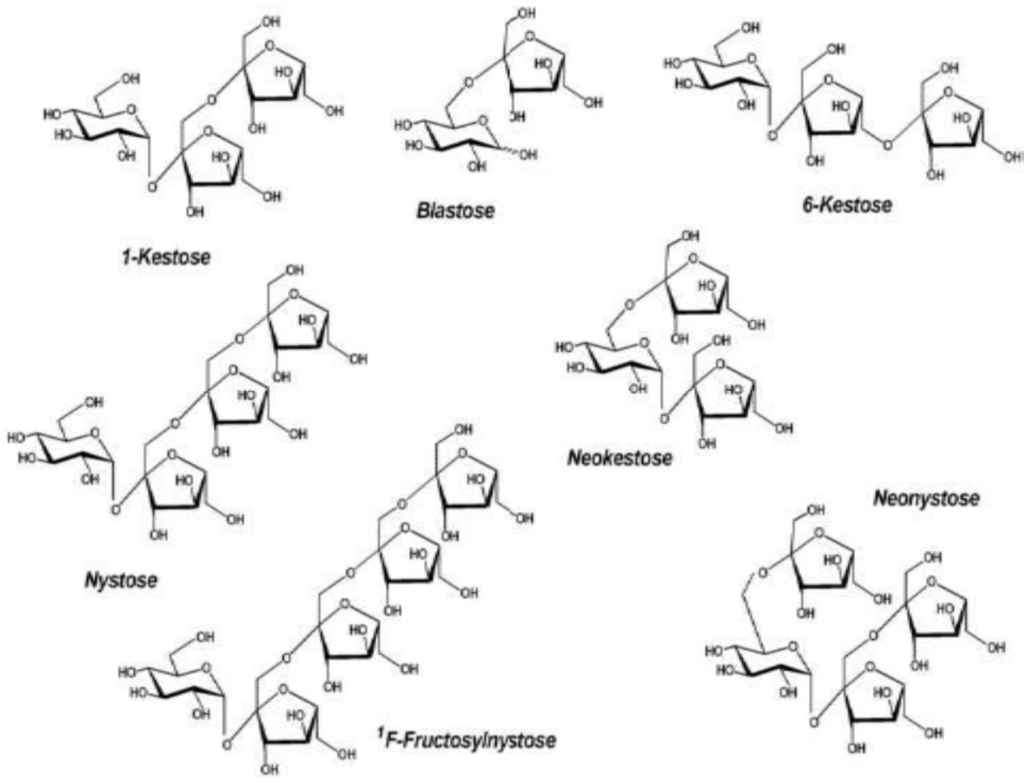


Figure 3. Structure of the fructooligosaccharides produced by P. sizovae and C. cladosporioides transfructosylating activity.

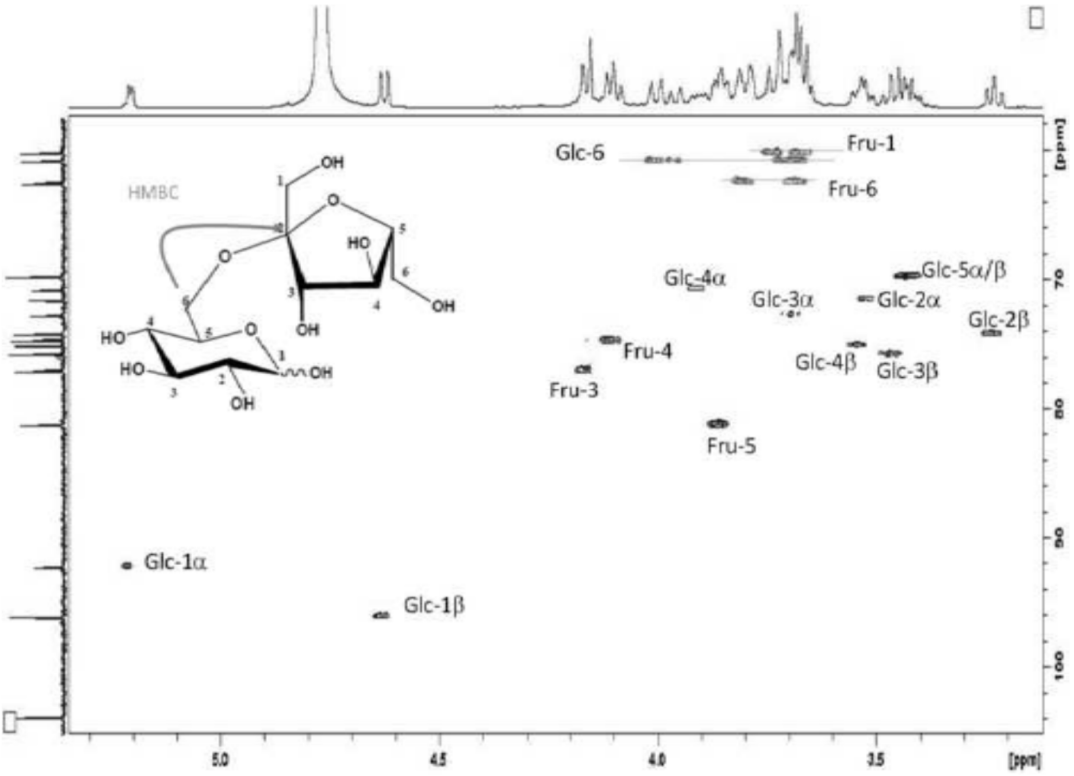


Figure 4. 2D-NMR DEPT-HSQC spectra of blastose [Fru-β(2→6)-Glc]. The signals are assigned and labelled. The key points for identifications are also shown.

Production of the different FOS

The FOS formation was analysed in detail using HPAEC-PAD. Figure 5 illustrates the profile of the biotransformation with lyophilised mycelium of *C. cladosporioides* starting from 600 g/L of sucrose. At the point of maximum FOS concentration (72 h), the FOS fraction was mainly composed of 1-kestose (158 g/L) and nystose (97 g/L), with formation of lower amounts of the disaccharide blastose (34 g/L), 1F-fructosylnystose (19 g/L), 6-kestose (12 g/L) and neokestose (10 g/L). Neonystose was only slightly detected at the end of the reaction (96 h). Such a complex mixture of 1F-FOS, 6F-FOS and 6G-FOS has only been described with the β- fructofuranosidase from Rhodotorula dairenensis (Gutierrez-Alonso P. et al 2013). The concentration of neokestose never surpassed 10 g/L throughout the reaction; however, blastose concentration was significantly higher (> 30 g/L) after 48h. This result suggests that blastose i 1 s not formed by hydrolysis of neokestose, but by the transfer of fructosyl moiety to the released glucose in the medium. In fact, the biosynthetic activity detected in the *C. cladosporioides* mycelium is not very efficient to hydrolyze the β(2→6) linkages between a fructose and a glucose, as the neoFOS concentration is not diminishing throughout the process, in contrast with 1F-FOS (Fig. 5).

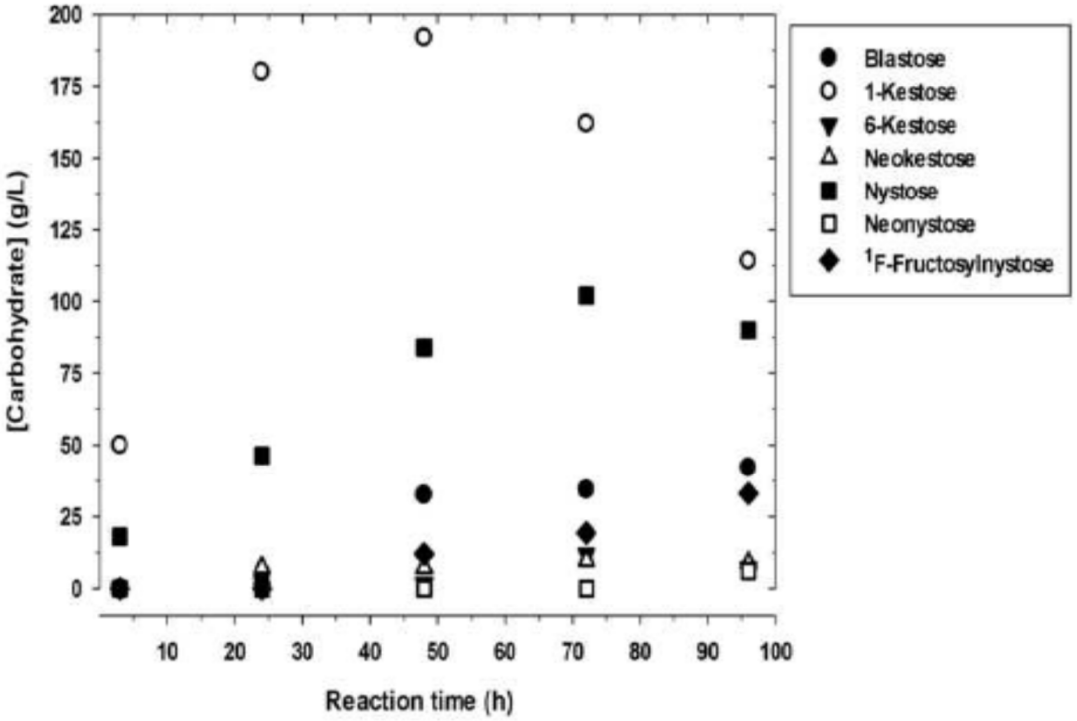


Figure 5. Kinetics of FOS formation using 600 g/L sucrose catalysed by lyophilised C. cladosporioides. Reaction conditions: 40 g/L lyophilised mycelium, 20 mM sodium acetate buffer (pH 6.0), 50ºC. Standard deviations were lower than 5%.

Figure 6 shows the formation of the different FOS with lyophilised mycelium of *P. sizovae*. At the point of maximum FOS yield (24 h), 156 g/L out of the total FOS concentration (184 g/L) corresponded to 1-kestose. The FOS fraction was completed with nystose (11 g/L), neokestose (6 g/L) and neonystose (11 g/L). The *P. sizovae* enzyme displays a more typical profile with major formation of 1F-FOS. However, its transglycosylation to hydrolysis ratio is less favourable than that of *C. cladosporioides.* It is interesting to note the negligible presence of blastose with the *P. sizovae* enzyme, which indicates its much lower tendency to use glucose as acceptor to form β(2→6) linkages. (Gutierrez-Alonso P. et al 2009)

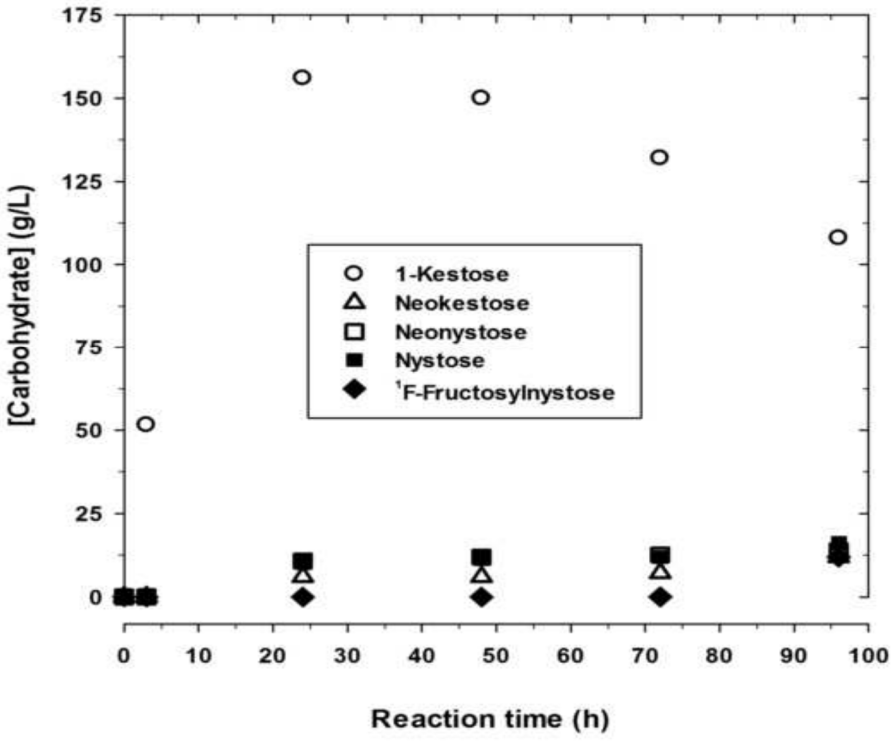


Figure 6. Kinetics of FOS formation using 600 g/L sucrose catalysed by lyophilised P. sizovae (40 g/L). Reaction conditions: 40 g/L lyophilised mycelium, 20 mM sodium acetate buffer (pH 6.0), 50ºC. Standard deviations were lower than 5%.

Conclusions

The main enzymes used for industrial production of FOS generally provide a mixture of molecules with the inulin-type structure, 1F-FOS, whereas those from yeasts usually form levan-type FOS (6F-FOS) or neoFOS (6G-FOS). In this work, two filamentous fungi (*Cladosporium cladosporioides* and *Penicilium sizovae*) showing mycelium-bound transfructosylating activity were isolated. Maximum FOS yields were 56% and 31% for *C. cladosporioides* and *P. sizovae* respectively. Interestingly, *C. cladosporioides* synthesised a mixture of 1F-FOS, 6F-FOS and 6G-FOS, including the presence of a non-conventional disaccharide (blastose). Considering that the FOS yield with *C. cladosporioides* is close to that obtained with typical *Aspergillus* or *Aureobasidium* enzymes, the formation of a mixture of FOS with different glycosidic linkages could give rise to certain benefits regarding their bioactivity.

References

Alvaro-Benito M., de Abreu M., Fernandez-Arrojo L., Plou F.J., Jimenez-Barbero J., Ballesteros A., Polaina J., Fernandez-Lobato M., 2007, Characterization of a β- fructofuranosidase from *Schwanniomyces occidentalis* with transfructosylating activity yielding the prebiotic 6-kestose. J Biotechnol; 132:75-81.

Linde D., Macias I., Fernandez-Arrojo L., Plou F.J., Fernandez-Lobato M., 2009, Molecular and biochemical characterization of a β-fructofuranosidase from *Xanthophyllomyces dendrorhous*. Appl Environ Microbiol; 75:1065-1073

Linde D., Rodriguez-Colinas B., Estevez M., Poveda A., Plou F.J., 2012, Fernandez-Lobato M. Analysis of neofructooligosaccharides production mediated by the extracellular β-fructofuranosidase from *Xanthophyllomyces dendrorhous*. Bioresour Technol; 109:123-130.

Gandolfi R., Marinelli F., Lazzarini A., Molinari F., 2000, Cell-bound and extracellular carboxylesterases from streptomyces: hydrolytic and synthetic activities. J Appl Microbiol; 89:870-875.

Gandolfi R., Converti A., Pirozzi D., Molinari F., 2001, Efficient and selective microbial esterification with dry mycelium of *Rhizopus oryzae*. J Biotechnol; 92:21-26

Converti A., Del Borghi A., Gandolfi R., Lodi A., Molinari F., Palazzi E., 2002, Reactivity and stability of mycelium-bound carboxylesterase from *Aspergillus oryzae*. Biotechnol Bioeng; 77:232-237.

Plou F.J., Alcalde M., Ghazi I., Fernandez-Arrojo L., Ballesteros A., 2009, Efficient fructooligosaccharide synthesis with a fructosyltransferase from *Aspergillus aculeatus*. In: Fessner WD, Anthonsen T editors. eds. Modern Biocatalysis: Stereoselective and enviromentally friendly reactions. Weinheim, Germany: Wiley-VCH ;. p. 153-71.

Fernandez R.C., Maresma B.G., Juarez A., Martinez J., 2004, Production of fructooligosaccharides by beta-fructofuranosidase from *Aspergillus sp* 27H. J Chem Technol Biotechnol; 79:268-272.

Sangeetha P.T., Ramesh M.N., Prapulla S.G., 2004, Production of fructo-oligosaccharidesby fructosyl transferase from *Aspergillus oryzae* CFR 202 and *Aureobasidium pullulans* CFR 77. Process Biochem; 39:753-758.

Flieger M., Kantorova M., Halada P., Kuzma M., Pazoutova S., Stodulkova E., Kolinska R., 2005, Oligosaccharides produced by submerged cultures of *Claviceps africana* and *Claviceps sorghi*. *Folia* Microbiol; 50:198-204

Homann A., Biedendieck R., Gotze S., Jahn D., Seibel J., 2007, Insights into polymer versus oligosaccharide synthesis: mutagenesis and mechanistic studies of a novel levansucrase from *Bacillus megaterium*. Biochem J; 407:189-198.

Gutierrez-Alonso P., Fernandez-Arrojo L., Plou F.J., Fernandez-Lobato M., 2009, Biochemical characterization of a β-fructofuranosidase from *Rhodotorula dairenensis* with transfructosylating activity. FEMS Yeast Res; 9:768-773.

Sweet-and-salty biocatalysis: fructooligosaccharides production using *Cladosporium cladosporioides* in seawater

Materials and methods

Chemicals

Sucrose, glucose, fructose, 3,5-Dinitrosalicylic acid (DNS) and were from Sigma Aldrich. 1-Kestose ND 1-nystose were from Fluka. 1-Fructofuranosylnystose was from Megazyme. Actilight© was from Beghin Meiji. Yeast extract was from Difco and barley malt flour from Diagermal. Malt extract was from Merck. All reagents and solvents were of the highest available purity and used as purchased.

Seawater (pH 7.5) was collected from the Camogli beach (Italy) and maintained at 4°C after microfiltration; water salinity of 35 PSU (Practical Salinity Units) was reported by ARPA (Agenzia Regionale Prevenzione e Ambiente) website for this area.

Culture production

C. cladosporium was previously isolated (Zambelli et al., 2014) and has been deposited at Mycotheca Universitatis Taurinenesis (MUT) as strain MUT 5506. *C. cladosporium* was routinely maintained on MYA slants (Barley malt flour 100 g/L yeast extract 5 g/L, agar 15 g/L, pH 5.6) at 4 °C. The strain, grown on MYA slants for 96 h at 28 °C, were inoculated into 1 L Erlenmeyer flasks containing 100 mL of the liquid medium MY and incubated on a reciprocal shaker (150 spm) for 96 h at 28 °C. Mycelia were recovered by centrifugation and lyophilised.

Calculation of the T/H index

The T/H index was calculated, using the following equation:

T/H = ([glucose] − [fructose])/[fructose].

Assays of fructofuranosidase activity

The enzymatic activity was assayed using sucrose as substrate by measuring the release of reducing sugars by the dinitrosalicylic acid (DNS) method adapted to a 96-well microplate scale (Rodriguez et al., 1995). A calibration curve was performed using fructose as standard (linearity range between 0.6 and 3 g/L of fructose). Lyophilised mycelia 10 g/L were incubated with 1 mL of a sucrose solution (100 g/L) in acetate buffer (20 mM pH 6.0) for 20 min at 50ºC and 90 rpm. 50 µL of the solution conveniently diluted to fit into the calibration curve, were added to each well. Then, 50 µL of 10 g/L DNS were added. Plates were incubated for 20 min at 80ºC to develop colour with a seal plate tape (GeneMate). After cooling 150 µL of water were added to each well, and the absorbance measured at 540 nm using a microplate reader (model Versamax, Molecular Devices). One unit (U) of activity was defined as that catalysing the formation of 1 µmol reducing sugar per minute under the above conditions. Lyophilised mycelia were incubated in the range of pH 3-9 and temperature 25-80 ºC to evaluate the maximum of activity. Residual activity of lyophilised mycelia was

measured after 24 h of incubation, before substrate addition, at pH and temperatures in the range of 3-9 and 40-80ºC respectively. The residual activity was measured at pH 6.0 acetate buffer 20 mM, 50ºC as described above.

Dried alginate entrapped (DALGEE) mycelium

The gel beads were prepared by ionotropic gelation as described elsewhere (Fernandez-Arrojo et al., 2013) with some variations. A 4% (w/v) sodium alginate solution was prepared in distilled water and stirred until a homogeneous clear solution was observed. The solution was let to settle for 2 h in order to eliminate all air bubbles. The alginate solution was then gently mixed in a ratio 1:1 (w/w) with 40 g/L of lyophilised mycelia in 20 mM sodium acetate buffer 6.0 after 5 cycle of sonication at 15 kHz for 1 min (Soniprep 150, MSE). The resulting mycelia–alginate mixture was used as previously described (Fernandez-Arrojo et al., 2013). Immobilisation efficiency - estimated with DNS test – was calculated as the ratio between the activity of DALGEEs and the total U/g of enzyme utilised in the immobilisation process. Recycle tests were carried out by repeated DNS-reactions using double washed immobilised mycelia at a concentration of sucrose of 600 g/L.

Biotransformation with DALGEE mycelium

40 g/L of DALGEEs were added to a solution of 600 g/L of sucrose in seawater or sodium acetate buffer 20 mM pH 6.0 in a final volume of 2 mL, incubated at 50°C in an orbital shaker at 90 rpm. Biotransformations were followed for 144 h and analysed with HPLC.

HPLC analysis

The samples were analysed by HPLC with a quaternary pump Delta 600, Waters coupled to a 5 µm Luna-NH_2 100 A column 4.6*250 mm (Phenomenex) with an isocratic elution in acetonitrile:water 78:22 at 1 mL/min. The detector was a refraction index 2410, Waters. Under these conditions the following retention times were observed: Fructose 7.3 min, Glucose 8.8 min, Saccharose 11.7 min, Blastose 13.4 min, Neo-kestose 16.5 min, 1-kestose 19.1 min, 6-kestose 23.2 min, Neo-nistose 25.2 min, 1-nistose 27.5 min, 1-Fructofuranosylnistose 39.3 min.

Results and Discussion

Activity of the mycelium-bound β-fructofuranosidase

The use of lyophilised mycelium of *Cladosporium cladosporioides* MUT 5506 in freshwater allowed for the production of FOS with maximum yields of 57% starting from 600 g/L of sucrose (Zambelli et al. 2014). Interestingly, *C. cladosporioides* synthesised a mixture of ^{1F}-FOS, ^{6F}-FOS and ^{6G}-FOS, including the presence of a non-conventional disaccharide (blastose). With the aim to explore the possibility to use "alternative" water sources, we decided to implement the whole FOS-production bioprocess in sea-water: *C. cladosporioides* was grown in MY medium (Zambelli et al. 2014) substituting deionised water with seawater and the activity of mycelium-bound β-fructofuranosidase was evaluated using different amounts of lyophilised mycelium in seawater (Table 1), using 600 g/L of sucrose as substrate at 30°C. Maximum FOS production was always registered after 96 h.

Table 1. Maximum FOS production and fructofuranosidase activity. FOS production calculated after 96 h of biotransformation with different amount of Cladosporium cladosporioides lyophilised mycelium in seawater (pH 7.5), with a initial sucrose concentration of 600 g/L.

Amount of mycelium (g/L)	Maximum FOS production (g/L)
10	108
20	164
30	188
40	208
50	194

The highest yields were obtained using 40 g/L of dry mycelium (208 g/L), and this biocatalyst concentration was used in the following experiments aimed at evaluating the effects of temperature. The reaction rates increased up to 60°C and then decreased rapidly due to thermal inactivation (Figure 1). Thermal stability in seawater was determined by measuring the residual activity after 24 h of incubation over a temperature range from 25 to 80°C (Figure 1). Mycelium-bound activity was fully maintained up to 50 ºC, above which a gradual decrease of stability was observed, with significant inactivation (40 % of lost activity) at temperatures above 80ºC. Thus, the best compromise between activity and stability was found at 50ºC.

Figure 1

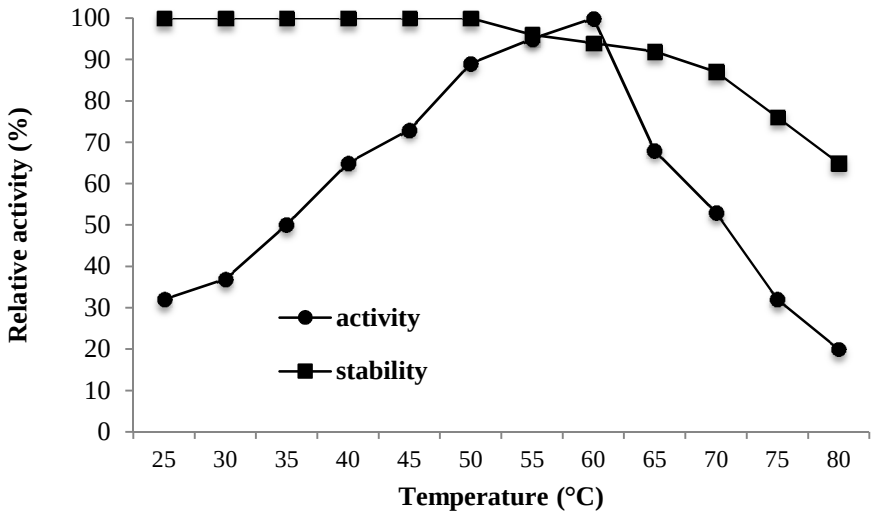


Figure 1. Effect of temperature on activity and stability of lyophilised mycelium of Cladosporium cladosporioides in seawater (pH 7.5). Stability is defined as residual activity determined at 50°C after pre-incubation of the lyophilised mycelia for 24 h in seawater at different temperatures.

The time-course of FOS production carried out under optimised conditions (40 g/L of lyophilised mycelium, 50°C, 600 g/L of sucrose) in seawater is reported in Figure 2. It should be pointed out that in all the experiments seawater was merely microfiltered with no further optimization/modification (pH, salt composition).

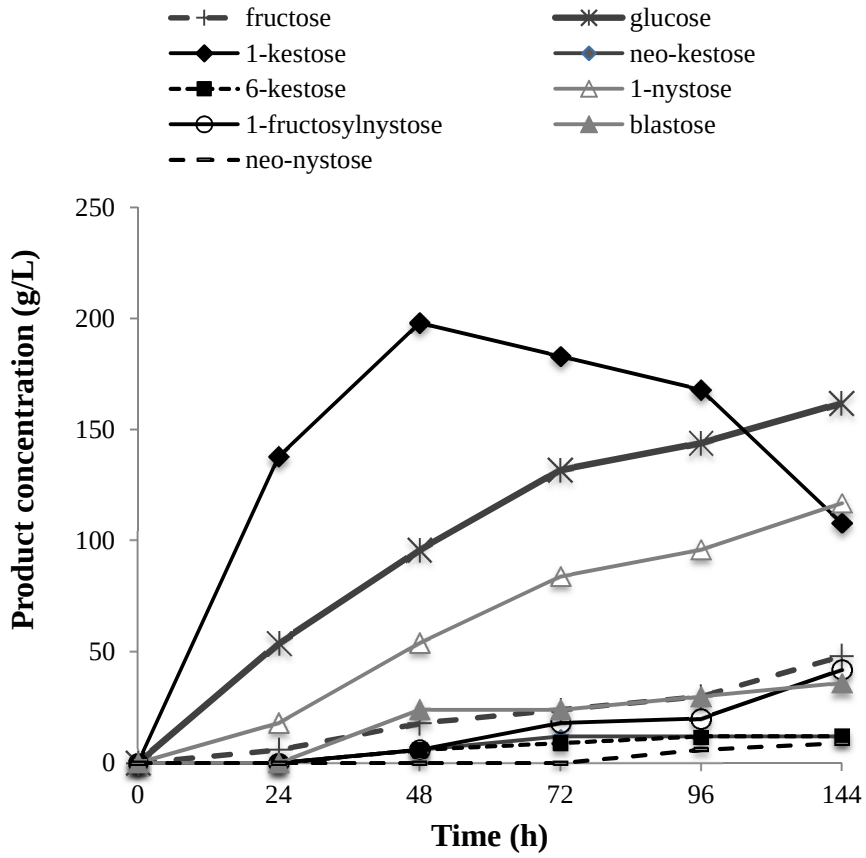


Figure 2. Time-course of FOS production with lyophilised mycelium of C. cladosporioides. Reaction conditions: 600 g/L sucrose, 40 g/L lyophilised mycelium, seawater (pH 7.5), 50°C. Results are the average of three triplicates, with standard deviations lower than 5%.

The highest amount of FOS was 344 g/L after 96 h. At this point the main reaction products were mostly $^{1F\text{-}FOS}$ derivatives (1-kestose 168 g/L, 1-nystose 96 g/L and 1-fructofuranosylnystose 20 g/L); concentrations below 3% of 6-kestose (12 g/L), neo-kestose (12 g/L), and neo-nystose (6 g/L) were also observed. The presence of the non-conventional disaccharide blastose was estimated in 30 g/L. The ratio between transfructosylating and hydrolytic activity varied from 4.5 (48 and 72 h) to 3.8 (at 96 h). Biotransformations were also carried out in synthetic seawater for comparison, showing similar results to the ones obtained in real seawater (data not shown).

Immobilisation studies

With the aim to further improve the system productivity *C. cladosporioides* was immobilised by employing a modification of the classical calcium alginate entrapment technique to overcome some of the limitations of the typical alginate-based biocatalysts, i.e., the effect of non-gelling cations occurring in seawater (mostly Na^+) on the stability of the alginate beads. Dried alginate entrapped (DALGEE) mycelium was obtained by dehydrating calcium alginate gel beads containing the entrapped mycelium, following the optimised procedure previously reported (Alcalde et al., 2013). The initial specific activity of the mycelium-bound β-fructofuranosidase of *C. cladosporioides* was 25.1 U/g, while, after the immobilisation, the specific activity was 16.4 U/g, with an apparent immobilisation efficiency of 65%. The highest amount of FOS (307 g/L) was observed after 96 h (1-kestose 139 g/L, 1-nystose 96 g/L and 1-fructofuranosylnystose 15 g/L; blastose 30 g/L; 6-kestose 9 g/L; neo-kestose 12 g/L and neo-nystose 6 g/L). Recycling of DALGEE-mycelium was studied in a batch reactor measuring the activity of the beads in a sequence of reaction cycles; reuse was carried out in seawater and in conventional buffer for comparison. Figure 3 illustrates the operational stability of the DALGEE-mycelium in 12 subsequent reaction cycles of 20 min each.

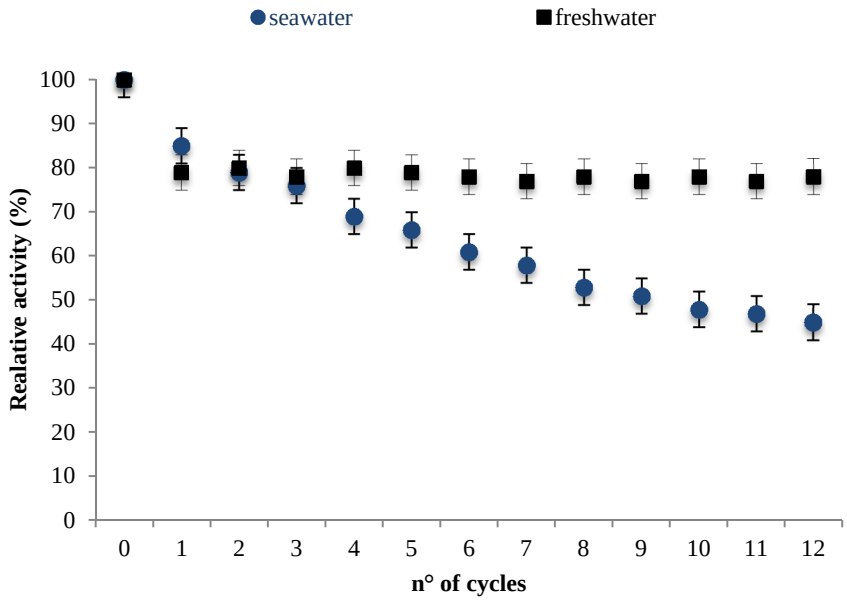


Figure 3. Reuse assay of DALGEE-mycelium of C. cladosporioides in successive batch reactions. After each reaction cycle (20 min), the DALGEE-mycelium was separated from the reaction medium and washed.

After 12 cycles of reutilisation, the DALGEE mycelium in seawater showed 46-48% of initial activity, whereas 78-80% residual activity was found when the beads were employed in acetate buffer. Although the DALGEE system partly prevents from the dissolution of the Ca-alginate beads due the non-gelling ions occurring in seawater, which also helps to prevent re-swollen of beads, a gradual loss of activity was observed, while the activity in a conventional buffer was very stable after the activity lost in the first re-use. Nervertheless this technology, given its characteristics in terms of cheapness, easiness of production and scalability, seems a promising strategy for the exploitation of *C. cladosporioides* as biocatalyst in continuous processes.

Conclusions

The prospect to perform bioprocesses avoiding the use of drinkable water seems a noticeable way for a sustainable use of natural resources. With this in mind in this work we reported the first production of alternative sweeteners entirely accomplished in seawater. Mycelium of *C. cladosporioides* MUT 5506, grown in a seawater-based medium, was able to promote the formation of 344 g/L (57% w/w) of total FOS from 600 g/L of sucrose in seawater, with performances slightly better than what observed in conventional buffers. Mycelium immobilization in dry alginate beads and recycle studies in repeated-batch mode were also studied, laying the groundwork for a future continuous process.

References

Fernandez-Arrojo, L., Rodriguez-Colinas, B., Gutierrez-Alonso, P., Fernandez-Lobato, M., Alcalde, M., Ballesteros, A. O., & Plou, F. J. 2013. Dried alginate-entrapped enzymes (DALGEEs) and their application to the production of fructooligosaccharides. Process Biochemistry, *48*, 677-682.

Chien, C. S., Lee, W. C., Lin, T. J., 2001. Immobilization of *Aspergillus japonicus* by entrapping cells in gulten for production of FOS. Enzyme Microb. Technol., 29, 252–257.

Dionisi, H.M., Lozada, M., Olivera, N.L., 2012. Bioprospection of marine microorganisms: biotechnological applications and methods. Rev. Argent. Microbiol. 44, 49–60.

Domínguez de María, P., 2013. On the use of ueawater as reaction media for large-scale applications in biorefineries. ChemCatChem 5, 1643–1648.

Ghazi, I., Fernandez-Arrojo, L., Garcia-Arellano, H., Plou, F. J., Ballesteros, A., 2007. Purification and kinetic characterization of a fructosyltransferase from *Aspergillus aculeatus.* J. Biotechnol. 128: 204–211.

Hayashi, S., Yoshiyama, T., Fuji, N., Shinohara, S., 2000. Production of a novel syrup containing neofructooligosaccharides by the cells of *Penicillium citrinum*. Biotechnol. Lett. 22, 1465–1469..

L'Hocine, L., Wang, Z., Jiang, B., Xu, S., 2000. Purification and partial characterization of fructosyl transferase and invertase from *Aspergillus niger* AS0023. J. Biotechnol. 81, 73–84.

Rodrıguez, J., Perez, J.A., Ruiz, T., Rodrıguez, L., 1995. Characterization of the invertase from *Pichia anomala*. *Biochem. J*. 306. 235–239.

Sabater-Molina, M., Larqué, E., Torrella, F., Zamora, S., 2009. Dietary fructooligosaccharides and potential benefits on health. J. Physiol. Biochem. 65, 315–328.

Sangeetha, P.T., Ramesh, M.N., Prapulla, S.G., 2004. Production of fructooligosaccharides by fructosyl transferase from *Aspergillus oryzae* CFR 202 and *Aureobasidium pullulans* CFR 77. Process Biochem. 39, 753–758.

Sangeetha, P.T., Ramesh, M.N., Prapulla, S.G., 2005. Recent trends in the microbial production, analysis and application of fructooligosaccharides. Trend Food Sci. Technol. 16, 442–457.

Trincone, A., 2011. Marine biocatalysts: enzymatic features and applications. Mar. Drugs 2010, 8, 608-628.

Zambelli, P., Fernandez-Arrojo, L., Romano, D., Santos-Moriano, P., Gimeno-Perez, M., Poveda, A., Gandolfi, R., Fernández-Lobato, M., Molinari, F., Plou, F.J., 2014. Production of fructooligosaccharides by mycelium-bound transfructosylation activity present in *Cladosporium cladosporioides* and *Penicilium sizovae*. Process Biochem. DOI: 10.1016/j.procbio.2014.09.021

An efficient continuous flow process for the synthesis of a *non*-conventional mixture of fructooligosaccharides

Materials and methods

Materials

Sucrose, glucose, fructose and p-anisaldehyde were purchased from Sigma-Aldrich. Standards of 1-Nystose and 1-kestose were purchased from Fluka (DE). A standard of 1-F-fructofuranosylnystose was purchased from Megazyme. Actilight® was kindly donated by Beghin Meiji. Gluzyme MONO10000 preparation was kindly donated by Novozymes. Yeast extract was from Difco (Difco, MD, USA) and barley malt flour were purchased from Diagermal (IT). Alginate beads of mycelia of MUT5506 were prepared as previously reported (Zambelli, et al., submitted**).**

Flow chemistry equipment

A commercially available R2+/R4 combination flow reactor commercially available from Vapourtec was used.

Growth and maintaining of the microorganisms

C. cladosporium, previously isolated and deposited at Mycotheca Universitatis Taurinenesis (MUT) as strain MUT 5506, was routinely maintained in MY medium at 28°C for 96h as previously described (Zambelli, et al., 2014).

Five probiotic *Lactobacillus* strains (*Lactobacillus paracasei* DG, *Lactobacillus rhamnosus* GG, L*actobacillus paracasei* SHIROTA, *Lactobacillus johnsonii* LC1, *Lactobacillus reuteri* ATCC55730) used in this study were cultivated in MRS broth (Difco) and incubated at 37°C for 24 hours. The strains were inoculated in triplicate at a final inoculum concentration of $4*10^5$ bacteria/mL (with the bacteria from cultures grown overnight to the stationary phase). The bacterial cell concentration of the overnight culture was determined microscopically with a Neubauer improved counting chamber (Marienfeld GmbH, Lauda-KÖnigshofen, Germany). The medium used to test the growth with different sugars was API 50 CHL medium without glucose, prepared at different dilutions. The medium was added with a 0.5% concentration of different sugars (di- and oligosaccharides): glucose (as positive control), inulin, blastose and a commercially available mixture of FOS (Actilight®) (Taveriniti et al., 2012). *In vitro* experiments were carried out in 384 well plates, filled by means of an automated pipetting system epMotion 5070 (Eppendorf, Germany). The microbial growth was monitored with a spectrophotometer (MicroWave RS2, Biotek, USA) programmed for 145-290 readings (OD 600 nm) every 10 min for 24-48 h at 37°C. At the end of the incubation, the Vmax and the final OD at 600 nm were calculated using the software Gen5 (Biotek, USA) and reported as the mean of tree independent measurements ± standard deviation (Arioli et al., 2014).

Analytical HILIC-HPLC

The samples were analysed by HPLC with a quaternary pump Delta 600, Waters coupled to a 5 µm Luna-NH_2 100 A column 4.6*250 mm (Phenomenex) with an isocratic elution in acetonitrile:water 78:22 at 1 mL/min. The detector was a refraction index 2410, Waters. Under these conditions the following retention times were observed: Fructose 7.3 min, Glucose 8.8 min, Saccharose 11.7 min, Blastose 13.4 min, Neo-kestose 16.5 min, 1-kestose 19.1 min, 6-kestose 23.2 min, Neo-nistose 25.2 min, 1-nistose 27.5 min, 1-Fructofuranosylnistose 39.3 min.

Semi-Preparative HILIC-HPLC

The semi-preparative purifications were performed with a commercially HPLC system composed by a Hitachi LaChrom L-7100 pump available from Merck equipped with a Sedex 75 evaporative light scattering detector (ELSD) available from Sedere. The chromatographic separation of products was performed with a Luna NH_2 100 Å column (250 x 10 mm, particle size 5 µm, Phenomenex, Aschaffenburg, Germany) kept at ambient temperature.

The following gradient of was used:

- 0’-30’ acetonitrile/water (80:20 v/v);

- 30’-35’ switching to acetonitrile/water (50:50 v/v)

- 35’-40’ acetonitrile/water (50:50 v/v)

- 40’-45’ switching to acetonitrile/water (80:20 v/v)

- 45’-60’ acetonitrile/water (80:20 v/v)

The flow rate was set to 4.7 mL/min. The temperature of detection was set to 52 °C. EZ Chrome Elite software by Agilent was used for data management.

Calculation of the T/H index

The T/H index was calculated, using the following equation:

T/H = ([glucose] − [fructose])/[fructose].

Eq. 1. Transfructosylation number (T.N.)

Productivity

The productivities for batch and flow biotransformations were calculated at the same degree of conversion using the following equations: (Tamborini L. et al 2012)

$$r_{\text{batch}} = \frac{n_P}{t \times m_E} (\mu\text{mol}/\text{min g})$$

Eq. 2. r_{batch}: batch productivity; np: amount of the product of interest (µmol); t: reaction time (min); m_E: mass of the catalyst used (g).

$$r_{\text{flow}} = \frac{[P] \times f}{m_E} (\mu\text{mol}/min\,\text{g})$$

Eq. 3. r_{flow}: flow productivity; [P]: concentration of the product of interest (µmol/µL); f: flow rate (µL/min); m_E: mass of the catalyst packed in the reactor (g).

Continuous flow biotransformation using dry alginate mycelia in a packed bed column

Dry Alginate beads (2 g) and celite (1:1 v/v) were packed into an Omnifit® glass Column (15 mm id x 150 mm length). Each gram of alginate beads, mixed with the same volume of celite, can fill about 6 mL of the inner volume of the column. The swelling of the dried beads was achieved with an acetate buffer solution (20 mM, pH 6) before connecting the column. A flow stream of 600 g/L sucrose solution in 20 mM sodium acetate buffer (pH 6) was pumped through the packed bed column, at different flow rates, in order to obtain residence times from 5 hours to 30 hours. The appropriate temperature was set by the R4 block heater. A 100 µL aliquot of the exiting flow stream was diluted with water (1:3), the sample was filtered on a 0.45 µm nitrocellulose filter and analysed by HLIC-HPLC.

Glucose oxidation with Gluzyme MONO 10000 BG

The mixture obtained under optimised conditions after 20 hours of residence time has been exposed to the biocatalytic activity of a Glucose Oxidase preparation from Novozymes (Gluzyme MONO 10.000 BG) in batch (Dey Chyi Sheu et al 2001). To a 600 g/L mixture (20 mL) in acetate buffer 20 mM (pH 6) Gluzyme powder (800 mg, 400 U/mL) and $CaCO_3$ (800 mg) were added. The resulted suspension was stirred at 35 °C insufflating air at a flow rate of 7 mL/min. The produced gluconic acid precipitated as calcium salt in the reaction flask. At different times, aliquots (100 µL) were withdrawn, diluted with water (1:3) and incubated for 10 min at 90 ºC to inactivate the enzyme.

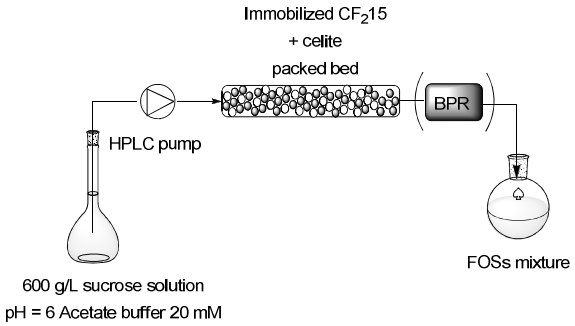
Each sample was filtered on a 0.45 µm nitrocellulose filter and analysed. After 24 h the whole suspension was centrifuged at 14000 rpm for 30 min, filtered on a cellulose filter paper and used for preparative HPLC purification.

Results and discussion

The use of lyophilised mycelium of *Cladosporium cladosporioides* MUT 5506 in freshwater allowed the production a high variety of FOS: kestose (1-, 6- and neo-), nystose (1-, 6-, and neo-), 1-fructofuranosylnystose and, interestingly, blastose. (Zambelli et al 2014; Zambelli P. et al 2014). We were interested to implement the production of the new mixture of FOS using the mycelium into a meso-flow reactor. Whole microbial cells can be packed into a glass column reactor as suchref or, in the case of difficult-to-handle microorganisms, they can be immobilised to avoid an excessive increase of flow resistance. Using mycelia of *C. cladosporioides*, we found necessary to immobilize the cells. Dried alginate entrapped (DALGEE)-mycelium was identified as the appropriate kind of immobilization because it appeared to be suitable for the application in a continuous flow reactor, due to the good stability over the time and during subsequent cycles of biotransformations (Tamborini L. et al 2012). Moreover, alginates are economic and ease to prepare (Fernandez-Arrojo et al., 2013). However, in the batch process, the higher conversion (total FOS amount ~ 54% (w/w)) was achieved after a prolonged reaction time (96 hours) and blastose appears only after 48 hours with a maximum (4.9% (w/w)) at 168 hours. In this contest, the use of a packed bed reactor could increase the speed and the productivity of the biotransformation

Optimization of reaction parameters in a packed bed continuous flow reactor

To rapidly optimize the reaction parameters, we considered the transfrucosylation/hydrolysis ratio (T/H index), which indicates the transglycosylation rate. A glass column (10 mm id x 100 mm length) was packed with the DALGEE-beads and a 600 g/L sucrose solution in 20 mM acetate buffer (pH 6.0) was flowed through it (Scheme 1) (Tamborini L. et al 2012). To avoid any unwanted and uncontrolled increase of the pressure, celite (alginate beads: celite = 1:1 v/v) was mixed to the alginates before the packaging. In this way, no over-pressure was observed over the time.



Scheme 1. Schematic representation the continuous system configuration used for pressure and temperature optimization. BPR: back pressure regulator.

First, the temperature effect on the T/H index was evaluated (Figure 2). The residence time was kept constant at 5 h and the temperature was varied between 25 °C and 70 °C. The highest T/H index was obtained at 35 °C, with a value of 4.0 ± 0.2.

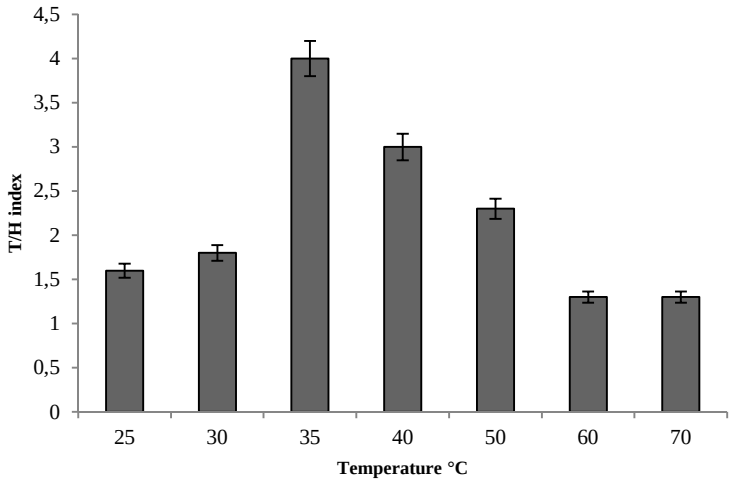


Figure 2. T/H index in function of the temperature. Conditions: [sucrose]: 600 g/L in acetate buffer 20 mM (pH 6); alginate beads: 1 g; celite: 1 g; residence time: 5 h; atmospheric pressure.

Then, the effect of the pressure on the biotransformation was evaluated, keeping the temperature constant at 35 °C. Pressurization of the system was easily achieved applying in-line different backpressure regulators (40, 75, 100 and 250 psi). No significant differences in the T/H index were observed (Figure 3) and, for this reason, we decided not to use any back pressure regulator to avoid any negative influence of the prolonged pressurization on the stability of the enzyme and/or alginates over the time

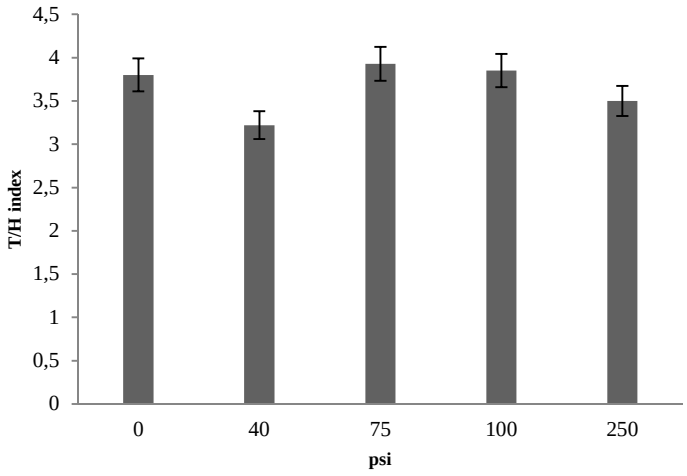


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Figure 3. T/H index in function of the pressure. Conditions: [sucrose]: 600 g/L in acetate buffer 20 mM (pH 6); alginate beads: 1 g; celite: 1 g; residence time: 5 h; T = 35 °C.

Stability of the packed bed alginate beads over time

To evaluate the stability of the packed bed alginate beads over time, the conversion at 5 h of residence time was evaluated during 7 days of continuous work. The reaction outcome was firstly checked after 5 hours of residence time and, then, every 24 hours. A similar concentration of residual sucrose was observed in the monitored reaction time (Figure 4). The same packed column was then washed with the acetate buffer 20 mM and conserved at 0 °C for two months. The column was used again under the same reaction conditions and a similar conversion was obtained.

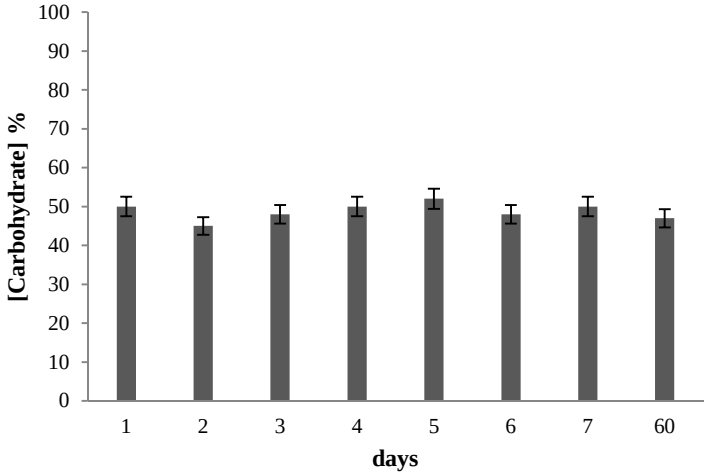

Figure 4. Sucrose residual concentration during 7 days of continuous work. Conditions: [sucrose]: 600 g/L in acetate buffer 20 mM (pH 6); alginate beads: 2 g; celite: 2 g; T = 35 °C; atmospheric pressure.

Kinetic profile of the packed bed continuous-flow biotransformation

In our previous works, we elucidated the kinetic profile of the biotransformation catalysed by lyophilised mycelia of *C. cladosporioides* in conventional buffer and by the corresponding alginates both in buffer and in seawater (Zambelli P. et al 2014). Considering the biotransformation catalysed by DALGEE-mycelium in acetate buffer (pH 6), the main products were 1-kestose, 1-nystose, 1-fructofuranosylnystose and blastose, with a maximum concentration of 138 g/L (72 h), 97 g/L (96 h), 15 g/L (96 h) and 30 g/L (168 h), respectively. Neo-kestose, neo-nystose and 6-kestose were found in later stage of the biotransformation (after 48 h of reaction time), with a concentration below 12 g/L (Zambelli P. et al 2014).

In flow, working at atmospheric pressure and at a temperature of 35 °C, we considered the residence time and we evaluated the conversion in terms of total amount of FOS formed (Figure 5). The maximum amount of FOS (52.3 % (w/w)) was reached at 20 h of residence time and remains constant up to 30 h. At 20 h of residence time, the concentration of FOS was 313 g/L, as represented in figure 5, with a concentration of 1-kestose, 1-nystose and 1-fructofuranosylnystose of 139 g/L, 86 g/L, 39 g/L, respectively and a concentration of neo-kestose, 6-kestose and neo-nystose of 9 g/L, 6 g/L and 6 g/L, respectively. It is worth noting that, in only 10 h of residence time, a 47.4 % (w/w) of conversion was reached and that the total amount of FOS slightly increases in the following 10 hours. A similar conversion (48% (w/w)) was obtained in batch after 72 hours of reaction (Zambelli P. et al 2014).

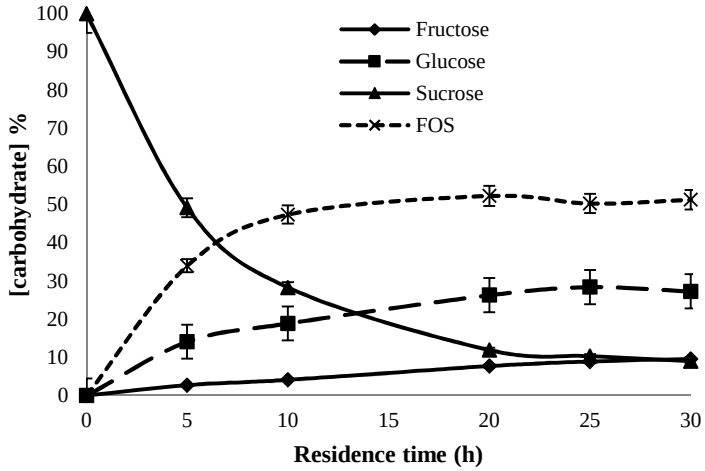

Figure 5: Total FOS amount at different residence times using a packed bed continuous flow reactor. Conditions: [sucrose]: 600 g/L in acetate buffer 20 mM (pH 6); alginate beads: 2 g; celite: 2 g; T = 35 °C; atmospheric pressure. The maximum amount of FOS was 310 g/L in 20 h.

After only 5 hours (residence time), blastose can be identified in the reaction flow stream (Figure 6). After 20 hours, it reaches a concentration (28 g/L) similar to the higher obtained in batch (30 g/L) after 96 hours. Moreover, in the following ten hours of residence time (30 hours total), the amount increases up to 31 g/L of the total solution. Neo-kestose, neo nystose and 6-kestose were formed in a concentration below of 12 g/L.

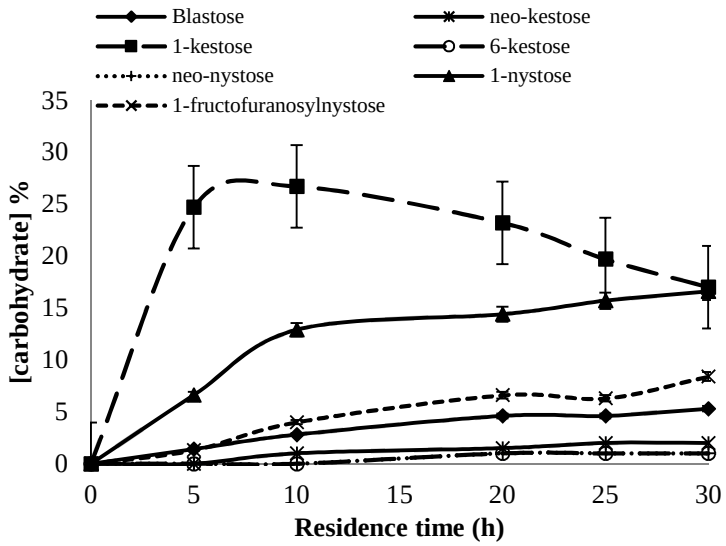

Figure 6. FOS composition at different residence times using a packed bed continuous flow reactor. Conditions: [sucrose]: 600 g/L in acetate buffer 20 mM (pH 6); alginate beads: 2 g; celite: 2 g; T = 35 °C; atmospheric pressure.

Productivity

To compare the batch and flow efficiencies for the FOS production, we determined the productivity values (Tamborini L. et al 2012) for both total FOS and blastose, at different reaction times, in these two different environments. We calculated the productivity for the batch biotransformation after 72 hours (total FOS ~ 48% (w/w)). In these conditions, we obtained a complex mixture of different FOS, with different degree of polymerization and structures, with a productivity of 1.82 mg/g*min. In flow, a similar FOS concentration (47.4%) was reached with a residence time of 10 hours. In these conditions, the productivity value was equal to 3.07 mg/g*min, about 1.7 times the productivity obtained in batch. Considering blastose, the maximum value (30 g/L) in batch was reached after 168 hours. This corresponds to a productivity 0.08 mg/g*min. In flow, a similar concentration of blastose was obtained after 10 hours of residence time: the productivity is 0.18 mg/g*min, about 2.3 times the productivity obtained in batch.

Continuous production of FOS using the packed bed continuous-flow system

After the optimisation of reaction parameters, we exploited the packed bed flow system for the continuous production of the new mixture of FOS. We selected a residence time of 20 h because the higher amount of total FOS is formed, including a 4.6% (w/w) of blastose, and we followed the production over 7 days of continuous work, as represented in figure 7. It is important to note that, using the flow system, once fixed the residence time, a constant mixture of FOS is produced. This

feature represents a good implement compared to the batch biotransformation in which the reaction needs to be stopped before the hydrolysis starts to overcome the transfructosylation. The total FOS amount remains in a range of 51.7-53 % (w/w) during the monitored reaction time..

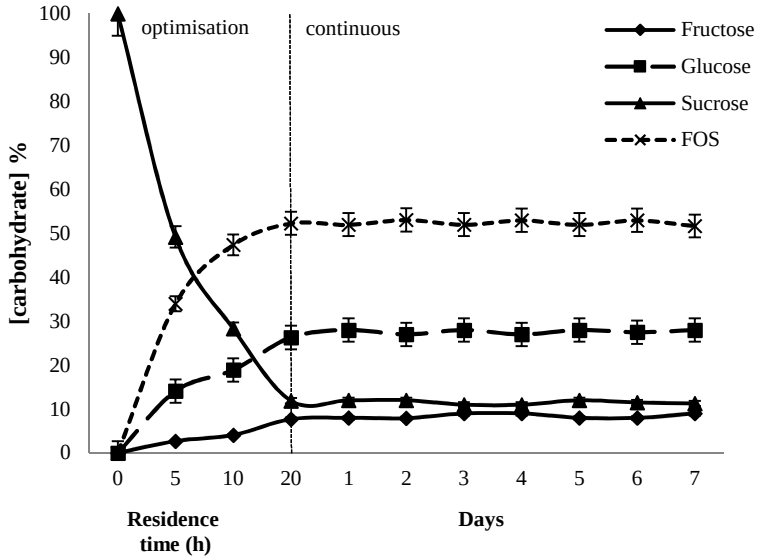

Figure 7. Continuous production of FOS under optimised conditions. Conditions: [sucrose]: 600 g/L in acetate buffer 20 mM (pH 6); alginate beads: 2 g; celite: 2 g; T = 35 °C; atmospheric pressure.

Removal of glucose from the FOS mixture and blastose purification

Glucose is the (undesirable) by-product in the synthesis of FOS catalysed by transfructofuranosydases (Dey Chyi Sheu et al 2001). In the exiting flow stream, glucose represents the 26% (w/w) of the mixture and it complicates the purification of the FOS mixture, and, in particular, of blastose. Therefore, we exploited an enzymatic method for glucose removal by oxidazing it to gluconic acid using a commercially available glucose oxidase; gluconate can be precipitated as calcium salt ans easily removed (Dey Chyi Sheu et al 2001). A 600 g/L mixture, obtained under optimised flow conditions, containing FOS (53% (w/w)), sucrose (11% (w/w)) and by-products (glucose 26% (w/w) and fructose 10% (w/w)), in acetate buffer (20 mM, pH 6), was exposed to the biocatalytic activity of a Gluzyme preparation in batch (Gluzyme® mono 10000 BG, from Novozymes). The biotransformation was followed during 24 hours and, after that time, glucose shows to be less than 5% of the mixture, whereas the absolute amount of the other saccharides remains unvaried. The reaction mixture was centrifuged at 14000 rpm for 30 min to

recover the supernatant without calcium gluconate. The obtained solution was filtered and purified by semi-preparative HPLC. From 3 mL of solution, 56 mg of blastose was obtained in 97% purity.

First *in vitro* evaluation of blastose prebiotic effect

In the last decade the concept of "prebiotic", substrate that selectively stimulate the growth and activity of health-promoting Lactobacilli and Bifidobacteria, has assumed much interest in terms of improving human host health (Gimeno-Perez et al., 2014). In order to investigate the ability of probiotic strains to use blastose as sole carbon source and thus its suitability for a potential application in symbiotic-prebiotic mixtures, an in vitro fermentation study was carried out on 5 well characterised probiotic Lactobacillus strains, easier to cultivate than Bifidobacteria: *L. paracasei* DG (Ferrario et al, 2014), *L. rhamnosus* GG (Segers and Leeber, 2014); *L. paracasei* SHIROTA (Aoki et al., 2014); *L. johnsonii* LC1 (Isobe et al., 2012); *L. reuteri* ATCC55730 (Di Nardo et al, 2014). In detail, the growth of the Lactobacillus strains in presence of glucose (considered as positive control) was compared to that obtained in presence of blastose or other well known prebiotic substrates, such as inulin and FOS, taking into consideration their growth kinetic parameters (Vmax values) and the final OD. The results are summarised in Table 1:

Table 1 Growth parameters (OD 600nm and Vmax) of probiotics during the in vitro prebiotic assay

	L. paracasei **DG**		***L. rhamnosus*** **GG**		***L. paracasei*** **SHIROTA**		***L. johnsonii*** **LC1**		***L. reuteri*** **ATCC55730**	
Carbohydrate	**OD**	**Vmax (1/h)**	**OD**	**Vmax (1/h)**	**OD**	**Vmax (1/h)**	**OD**	**Vmax (1/h)**	**OD**	**Vmax (1/h)**
Glucose	0.63	0.78	0.63	0.99	0.63	0.72	0.52	0.5	1.5	0.1
FOS	0.74.	0.84	nd	nd	0.24	0.13	0.39	0.35	0.15	0.21
Inulin	0.76	0.79	0.30	0.32	0.25	0.21	0.24	0.25	nd	nd
Blastose	0.78	1.20	0.32	0.60	0.37	0.57	0.47	1.13	nd	nd

nd: not detected growth after 48h of incubation at 37°C

Standard deviation below 5%

All the Lactobacillus probiotics were able to growth in presence of blastose as carbon source, except for *L. reuteri* strain, unable to growth even in presence of inulin, and showing a poor efficient growth on FOS, as clearly deduced by the OD and Vmax values. Among the probiotics tested, *L. paracasei* DG showed the most efficient growth in presence of the non-conventional disaccharide, with an increase of the final OD of 20% and a higher Vmax (>36%) respect to the positive control glucose, thus suggesting a growth stimulation effect of the blastose. Analogous result was obtained for *L. johnsonii* LC1, but in this case only the Vmax value was positively affected, suggesting an efficient intake and catabolism of the sugar, but with a lower efficiency compared to the glucose. The growth of the other microorganisms in presence of blastose was generally less efficient than the growth of the positive control, but comparable with the other prebiotic substrates, in terms of final biomass produced and Vmax reached during the growth. These positive results provide a first, preliminary evidence on the potential in vitro prebiotic effect of the newly isolated blastose. Neverthless, further in vitro and in vivo studies are needed in order to assess the effect of the new compound on other probiotic microorganisms and its possible use in prebiotic formulations.

Conclusions

A continuous production of a new mixture of fructooligosaccharides was performed exploiting the innovative combination of immobilised whole cells with a continuous flow chemistry reactor. The non-conventional disaccharide blastose was produced and isolated by HPLC in a pure form to preliminary evaluate the in vitro prebiotic effect. The technological transfer operated moving from a classical batch system to an innovative flow environment allows the obtainment of significant improvement, in terms of FOS (1.7 times) and blastose (2.3 times) productivities.

Blastose presents prebiotic properties similar or even better than that of the reference prebiotics, i.e. inulin and a commercialised FOS mixture (Actilight). This result represents the first indication that blastose has an influence on the growth of non-pathogenic microorganisms and this can justify in vivo assays conducted on animal models or human volunteers to ensure its prebiotic properties.

References

Zambelli P, Serra I, J. Plou F., Fernandez-Arrojo L., Tamborini L., Contente M.L., Conti P., Romano D., and Molinari F., 2014. Sweet-and-salty biocatalysis: fructooligosaccharides production using *Cladosporium cladosporioides* in seawater, marine chemistry, submitted 4/11/14

Zambelli P., Fernandez-Arrojo L., Romano D., Santos-Moriano P., Gimeno-Perez M., Poveda A., Gandolfi R., Fernandez-Lobato M., Molinari F. and J. Plou F.. Production of fructooligosaccharides by mycelium-bound transfructosylation activity present in *Cladosporium cladosporioides* and *Penicilium sizovae*. Process Biochem (2014), http://dx.doi.org/10.1016/j.procbio.2014.09.021

Tamborini L., Romano D, Pinto A., Bertolani A., Conti P. and Molinari F., 2012, An efficient method for the lipase-catalysed resolution and in-line purification of racemic flurbiprofen in a continuous-flow reactor J. Mol. Catal. B-Enzym, 84, 78–82.

Dey Chyi Sheu, Po Jang Lio, Shin Tse Chen, Chi Tsai Lin and Kow Jen Duan, 2001. Production od fructooligosaccharides using a mixed enzyme system of Fructofuranosidase and glucose oxidase. Biotechnology Letters 23, 1499-1503

Taverniti L., Minuzzo M., Arioli S., Junttila I., Hämäläinen S., Turpeinen H., Mora D., Karp M., Pesu M., and Guglielmetti S., 2012. In Vitro Functional and Immunomodulatory Properties of the *Lactobacillus helveticus* MIMLh5-Streptococcus salivarius ST3 Association That Are Relevant to the Development of a Pharyngeal Probiotic Product. Appl. Environ. Microbio 78(12):4209.

Arioli, S., Guglielmetti, S., Amalfitano, S., Viti, C., Marchi, E., Decorosi, F., Govannetti, L., & Mora D. (2014). Characterization of *tetA*-like gene encoding for a MFS efflux pump in Streptococcus thermophilus. *FEMS Microbiology Letters*, 355, 61-70

Aoki T., Asahara T., Matsumoto K., Takada T., Chonan O., Nakamori K., Nonaka C., Yamaji I., Hisamoto T., Sato M., Matsuda T., Nomoto K. (2014) Effects of the continuous intake of a milk drink containing *Lactobacillus casei* strain Shirota on abdominal symptoms, fecal microbiota, and metabolites in gastrectomized subjects. Scandinavian Journal of Gastroenterology 49(5):552-63.

Valeur N., Engel P., Carbajal N., Connolly E., and Ladefoged K. (2004) Colonization and Immunomodulation by *Lactobacillus reuteri* ATCC 55730 in the Human Gastrointestinal Tract. Applied and Environmental Microbiology 1176-1181.

Ferrario C., Taverniti V., Milani C., Fiore W., Laureati M., De Noni I., Stuknyte M., Chouia B., Riso P., Guglielmetti (2014) Modulation of Fecal Clostridiales Bacteria and Butyrate by

Probiotic Intervention with *Lactobacillus paracasei* DG Varies among Healthy Adults. The Journal of Nutrition 144(11):1787-1796.

Isobe H., Nishiyama A., Takano T., Higuchi W., Nakagawa S., Taneike I., Fukushima Y., Yamamoto T. (2012) Reduction of overall Helicobacter pylori colonization levels in the stomach of Mongolian gerbil by *Lactobacillus johnsonii* La1 (LC1) and its in vitro activities against *H. pylori* motility and adherence. Bioscience, Biotechnology and Biochemistry 76(4):850-2

María Gimeno-Pérez & Dolores Linde & Lucía Fernández-Arrojo & Francisco J. Plou & María Fernández-Lobato (2014) Heterologous overproduction of β-fructofuranosidase from yeast *Xanthophyllomyces dendrorhous*, an enzyme producing prebiotic sugars. Applied Microbiology and Biotechnology In press DOI 10.1007/s00253-014-6145-1

Marijke E Segers Sarah Lebeer (2014) Towards a better understanding of *Lactobacillus rhamnosus* GG - host interactions. Microbial cell factories 13(Suppl 1):S7

Purification and biochemical characterisation of β-fructofuranosidase from *C. cladosporioides*

Materials and methods

Chemicals

Sucrose, glucose, (D-)fructose, DEAE Cellulose DE52, Sephadex G50, Tetrazolium chloride (TTC), 3,5-Dinitrosalicylic acid (DNS) and Protease Inhibitor Cocktails were from Sigma Aldrich. 1-kestose, 1-nystose were from Fluka. 1-Fructofuranosylnystose was from Megazyme. Actilight® was from Beghin Meiji. Yeast extract was from Difco and barley malt flour from Diagermal. Malt extract was from Merck. All other reagents and solvents were of the highest available purity and used as purchased.

Culture production

C. Cladosporium was routinely maintained on MYA slants (Barley malt flour 100 g/L, yeast extract 5 g/L, agar 15 g/L, pH 5.6) at 4 °C. The strain, grown on MYA slants for 96 h at 28 °C, were inoculated into 1 L Erlenmeyer flasks containing 100 mL of the liquid medium MYA and incubated on a reciprocal shaker (150 rpm) for 96 h at 28 °C. After centrifugation, the cultures were lyophilised.

Test of activity

The enzymatic activity was measured toward sucrose by measuring the release of reducing sugars by the dinitrosalicylic acid (DNS), method adapted to a 96-well microplate scale (Heyer G. and Wenderburg R., 2001). A calibration curve was performed with a 3 g/L fructose solution. Lyophilised mycelia 10 g/L were incubated with 1 mL of a sucrose solution 100 g/L in buffer acetate 20 mM pH 6.0 for 20 min at 50ºC and 90 rpm. 50 µL of the solution conveniently diluted to fit into the calibration curve, were added to each well. Then, 50 µL of 10 g/L DNS were added. The plate was incubated for 20 min at 80ºC to develop colour with a seal plate tape (GeneMate). Then, the microplate was cooled, 150 µL of water added to each well, and the absorbance measured at 540 nm using a microplate reader (model Versamax, Molecular Devices). One unit (U) of activity was defined as that catalysing the formation of 1 µmol reducing sugar per minute under the above conditions.

Cellular extract preparation

40 g (wet weight) of mycelia were diluted 1:10 (w/v) with buffer TRIS 50 mM pH 7.4 with 30 mM of NaCl and 1 mg/L of Protease Inhibitor Cocktails, after homogenisation a cycle of French press at 37 Kpsi was performed to extrapolate the maximum amount of the total proteins.

Protein purification

The resin utilised for the purification of enzyme was DEAE Cellulose DE52 anionic exchange; equilibrated with TRIS 50 mM pH 7.4 and eluted with a step gradient (0-10%-25%-50%-100%) of

TRIS 50 mM 1 M NaCl. The HPLC chromatography for the purification was performed with pump waters 600 E and Waters double U.V. detector 2489. The columns used were a Mono Q/S 5/50 Amersham Bioscience anionic exchange equilibrated with TRIS 50 mM pH 7.4 and eluted with a linear gradient (0-100%) of TRIS 50 mM 1 M NaCl and a Superose 12 GE Healthcare Life Sciences gel filtration eluted with isocratic TRIS 50 mM pH 7.4 0.15 M NaCl. The active fraction was dialysed and stored at -20 ºC. Coomassie blue-stained sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of the samples confirmed the purity of the β-fructofuranosidase. Full range protein markers (Bio-Rad) were used as a control. The samples were concentrated using the Ultracel Ultrafiltration Disc (Millipore) 76, 44, 23 mm 10 kDa cut off. β-fructofuranosidase activity was detected in situ by native electrophoresis 8% polyacrylamide gels, using Tetrazolium chloride (TTC) method as described previously (Fernandez-Arrojo L, et al 2013). Invertase from *S. cerevisiae* was used as positive control. The native molecular weight of the purified enzyme was estimated by gel filtration HPLC with Superose 12 GE Healthcare Life Sciences eluted with isocratic TRIS 50 mM pH 7.4 0.15M NaCl. Ferritin (450 kDa), aldolase (158 kDa), ovoalbumine (44 kDa) and cytochrome c (12.5 kDa) were used for column calibration.

Kinetic analysis

The Michaelis–Menten kinetic constants were determined using purified protein with sucrose (0–1.2M) and 1-kestose (0-0.8 M). The plotting and analysis of the curves was carried out using the SIGMAPLOT program (version 10.0). Kinetic parameters were calculated by fitting the initial rate values to the Michaelis–Menten equation V= Vmax[S] Km[1/S].

Mass spectrometry analysis

The unknown carbohydrate was analysed by MALDI-TOF mass spectrometry (Bruker, model Ultraflex III TOF-TOF) using 2,5-dihydroxybenzoic acid doped with sodium iodide as matrix, in positive reflector mode. The identification of the purified protein was carried out on a PerSeptive Biosystems (Framingham, MA, USA) Voyager DE-PRO instrument equipped with an N_2 laser (337 nm 3 ns pulse width). The sample was loaded on a stainless steel plate together with 1 mL of CHCA matrix (10 mg in 1 mL aqueous 50% ACN). Mass spectra acquisition was performed in both positive linear and reflectron mode.

Results and Discussion

Protein purification

The initial concentration of proteins was 0.55 g/L in a final volume of 0.4 L, with a β-fructofuranosidase activity of 3 U/L (1200 total units). The first purification step was an anionic exchange gravimetrical chromatography with a DE52 resin, the U/L was 78 in a volume of 12 mL and the total enzymatic units were 978. Two steps of HPLC with column anionic exchange Mono Q 5/50 were performed, these results in an activity of 126 U/L in a volume of 2 mL, the total units of enzyme were 252. The last step was a HPLC with column size exclusion Superose 12. The total units were 51 with an activity of 3 U/L in 17 mL of final volume. Purified fractions of enzyme from *C. cladosporioides* shown a single homogenous peak by analytical size exclusion chromatography, which corresponds to an estimated molecular mass of 60 kDa as show in Figure 1.

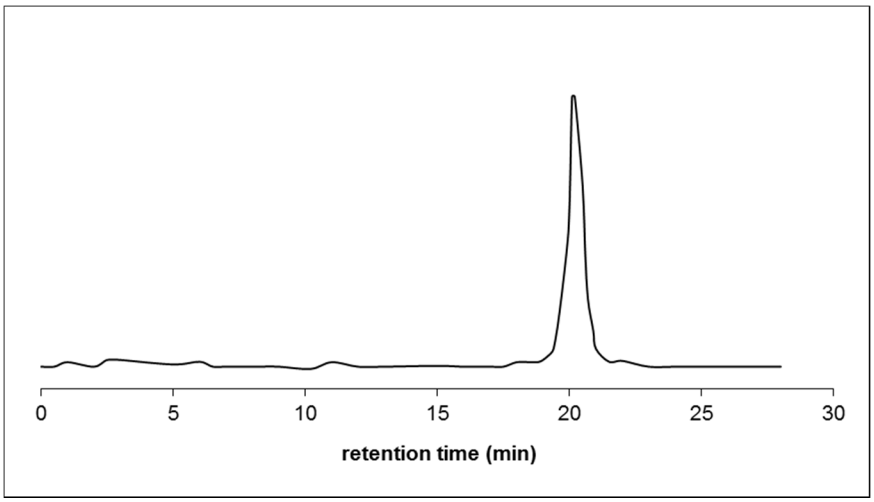

Figure 1: Cladosporium cladosporioides estimation of native enzyme size. Purified β-fructofuranosidase, was analysed by gel filtration HPLC isocratic TRIS 50 mM pH 7.4 0.15 M NaCl. Chromatograms indicates UV absorbance (AU) referred to time. Peaks eluted at 20.1 min

Table 1: purification steps and quantification of activity and proteins

Purification step	Bradford (mg/mL)	Total volume (mL)	Total proteins (mg)	U/mg	U/mL	U tot
French press	0.55	400	220	1.11	3	1200
DEAE	2.33	12	29	33.71	78	936
HPLC Mono Q 1	1.32	12	17	48.23	63	756
HPLC Mono Q 2	1.61	2	3	78.87	126	252
HPLC Superose 12	0.06	17	1	94.57	3	51

An electrophoresis SDS-PAGE was performed with the purified fraction and only one band of about 50 kDa appeared with Blue Coomassie coloration.(Figure 2)

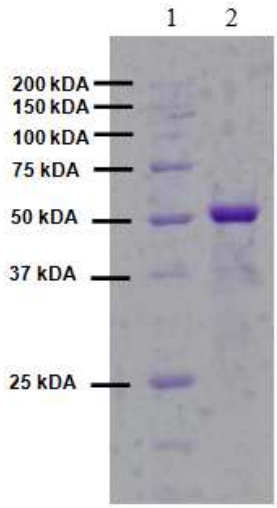

Figure 2: SDS-PAGE analysis of the purified enzyme. After purification the active fracion was subjected to SDS-PAGE 10 % polyacrylamide. Lane 1, full range protein markers (Bio-Rad). Line 2, active purified fraction: only a band about 50 KDa was revealed (Coomassie)

Other invertases, such as *Pichia anomala* (Rubio M.C. et al 2002) and *X. dendrorhous* (Heyer G. and Wenderburg R., 2001) are dimeric or multimeric enzymes, which have an average molecular weight of 60–65 kDa for the nonglycosylated-monomeric form. Several enzymes have been described such as that from *R. glutinis* (around 47 kDa) (Boer E. et al, 2004) and *Arxula adeninivorans* (about 100 kDa) (Sanjay G. et al 2005). The active forms of these two enzymes exist as a dimer (100 kDa), and a hexamer (600 kDa), respectively.

Kinetic properties

The kinetic constant Km was calculated using sucrose and 1-kestose as substrates. The results shown a Km value with sucrose of 129 ± 6 mM and a Vmax of 2.2307 ± 0.04 U/mL. Data are presented in Figure 2. The value of Kcat was calculated in 2.88 ± 0.04 1/s and Kcat/Km 22.3 ± 1.4 1/M*s. A similar value (130 mM) of Km was obtained with the yeast *Saccharomyces cerevisiae* with immobilised enzyme, pH 5.0, 30°C (Rubio M.C. and Maldonado M.C. 1995) while *Aspergillus niger* shown a Km value of 62.5 mM with sucrose (Martel C.M. et al 2010). The other substrate tested was 1-kestose and the Km value for *C. Cladosporioides* was calculated in 268 ± 6 mM as shown in figure 3.

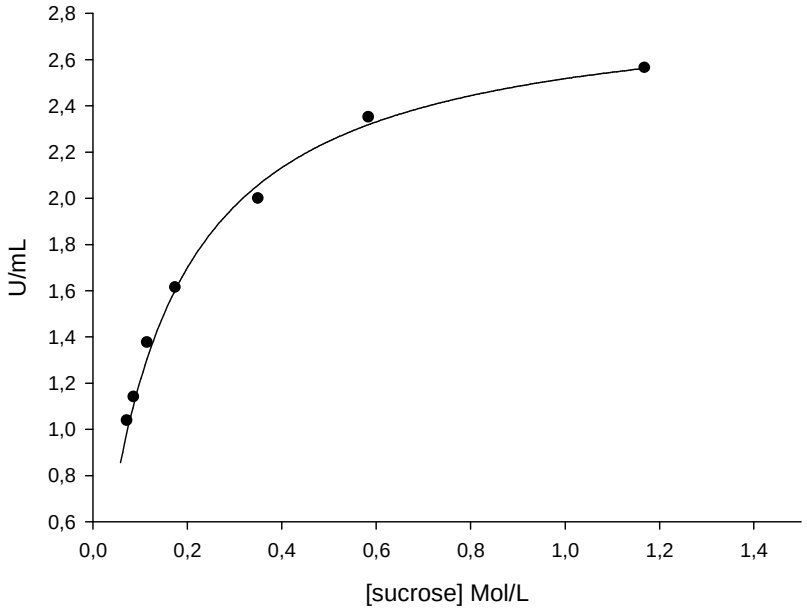

*Figure 2: kinetic analysis of C. Cladosporioides with sucrose [0-1.2 M]. Km 129 ± 6 mM, Vmax 2.2307 ± 0.04 U/mL and Kcat 2.88 ± 0.04 1/s and Kca t/Km 22.3 ± 1.4 1/M*s*

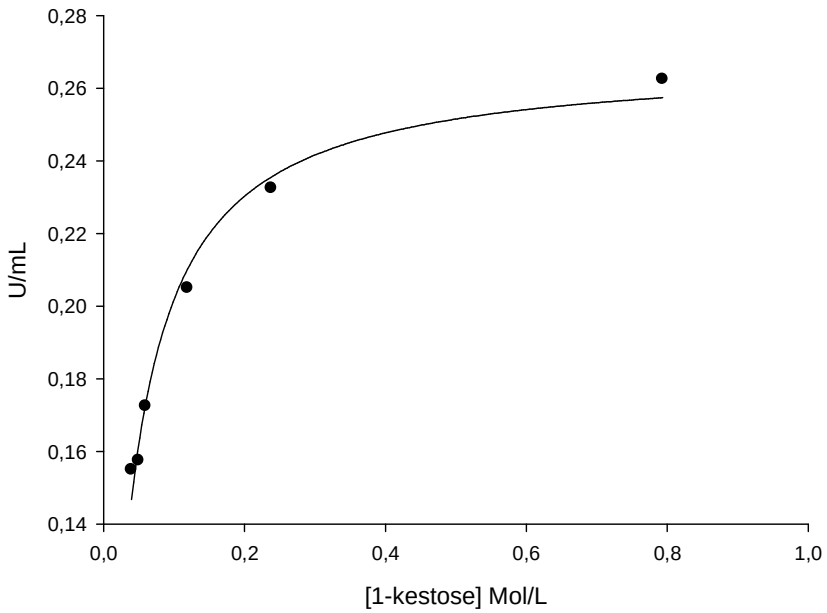

*Figure 3: kinetic analysis of C. Cladosporioides with 1-kestose [0-0.8 M] Km 268 ± 6 mM, Vmax 0.0328 ± 0.0023 U/mL, Kcat was estimated in 0.0334 ± 0.003 1/s and Kcat/Km 0.124 ± 0.014 1/M*s*

The values of Km from *Lactobacillus paracasei* and *Aureobasidium pullulans* with 1-kestose were of 49 mM (Yoshikawa J. et al 2007) and 700 mM (Goosen C.et al 2007) respectively. We can deduce from these data a pronounced variability in the affinity with 1-kestose. The Vmax calculated result in 0.0328 ± 0.0023 U/mL and the value of Kcat was estimated in 0.0334 ± 0.003 1/s. Kcat/Km results in 0.124 ± 0.014 1/M*s. Table 2 resume the kinetic value.

Table 2: kinetic parameters with different substrates

	Km (mM)	**Vmax (U/mL)**	**Kcat (1/s)**	**Kcat/Km (1/M*s)**
sucrose	129 ± 6	2.83 ± 0.04	2.88 ± 0.04	22.3 ± 1.4
1-kestose	268 ± 6	0.0328 ± 0.003	0.0334 ± 0,003	0.124 ± 0.014

Mass spectrometry analysis

A mass spectrometry MALDI-TOF analysis study was performed on the protein, showing a MW of 61178 Da. A trypsin digestion was performed and the fragments analysed but we did not found match in databases. The molecular study of the protein was stopped until protein sequence elucidation.

References

Heyer G., Wenderburg R., 2001, Gene Cloning and Functional Characterization by Heterologous Expression of the Fructosyltransferase of *Aspergillus sydowi*. Appl Environ Microb 67: 363-370

Fernandez-Arrojo L., Rodriguez-Colinas B., Gutierrez-Alonso P., Fernandez-Lobato M., Alcalde M., Ballesteros A.O., Plou F.J., 2013, Dried alginate-entrapped enzymes (DALGEEs) and their application to the production of fructooligosaccharides. Process Biochem 48: 677-682

Rubio M.C., Runco R., Navarro A.R., 2002, Invertase from a strain of *Rhodotorula glutinis*. Phytochemistry 61: 605–609.

Boer E., Wartmann T., Luther B., Manteuffel R., Bode R., Gellissen G., Kunze G., 2004, Characterization of the AINV gene and the encoded invertase from the dimorphic yeast *Arxula adeninivorans*. Antonie van Leeuwenhoek 86: 121–134.

Sanjay G., Sugunan S., 2005, Enhanced pH and thermal stabilities of invertase immobilised on montmorillonite K-10. Food Chem. 94: 573-579

Rubio M.C., Maldonado M.C., 1995, Purification and characterization of invertase from *Aspergillus niger* (0.0625). Curr. Microbiol. 31: 80-83

Martel C.M., Warrilow A.G., Jackson C.J., Mullins J.G., Togawa R.C., Parker J.E., Morris M.S., Donnison I.S., Kelly D.E., Kelly S.L., 2010, Expression, purification and use of the soluble domain of *Lactobacillus paracasei* beta-fructosidase to optimise production of bioethanol from grass fructans Biores. Technol. 101: 4395-4402

Yoshikawa J., Amachi S., Shinoyama H., Fujii T.J., 2007 Purification and some properties of beta-fructofuranosidase I formed by *Aureobasidium pullulans* DSM 2404. Biosci. Bioeng. 103: 491-493

Goosen C., Yuan X.L., van Munster J.M., Ram A.F., van der Maarel M.J., Dijkhuizen L., 2007, Molecular and biochemical characterization of a novel intracellular invertase from *Aspergillus niger* with transfructosylating activity. Eukaryot. Cell 6: 674-681

Appendix 1

DOI: 10.1016/j.procbio.2014.09.021, accepted. Process Biochemistry Journal.

Title: Production of fructooligosaccharides by mycelium-bound transfructosylation activity present in *Cladosporium cladosporioides* and *Penicilium sizovae*

Article Type: Full Length Article

Keywords:

fructooligosaccharides; transfructosylation; prebiotics; bioactive oligosaccharides; blastose

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Abstract:

Different filamentous fungi isolated from molasses and jams (kiwi and fig) were screened for fructooligosaccharides (FOS) producing activity. Two strains, identified as *Penicilium sizovae* (CK1) and *Cladosporium cladosporioides* ($CF_2$15), were selected on the basis of the FOS yield and kestose/nystose ratio. In both strains the activity was mostly mycelium-bound. Starting from 600 g/L of sucrose, maximum FOS yield was 31 % w/w and 56 % w/w for *P. sizovae* and *C. cladosporioides*, respectively. Interestingly, the highest FOS concentration with C. *cladosporioides* was reached at 93% sucrose conversion, which indicated a notable transglycosylation to hydrolysis ratio. The main FOS in the reaction mixtures were identified by HPAEC-PAD chromatography. *C. cladosporioides* synthesised mainly 1-kestose (158 g/L), nystose (97 g/L), 1F-fructosylnystose (19 g/L), 6-kestose (12 g/L), neokestose (10 g/L) and a disaccharide (34 g/L) that after its purification and NMR analysis was identified as blastose [Fru-β(2-6)-Glc]. *P. sizovae* was very selective for the formation of 1F-FOS (in particular 1-kestose) with minor contribution of neoFOS and negligible of levan-type FOS.

Manuscript submitted to Marine Chemistry Journal.

Sweet-and-salty biocatalysis: fructooligosaccharide production using *Cladosporium cladosporioides* in seawater

Article Type: Full length article

Keywords:

Fructooligosaccharides, fructofuranosidase, *Cladosporium cladosporioides*, Dried Alginate Entrapped Enzyme (DALGEE), immobilisation, marine, seawater

First author: Paolo Zambelli

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Corresponding author: Diego Romano,

Abstract:

The halophilic fungus *Cladosporium cladosporioides* MUT 5506 was used for the preparation of fructooligosaccharides (FOS) from sucrose in seawater.

C. cladosporioides was grown in a seawater-based medium and mycelium displayed an optimal activity at 50-60ºC, being stable up to 60 ºC. Under optimised conditions in seawater (50°C, sucrose 600 g/L, lyophilised mycelium 40 g/L), *C. cladosporioides* gave a maximum FOS yield of 344 g/L with a preponderance of $^{1F\text{-}FOS}$ derivatives (1-kestose 168 g/L, 1-nystose 96 g/L and 1-fructofuranosylnystose 20 g/L), with the noteworthy presence of the non-conventional disaccharide blastose (30 g/L). An immobilisation study was performed using Dried Alginate Entrapped (DALGEE)-mycelium, showing 51% conversion of total sugars into FOS using DALGEE-mycelium of *C. cladosporioides* in seawater after 96 h.

Manuscript submitted to Food Chemistry Journal.

An efficient continuous flow process for the synthesis of a non-conventional mixture of fructooligosaccharides

Article Type: Full Length Article

Keywords:

Flow chemistry biocatalysis, fructooligosaccharides, fructofuranosidase, *Cladosporium cladosporioides*, Dried Alginate Entrapped Enzymes (DALGEE), blastose, lactobacillus

First author: Paolo Zambelli

Order of authors: Paolo Zambelli, Samuele Cazzamalli Diego Romano, Lucia Fernandez-Arrojo, Andrea Pinto, Paola Conti, Lucia Tamborini and Francesco Molinari,

Corresponding author: Francesco Molinari

Abstract:

A sustainable and scalable process for the production of a new mixture of fructooligosaccharides (FOS) was developed by using a continuous-flow approach based on an immobilised whole cells-packed bed reactor. The technological transfer from a classical batch system to an innovative flow environment allows the obtainment of a significant improvement, in terms of productivities. The FOS mixture contains the unconventional disaccharide blastose, which was isolated by semi-preparative HPLC and tested on eight different *Lactobacillus* strains to preliminary evaluate its prebiotic effect.

Gordon research conference, 7-11 July 2014, Bryant University, Rhode Island, MA, U.S.A.

Development of new Biocatalytic Processes for Fructooligosaccharides Production

Paolo Zambelli, Lucia Tamborini, Lucia Fernandez-Arrojo, Diego Romano, Samuele Cazzamalli, Martina Letizia Contente, Francisco J. Plou and Francesco Molinari.

Abstract

Commercial fructooligosaccharides are a mixture of oligomers with units of fructose linked in β 2-1 position of sucrose: kestose with three monomeric units (GF2), nystose with four monomeric units (GF3) and fructo furanosyl nystose with five monomeric units (GF4) (Maiorano A. E., 2008). FOS have shown interesting properties such as: low caloric intake (2 kcal/g), low glycemic impact, prebiotic action favouring the development of Bifidobacteria and Lactobacillus, promotion of the absorption of Ca^{2+} and Mg^{2+}, lowering blood lipid levels and reduction total cholesterol and triglycerides (Katapodis P., 2004). Enzymes able to produce FOS are mostly fungal fructofuranosidase (FF) or fructofuranosyltransferases (FT).

VII Workshop on Biocatalysis and Biotransformations, Ferradura resort, Bùzios, Brazil September 23th-26th

"Flowcells and flowzymes":biocatalysis in flowchemistry reactors

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Abstract

Biocatalys is has a number of advantages, but applications are often hampered by low productivity and difficulties in scaling up. A logical step for improving the performances of enzymes and/or whole-cell systems is to use them in flow chemistry reactors, where productivities can be largely improved. Flow-based applications has potential advantages, such as: increased mixing efficiency, controlled scaling factors, improved safety ratings and continuous processing capabilities. In a flow reactor, reaction conditions (flow rate, temperature, pressure) can be independently varied and precisely controlled. This leads to high reproducibility and greatly facilitates optimization allowing different conditions to be rapidly investigated. Flow processes are readily scalable either by running the flow reactor for an extended time or by employing multi-channel parallel reactors.

RESULTS AND DISCUSSION

We are developing flow-based bioconversions, where immobilised enzymes and whole microbial cells are employed. We suggest to naming these processes as "flowzymes" and "flowcells", respectively. Immobilised lipase B from *Candida Antarctica* (flowzyme) and dry mycelia of *Aspergillus oryzae* (flowcell) were used for the enantioselective direct esterification of racemic flurbiprofen in organic solvent. The use of flowzyme and flowcell dramatically improved the productivity of the batch biotransformation, with beneficial effects also on the enantioselectivity. The overall process can be implemented by adding an in-line purification step of the exiting solution, consisting in a catch and release protocol, which allows the easy separation and recovery of (R)-flurbiprofenethyl ester and (S)-flurbiprofen, the latter was racemised and recycled. Production of fructooligosaccharides (FOS) was accomplished with a flowcell system using Dried Alginate Entrapped (DALGEE) mycelium of *Cladosporium cladosporioides*. Improvement of the reaction rates was observedusing the flowcells system, with a maximum yield of FOS (52%) in 24h, while in batch mode a similar conversion was observed only after96h. DALGEE-mycelium of *C. cladosporioides* was stable over 4 months in the flowcell system. Interestingly, the unconventional disaccharide blastose ([Fru-β(2→6)-Glc]) was produced among nystose and kestose. Glucose oxidation using commercially available glucose oxidase was carried out in different flow reactors to assess configurations enabling the more efficient O_2 transfer to the biocatalyst, allowing for high enzymatic activity. This system was exploited to set up a continuous biotransformation for the production of FOS, where the by-product (glucose) could be in situ removed by its oxidation. Finally, multienzymatic catalysed processes using cascade reactions (including redox reactions)for preparing prostaglandin intermediates are now under study for fully exploit the potential of flowzymes and flowcells for practical applications.

CONCLUSION

The performances of biotransformations can be improved by employing flowzymes and/or flowcells; operational requirements of different enzyme-catalysed processes (i.e., organic solvents, oxygen availability, substrate/product solubility) need to be adjusted for obtaining high rates and productivity. Flowzymes and flowcells appear to be like optimal solutions for many biocatalytic applications

Development of chemoenzymatic continuous flow processes for the preparation of biologically active compounds

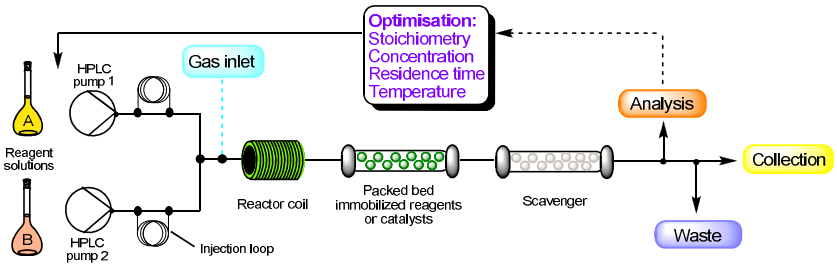
Lucia Tamborini, Federica Mastronardi, Samuele Cazzamalli S. and Paolo Zambelli.

Abstract

Flow reactor technology represents one of the new strategies introduced in recent years to advance the sustainability of organic synthesis and shows many advantages compared with the classical batch methods, such as increased safety, high control of reaction parameters, possibility of automation, reduced manual handling, in-line purifications, reaction telescoping and increased contact surface between phases in biphasic or triphasic systems.1

Over the last few years, one of our research lines has been focused on devising efficient continuous-flow synthetic procedures to generate chemical complexity and produce pharmaceutically and nutraceutically relevant compounds, exploiting the use of immobilised reagents, scavengers and catalysts in flow reactors (Scheme 1). This strategy led to the obtainment of a small library of variously decorated thiazoles and imidazoles,2 to the multi-step synthesis of N-Boc-3,4-dehydro-L-proline methyl ester, the precursor of a number of non-natural amino acids active on the glutamatergic system,3 and to the obtainment of a series of bicyclic-Δ2-isoxazolines, intermediates in the synthesis of novel DNA methyltransferase 1 inhibitors.4

Recently, we also explored the innovative combination of biocatalysis with the flow chemistry facilities, using both immobilised enzymes (e.g. Novozyme 435) and whole cells.5,6 The application of whole cells in a flow reactor combines the advantages of an easy to produce biocatalyst, that can be employed even without immobilization, with a process-intensification technology, that can dramatically improve the performances of biotransformations, in particular in terms of productivity. At present, we are studying multi-phase enzymatic reactions in presence of immiscible liquids or gases (unpublished results).



XVII Workshop on the *Developments in the Italian PhD Research on Food Science Technology and Biotechnology,* University of Bologna, Cesena, 19-21 September, 2012

Development of new Biocatalytic Processes for Fructooligosaccharides Production

Paolo Zambelli

Abstract

This PhD research project is aimed at the development of new biocatalytic processes to produce natural sugars by selection and characterisation of new enzymes able to produce fructooligosaccharides. Molecular and biochemical studies will be performed to obtain information on the mechanism of action and understand the structural elements that define the activity. The enzymes will be evolved to obtain more efficient catalysts by protein engineering. Separation methods of the transformation products will be also assessed, in order to obtain FOS in purified form.

18th Workshop on the *Developments in the Italian PhD Research on Food Science Technology and Biotechnology,* University of Padova, Conegliano veneto 25-27 september 2013

Development of new Biocatalytic Processes for Fructooligosaccharides Production

Paolo Zambelli

Abstract

This PhD research project is aimed at the development of new biocatalytic processes based on the selection and characterisation of new enzymes able to produce fructooligosaccharides (FOS). Molecular and biochemical studies will be performed to obtain information on the mechanism of action and understand the structural elements that define the activity. Separation methods of the transformation products will be also assessed, in order to obtain FOS in purified form.

19th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, University of Bari, 24-26 september 2014

Development of new Biocatalytic Processes for Fructooligosaccharides Production

Paolo Zambelli

Abstract

This PhD research project aims at the development of new biocatalytic processes based on the selection and characterisation of new enzymes able to produce fructooligosaccharides (FOS) from sucrose. Molecular and biochemical studies will be performed to obtain informations on the mechanism of action and to understand the structural elements that define the activity. Separation methods of the transformation products will be also assessed, in order to obtain FOS in purified form