**UNIVERSIDADE DE LISBOA**

**FACULDADE DE CIÊNCIAS**

**DEPARTAMENTO DE BIOLOGIA VEGETAL**


# Isolation, screening and characterization of microorganisms with potential for biofuels production

Pedro Jorge Dias Teixeira

**MESTRADO EM MICROBIOLOGIA APLICADA**

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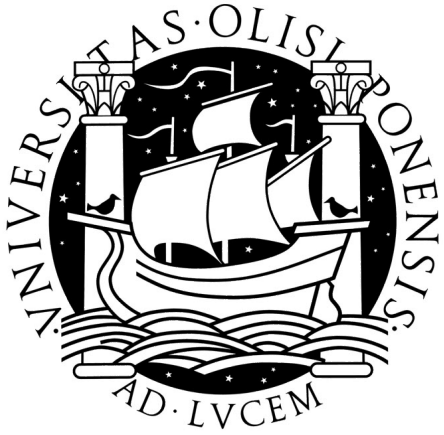


# Isolation, screening and characterization of microorganisms with potential for biofuels production

Dissertation oriented by **Dr. Susana Maria Teixeira Paixão Alves** (LNEG) and Professor **Dr. Maria Manuela Carolino** (FCUL)

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**MASTER THESIS**

**2012**

This thesis was fully performed at the Laboratório Nacional de Energia e Geologia (LNEG), campus of Lumiar, Lisbon (Portugal), namely in the Laboratório Nacional de Energia - Unidade de Bioenergia, under the direct supervision of the researchers Dr. Susana Maria Teixeira Paixão Alves and Dr. Luís M. Gonçalves Alves.

Prof. Dr. Manuela Carolino was the internal designated supervisor in the scope of the *Master in Applied Microbiology* of Faculdade de Ciências da Universidade de Lisboa.

**Acknowledgements**

I would like to thank my direct supervisors, Dr. Susana Maria Teixeira Paixão Alves and Dr. Luís M. Gonçalves Alves, for their guidance and integration in laboratory research life. Thank you for your help and support for the elaboration and correction through the thesis process.

A special thanks to my internal supervisor, Prof. Dr. Maria Manuela Carolino, for the support, availability to any question, constructive critics and valuable advices.

I would like to thank Dr. Francisco Gírio, director of Bioenergy Unit in LNEG, for consenting in the realization of this thesis work in these facilities. I also would like to thank the Faculdade de Ciências da Universidade de Lisboa for the opportunity to develop this thesis work.

I would like to express my gratitude to all colleagues with who I worked, directly or not, in LNEG which made this thesis work a much easier task. To Amélia Marques, thank you for your patience, sympathy and availability to always get the necessary material for the laboratory work, and to Margarida Monteiro, for sharing your experience in FISH technique and the skilled necessary method of photography using the available equipment. To Alexandra Teixeira, thank you for your help and friendship, and encouraging words even when I thought I could no longer laugh. To Tiago Silva, thank you for introducing me my supervisors and the LNEG facilities, and for the support along this project. To Carla Ferreira, thanks for the warnings and depressing thoughts (you were right about writing this). To all other PHD students Nuno Faria, Carla Lara, Raquel Cadete and researchers Patricia Branco, Diana Francisco, Ana Clemente, Maria Leonor and all other co-workers for all the lunch and other breaks, thank you all for helping me keep my sanity!

A special thanks to all my family, my father, mother and brothers, who were always concerned about me and kept asking what I was doing, though not even I was sure about it. Thank you, not only for the obvious financial support, but primarily for put up with me even when I was harsh and impatient and for never stop believing in me.

For last but not the least, a special thanks to Carla Silvestre, for your special support, confidence in me and the tremendous amount of patience necessary to keep me moving. It would be impossible for me to do this without you. Thank you.

**Abstract**

Rapid global population growth has increased the demand for food and energy supply. The limited oil reserves, pollution concerns, global warming and political instability and disagreements, lead to an increased financial support for sustainable and environmental sources of energy, biofuels. In the last decades there is an increasing interest in the development of the bioethanol production from lignocellulosic residues, which do not compete directly with food. However, the low efficient conversion of cellulosic biomass to biofuels hinders its success. Alternative substrates are inulin containing plants, as Jerusalem artichoke, representing a renewable and inexpensive raw material for industry and biofuel production. In this work, the main goal was to search for new microorganisms, with high potential to produce bioethanol, due to the presence of better ethanologenic characteristics or ability to produce relevant hydrolytic enzymatic machinery. From the isolation and screening of 98 novel strains, 7 were selected and further characterized. A preliminary identification was performed using FISH. Three isolates which showed inulinase capacity gave a putative identification as *Z. bailii* strains, and the best (Talf1) was optimized and characterized for inulinase production. Talf1 enzymatic extract presented maximum activity (8.7 U/ml) at 45 ºC and pH 5.5, and high stability at 30ºC. Talf1 isolate was used in a Consolidated Bioprocessing (CBP) and its enzymatic extract in a Simultaneous Saccharification and Fermentation (SSF) process, for bioethanol production, obtaining an ethanol yield of 45% and 47% from pure inulin; and a yield of 51% and 48% from Jerusalem artichoke juice, respectively. Four selected isolates from strawberry tree fruit (STF) were used in a fermentation assay using STF juice, producing 86 - 100 g/l of ethanol from this raw material, at a very high yield (47-50%). These results show the enormous potential of inulin and Jerusalem artichoke as substrates for bioethanol production and the application of these novel yeasts as ethanol and/or inulinase producers.

**Key words**: Bioethanol; Bioprocesses; Inulinases; Jerusalem artichoke; Fluorescence *in situ* hybridization.

**Resumo**

A exploração de novos recursos energéticos foi crucial para a revolução tecnológica que ocorreu no início do século 19. Já nesse século se conhecia o enorme potencial do álcool como fonte de energia, visto que o primeiro protótipo de motor de ignição foi desenhado para funcionar com este combustível. No início do século 20, a produção de etanol foi substituída pela gasolina, devido ao baixo custo de extração. Os combustíveis fósseis têm sido, desde então, a principal fonte de energia utilizada. O rápido aumento da população tem intensificado a necessidade do aumento de produção alimentar e energética.

As limitadas reservas de petróleo, preocupações ambientais, o aquecimento global e a instabilidade política renovaram o interesse e, consequentemente, o apoio financeiro direcionada para o desenvolvimento de fontes renováveis de energia, como os biocombustíveis. Vários tipos de biocombustíveis têm sido estudados, mas apenas dois são produzidos à escala industrial: o biodiesel e o bioetanol. O bioetanol apresenta as melhores qualidades para a sua utilização nos atuais motores, podendo ser utilizado como aditivo na gasolina, sendo por isso, o biocombustível mais utilizado a nível mundial (Antoni *et al*., 2007).

A produção mundial de bioetanol à escala industrial utiliza principalmente duas fontes naturais: *Saccharum officinarum* (cana-de-açúcar) e *Zea mays* L. (milho). A cana-de-açúcar é sobretudo utilizada no Brasil, sendo o segundo maior produtor mundial de bioetanol, enquanto o milho é a principal fonte natural utilizada nos E.U.A., o maior produtor mundial. Estes dois países representam 88% da produção global (REN21).

Na produção de bioetanol ocorre a fermentação alcoólica dos substratos naturais, diretamente a partir da cana-de-açúcar (que contém principalmente sacarose); para a bioconversão do milho (composto principalmente por amido) há necessidade de um passo prévio de hidrólise enzimática para a sua conversão em açúcares simples. O bioetanol obtido a partir de culturas agrícolas produzidas exclusivamente para esse fim, ocupando assim área cultivável, denomina-se Bioetanol de 1ª Geração (1G). Este tipo de produção levanta questões morais e éticas porque, deste modo, o bioetanol compete diretamente com a produção alimentar (Luo *et al*., 2009).

Para ultrapassar este problema, tem sido proposto a utilização de resíduos lenhocelulósicos, como substratos, para produção de Bioetanol de 2ª Geração (2G). Este bioetanol não compete diretamente com a produção alimentar, apesar de consumir recursos agroindustriais. Atualmente a conversão de biomassa lenhocelulósica em bioetanol é ainda um procedimento caro e de baixa eficiência, sendo economicamente desfavorável a sua aplicação (Gibbons and Hughes, 2009). O principal obstáculo é o custo do pré-tratamento para conversão dos vários polímeros (celulose, hemicelulose, xilano, etc.) em açucares simples (glucose e xilose principalmente) que esta biomassa necessita, quer seja por degradação enzimática (com custos associados de produção de extratos enzimáticos); quer seja a aplicação de métodos químicos, como hidrólise ácida (que para além do seu custo, leva à produção de composto inibidores do crescimento microbiano).

Existem vários bioprocessos propostos para a produção em larga escala de 2G nomeadamente a hidrólise separada da fermentação (SHF), em que o passo de hidrólise da biomassa lenhocelulósica antecede a fase de produção de bioetanol; a fermentação e sacarificação simultâneas (SSF), neste caso há a adição exógena de enzimas ou a co-fermentação de biomassa com um microrganismo produtor das enzimas necessárias à conversão dos polímeros nos seus monómeros, disponibilizando assim os açúcares simples para o microrganismo etanologénico os converter em etanol; e o bioprocesso consolidado (CBP), em que a produção de enzimas para a hidrólise enzimática e a produção de etanol ocorrem por ação do mesmo microrganismo (considerado o melhor processo conceptual) (Lynd, 1996).

Infelizmente não está descrito nenhum microrganismo que, simultaneamente, seja capaz de produzir a maquinaria enzimática necessária para a hidrólise da biomassa lenhocelulósica e que a produção de etanol atinja valores de rendimento e produtividade economicamente viáveis.

Dadas as dificuldades de implementação à escala industrial do 2G e movidos por preocupações ambientais, de utilização de solos cultiváveis e o desequilíbrio atual no ciclo de carbono, levou alguns cientistas a desenvolver o Bioetanol de 3ª Geração (3G). Este bioetanol recorre a microalgas com elevado conteúdo nutritivo para a sua produção. Ao invés de aproveitar resíduos agroindustriais como substrato, as algas são produzidas explorando os recursos hídricos para a produção de biomassa. Desta forma, não há competição com produção alimentar nem área cultivável e não há utilização de recursos agroindustriais. No entanto, o baixo rendimento de produção de biomassa torna este processo, ainda, economicamente inviável à escala industrial (Goh and Lee, 2010).

Uma alternativa a estas opções é a utilização de plantas produtoras de inulina, como *Helianthus tuberosus* (tupinambo), que representam um recurso renovável, barato e abundante para a produção de bioetanol. O tupinambo tem várias características importantes que justificam a sua utilização, nomeadamente a tolerância a frio, à seca, ao vento e a terrenos arenosos e/ou salinos, com uma alta taxa de fertilidade e de resistência a pestes e doenças, não necessitando obrigatoriamente de terrenos férteis para se desenvolver. Estas características tornam-no numa fonte apetecível de biomassa para conversão em bioetanol que não compete pelos terrenos cultiváveis utilizados para produção alimentar (Neagu and Bahrim, 2011; Chi *et al*., 2011; Bajpai and Margaritis, 1982).

Este trabalho teve como objetivo a procura de novos microrganismos, especialmente leveduras, a partir de isolamentos de diferentes fontes naturais, nomeadamente dos frutos de alfarrobeira (*Ceratonia síliqua*), *ameixeira* (*Prunus domestica*), cerejeira (*Prunus avium*), figueira (*Ficus carica*), medronheiro (*Arbutus unedo*) e pessegueiro (*Prunus persica*) e tubérculos de tupinambo (*Helianthus tuberosus*). Os microrganismos foram selecionados com base nas suas características etanologénicas (maior tolerância a elevadas concentrações de etanol, pH e temperatura e, por isso,

capazes de fermentações alcoólicas mais longas); e/ou na capacidade de produção de enzimas relevantes, como inulinases, para posterior aplicação em bioprocessos industriais.

A partir de 98 isolados, dos quais 90 eram leveduras e 8 bactérias, foram selecionados 7 isolados diferentes. Destes, 3 isolados foram selecionados porque apresentaram capacidade de converter inulina purificada em etanol; os outros 4 isolados foram selecionados porque foram capazes de produzir etanol mais rapidamente, utilizando sumo de medronho como meio completo.

Às 7 estirpes foi aplicado um procedimento de identificação preliminar, utilizando sondas marcadas com um fluoróforo, para hibridação de fluorescência *in situ* (FISH), especificas para várias espécies de leveduras vínicas, nomeadamente: *Hanseniaspora uvarum*, *Kluyveromyces marxianus*, *Lachancea thermotolerans*, *Metschnikowia pulcherrima*, *Saccharomyces cerevisiae*, *Torulaspora delbrueckii* e *Zygosaccharomyces bailii*.

As 7 estirpes apresentaram um resultado positivo para apenas uma destas sondas, distribuindo-se por três espécies diferentes: *Lachancea thermotolerans* (AP1), *Saccharomyces cerevisiae* (DP2 e GluP4) e *Zygosaccharomyces bailii* (GerP3, Calf2, Talf1 e Talf2). Foi realizado um controlo positivo em cada experiencia de FISH para cada sonda testada, utilizaram-se estirpes identificadas e existentes no laboratório, nomeadamente: CBS 314 (*Hanseniaspora uvarum*), *Kluyveromyces marxianus* 516F, CBS 2803 (*Lachancea thermotolerans*), NRRL Y-987 (*Metschnikowia pulcherrima*), *Saccharomyces cerevisiae* CCMI 885, PYCC 4478 (*Torulaspora delbrueckii*) e *Zygosaccharomyces bailii* 518F.

Foi também utilizada uma sonda universal para células eucariotas (EUK516) em todas as células utilizadas, como controlo positivo, de forma a assegurar a presença de RNA acessível nas células após o procedimento de fixação, essencial na técnica de FISH. Esta identificação preliminar deverá ser validada com técnicas moleculares mais precisas, complementadas com estudos completos de morfologia e fisiologia.

Das estirpes preliminarmente identificadas como *Z. bailii*, três (Calf2, Talf1 e Talf2) apresentaram capacidade de produção de inulinases, uma caraterística não referenciada em estirpes desta espécie (Kurtzman *et al*., 2011). Foi traçado um perfil metabólico das estirpes, Calf2, Talf1 e Talf2, em confronto com a estirpe *Z. bailii* 518F utilizando duas galerias API distintas: API ZYM and the API 20C AUX. Conclui-se que as três estirpes têm perfiz metabólicos semelhantes entre si e com *Z. bailii* 518F, revelando características fisiológicas importantes para aplicação futura.

A produção de inulinases é uma característica essencial para a fermentação de inulina em etanol, permitindo a utilização de matérias-primas ricas em inulina como substrato para produção de bioetanol. Com esta finalidade, foi avaliada a produção de inulinases extracelulares das 3 estirpes, utilizando dois substratos indutores: a inulina (extraída de chicória) e o sumo de tupinambo. A estirpe Talf1 apresentou maior atividade enzimática extracelular quando induzida com sumo de tupinambo (8,7 U/ml) do que a estirpe Calf2 e Talf2, que atingiram 4,1 e 7,8 U/ml respetivamente.

Utilizando inulina purificada como substrato indutor, todas as estirpes atingiram aproximadamente 0,6 U/ml de atividade enzimática no extrato celular, o que demonstra a fraca capacidade de indução por parte da inulina purificada.

De forma a otimizar a produção de inulinases, utilizando a estirpe Talf1, foram testados outros substratos como indutores de inulinases: duas inulinas comerciais, extraídas de fontes naturais diferentes; e três matérias-primas: raízes de acelga, tubérculos de dália e o resíduo sólido de tupinambo, obtido após extração do sumo. Conclui-se que as inulinas comerciais purificadas são fracos indutores, não atingindo valores superiores a 0,6 U/ml, enquanto todas as matérias-primas naturais induziram positivamente a produção de inulinases, obtendo 1,9 U/ml com raízes de acelga, 1,5 U/ml com tubérculos de dália e 5,9 com o resíduo sólido de tupinambo. Concluiu-se que o tupinambo, em particular o sumo, é o melhor substrato indutor, para a produção de inulinases extracelulares utilizando a estirpe Talf1.

Foi feita a caracterização bioquímica do extrato enzimático desta estirpe, revelando que a temperatura e o pH ótimos de atividade são 45 ºC e 5,5 respetivamente. Foi determinada a estabilidade à temperatura e ao pH; o extrato enzimático manteve 57% da sua atividade inicial ao fim de 24 dias, quando mantido a 30 ºC e ao pH natural (5,5). No entanto alterações de pH diminuem drasticamente a estabilidade do extrato (a 30 ºC). As características do extrato enzimático da estirpe Talf1 são promissoras para a sua posterior utilização em bioprocessos que utilizem inulina ou fontes ricas em inulina como substrato desde que ocorram a pH ácido (aproximadamente 5,5) e à temperatura de 30 ºC.

Dadas as características descritas, a estirpe Talf1 foi utilizada num bioprocesso consolidado (CBP) e o respetivo extrato enzimático utilizado num processo de fermentação e sacarificação simultâneas (SSF) para produção de bioetanol. Para o processo SSF foi utilizada a estirpe etanologénica *S. cerevisiae* CCMI 885 e o meio foi suplementado com o extrato enzimático produzido por Talf1.

Na utilização de inulina como única fonte de carbono, o processo SSF apresentou maior rendimento e produtividade máximos de etanol (47% e 2,75 $g.l^{-1}.h^{-1}$) e obteve-se maior concentração de etanol no meio (78 g/l), enquanto no processo CBP produziram-se 67 g/l de etanol, com um rendimento e produtividade máximos de 45% e 1,70 $g.l^{-1}.h^{-1}$ respetivamente.

Foi realizado, em paralelo, um crescimento controlo com *S. cerevisiae* CCMI 885 e inulina como única fonte de carbono. Nestas condições, foram produzidas apenas 50 g/l de etanol, o que demonstra que a adição do extrato enzimático levou à hidrólise de polímeros de inulina que não são utilizados naturalmente por *S. cerevisiae*, apesar desta estirpe conseguir produzir enzimas que degradam parcialmente polímeros de inulina.

A produção de bioetanol a partir diretamente de sumo de tupinambo foi testada pelos dois bioprocessos (SSF e CBP). Neste caso obtiveram-se melhores resultados utilizando a levedura

Talf1 no bioprocesso consolidado. A estirpe Talf1 atingiu melhor produtividade (3,62 $g.l^{-1}.h^{-1}$,), rendimento (51%) e concentração máximas de etanol (67 g/l), do que a estirpe *S. cerevisiae* CCMI 885 em sumo de tupinambo (SSF), suplementado com o extrato enzimático contendo inulinases, que produziu 62 g/l de etanol, com um rendimento e produtividade máximos de 48% e 2,40 $g.l^{-1}.h^{-1}$, respetivamente.

No ensaio controlo, utilizando a levedura *S. cerevisiae* CCMI 885 sem adição de qualquer enzima, obtiveram-se menores resultados de rendimento e produtividade máxima (42% e 2,07 $g.l^{-1}.h^{-1}$) e apenas 55 g/l de etanol produzido, um valor inferior aos resultados obtidos com os anteriores bioprocessos. Estes resultados são consistentes com os obtidos apenas com inulina como fonte de carbono, reforçando a hipótese de que esta estirpe, *S. cerevisiae* CCMI 885, seja capaz de hidrolisar algumas cadeias de inulina, mas não utilizar todos os açúcares presentes no sumo de tupinambo.

Estes resultados mostram o potencial da inulina e fontes naturais ricas em inulina, como o tupinambo, para fontes alternativas na produção de bioetanol. No entanto para a aplicação industrial das estirpes descritas neste trabalho, será necessária posterior otimização de todo o processo.

As 4 leveduras selecionadas a partir do rastreio inicial de fermentação de sumo de medronho (*L. thermotolerans* AP1, *S. cerevisiae* DP2, *S. cerevisiae* GluP4 e *Z. bailii* GerP3), foram utilizadas num ensaio de fermentação de sumo de medronho, em comparação com *S. cerevisiae* CCMI 885. O melhor resultado foi obtido com a estirpe *S. cerevisiae* CCMI 885, atingindo-se 108 g/l de etanol, com um rendimento máximo de 51%, igual ao teórico possível, e uma produtividade máxima de 1,29 $g.l^{-1}.h^{-1}$.

No entanto, a estirpe de *Z. bailii* GerP3, com valores máximos de etanol, produtividade e rendimento máximos de 100 g/l, 1,11 $g.l^{-1}.h^{-1}$ e 50%, respetivamente, são próximos daqueles obtidos com a levedura *S. cerevisiae*. A estirpe *Z. bailii* GerP3 obteve, por isso, os resultados mais promissores para a aplicação num sistema de fermentação em estado sólido diretamente a partir de medronho.

O desenvolvimento de um processo eficiente e economicamente viável para produção de bioetanol é crucial para a sociedade atual, servindo como alternativa à utilização de combustíveis fósseis, para uma população mundial que requer cada vez mais energia.

A utilização de fontes naturais como o tupinambo e o medronho, para a produção de bioetanol, pode ajudar a reduzir a dependência energética dos combustíveis fósseis. No entanto, para produzir industrialmente bioetanol a partir destas fontes naturais renováveis, e ainda necessária a otimização do processo a escala industrial.

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**Abbreviations**

| | |
|---|---|
| % (v/v) | Percentage (ml/ml) |
| % (w/v) | Percentage (g/ml) |
| % (w/w) | Percentage (g/g) |
| 1G | 1st Generation Bioethanol |
| 2G | 2nd Generation Bioethanol |
| 3G | 3rd Generation Bioethanol |
| API | Analytical Profile Index |
| C5 | Pentoses |
| C6 | Hexoses |
| ca | Circa |
| CBP | Consolidated bioprocessing |
| CCMI | Culture Collection of Industrial Microorganisms, INETI, Lisbon, Portugal |
| Cy | Carbocyanine |
| DNA | Deoxyribonucleic acid |
| DP | Degree of polymerization |
| e. g. | *exempli gratia* (Latin); for example |
| ETBE | Ethyl tert-butyl ether |
| FISH | Fluorescence *in situ* hybridization |
| FITC | Fluorescein IsoThioCyanate |
| GC (%) | Guanine and cytosine percentage |
| HFS | High fructose syrup |
| i.e. | *id est* (Latin); (in other words) |
| IOS | Inulo-oligosaccharides |
| JA | Jerusalem artichoke |
| JAJ | Jerusalem artichoke juice |
| MTBE | Methyl tert-butyl ether |
| RCB | Rose bengal chloramphenicol agar |
| rpm | Rotations per minute |
| rRNA | Ribosomal ribonucleic acid |
| SDS | Sodium dodecyl sulfate |
| SSF | Simultaneous saccharification and fermentation |
| STF | Strawberry tree fruit |
| STFJ | Strawberry tree fruit' juice |
| TAMRA | Tetramethylrhodamine |
| Tm | Temperature of melting |
| TRICT | Tetramethylrhodamine-isotiocianate |
| Tris/HCl | 2-Amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride |
| TSB | Tryptone soy broth |
| YMA | Yeast malt agar |

| | |
|---|---|
| YMB | Yeast malt broth |
| $\mu_{max}$ | Maximum growth rate |

# 1. Introduction

## 1.1. Biofuels

Throughout history, mankind has relied on renewable energy sources like wood, windmills, water wheels and animals for sustainability. The development of new energy resources was crucial for the technological revolution occurred in the 19th century. Early in that century, alcohols were repeatedly reported as biofuels, alongside combustion, they were transformed into solvents, greases, cleaners or simple chemicals for the emerging chemical industry, ending with fossil fuel breakthrough. The first prototype for a spark ignition engine, by Nikolaus August Otto in the 1860s, was designed for ethanol and sponsored by the sugar factory of Eugen Langen. Later, in 1902, one third of Eugen Langen new company (Deutz Gas Engine Works) heavy locomotives ran on pure ethanol (Antoni *et al*., 2007). During the beginning of the 20th century, ethanol was recognized as an anti-knocking additive for internal combustion engines and was added to gasoline between 1925 and 1945. Even Henry Ford, a well known automotive inventor, believed the "fuel of the future" was ethyl alcohol made from farm products and cellulosic materials (Kovarik, 1998). He was involved in the chemurgy movement, which proposed the use of farm products for chemicals and energy production (Finlay, 2004). In the 1940s, ethanol production for fuel purposes was overthrown by gasoline's low price in USA. Fossil fuel has been, since then, the major energy source used.

In the past few decades, the rapid global population growth has increased the demand for higher food and energy production (Goldemberg, 2007; Nass *et al*., 2007; Ragauskas *et al*., 2006). The world's fossil fuel reservoir and its extraction will not keep pace with the society's need for petrochemical fuels. Several factors led to a renewed interest for biofuels, namely, the limited oil reserves, pollution concerns, global warming and political instability and disagreements, leading to an increased financial support for more sustainable and environmental friendly sources of energy (Antoni *et al*., 2007; Ragauskas *et al*., 2006).

Biofuel refers to fuel source generated from agricultural products, as crops or other organic material, obtained from other industries residues, such as crop residues, perennial grasses or alternative crops with higher productivity in impoverished soils. In Table 1 are compiled most recent biofuels and research status for its application. The biodiesel and bioethanol research areas are the only reproduced in industrial scale. Counting with ETBE (Ethyl Tert-Butyl Ether), which is primarily made by bioethanol, these 3 fuels compose 90% of all biofuel market. The main component for biodiesel is a monoalkyl ester of fatty acids from vegetable oil and is presently produced by catalytic transesterification with petro-chemically derived methanol (Ma and Hanna, 1999). Although biodiesel is not microbially produced, several research groups have focus on the utilization of microalgae, grown in photobioreactors, which reduces the specific demand of land area needed for oil derived from plants (Chisti, 2007); another potential approach followed for biodiesel bacterial production is microdiesel (Kalscheuer *et al*., 2006) where bacterial strains are selected and/or improved to increase fatty acids content of cells, which yields higher production rates for the biodiesel extraction directly from cells, and there by allow the use of different carbon sources or raw materials to serve as growth medium.

**Table 1.** List of selected biofuels with a potential microbial production route, the status of their use and the engine application (adapted from Antoni *et al*., 2007).

| Biofuel | Process | Status | Engine application |
|---|---|---|---|
| Biomethanol | Thermochemical/microbial | Pilot plant | [Pure/blend] MTBE/biodiesel |
| Bioethanol | Microbial | Industrial | Pure/blend |
| Biobutanol | Microbial | Industrial (until ca 1990) | Pure/blend |
| ETBE | Chemical/microbial | Industrial | Blend |
| Biomethane | Microbial | Industrial | Pure/blend |
| Biohydrogen | Microbial | Laboratory | Bioethanol (Syngas)/pure |
| Biodiesel | Physical/chemical (enzymatic) | Industrial (laboratory) | Pure/blend |

Although additional types of biofuels are being investigated, such as methane, hydrogen, *n*-Butanol, acetone and others, bioethanol fermentation is by far the largest scale microbial process, with a strong history of productivity in several countries (Antoni *et al*., 2007). The fermented beverages produced through yeast's activities have contributed significantly to the worldwide advancement and sustainability of human societies (Legras *et al*., 2007). Bioethanol does not need to be rectified to high purity, being directly used in Brazil as AEHC (*Álcool Etílico Hidratado Combustível*) (Antoni *et al*., 2007). Given the appropriate chemical characteristics of bioethanol as a liquid fuel for current engines, it is the most used biofuel worldwide.

### 1.2. Bioethanol

Ethanol can be produced synthetically from petrochemical materials (crude oil, gas or coal) or biologically by fermentation of sugars (bioethanol). In 1995, only 7% of world ethanol was produced by the synthetic method, the remaining 93% was produced by microbial fermentation (Kádár, 2005). Bioethanol as a biofuel regained importance in the 1970s, caused by a gasoline depletion which made the costs increase tremendously. Brazil was the first country to approve governmental support to implement bioethanol as current biofuel in that decade (Goldemberg, 2007). Today, the USA and EU have defined political objectives for increasing the use of bioethanol as alternative to fossil fuel, namely: in the Energy Independence and Security Act (EISA) and the European Commission White Paper, respectively. In 2010, the USA was responsible for 57% of global bioethanol production and Brazil contributed with almost 31%. The third producer worldwide was China, representing 2.3%. The global production of fuel ethanol reached 86 billion liters, an increase of 17% over 2009 (REN21). In Table 2 are described the main feedstocks used for bioethanol production. Bioethanol is mainly produced from two types of feedstocks: sugar and starch rich biomass. Sugar can be used for direct fermentation of ethanol. Sugarcane (*Saccharum officinarum*) and sugar beet (*Beta vulgaris*) are the major sugar-producing plants. Sugarcane is adapted to warm temperate to tropical areas, used in Brazil, whereas sugar beet is grown in temperate areas, mainly in Europe. Therefore the two sugar crops occupy different geographical niches (Yuan *et al*., 2008a). In the USA, corn (*Zea mays* L.) is the starch source for bioethanol production. For the alcoholic fermentation process, a saccharification step (conversion of polymers, like starch, into simple sugars) is required prior to its industrial batch.

**Table 2.** Bio-ethanol production and land use by major producing countries, 2006/07 (adapted from Balat and Balat, 2009).

| Country | Feedstocks used | Ethanol yield (l/hectare) | Feedstock share (%) | Arable land (Million hectare) | |
|---|---|---|---|---|---|
| | | | | Total area | Implied feedstock area |
| Brazil | Sugar cane | 6641 | 100 | 59 | 2.99 |
| USA | Corn | 3770 | 98 | 174 | 6.35 |
| | Sorghum | 1365 | 2 | - | 0.28 |
| China | Corn | 2011 | 70 | 143 | 0.65 |
| | Wheat | 1730 | 30 | - | 0.32 |
| EU-27 | Wheat | 1702 | 48 | 114 | 0.53 |
| | Sugar beet | 5145 | 29 | - | 0.12 |
| Canada | Corn | 3460 | 70 | 46 | 0.12 |
| | Wheat | 1075 | 30 | - | 0.16 |

The bioethanol produced exclusively from crops that use farmland is named 1$^{st}$ Generation Bioethanol (1G). The use of edible crops and farmland has raised morality and ethics issues, since fuel is directly competing with food resources. In order to overcome this issue, bioethanol produced from lignocellulosic biomass (e.g. trees, forestry and crop residue, grasses, and municipal solid waste), named 2$^{nd}$ Generation Bioethanol (2G), offers a great option which is compatible with economic growth and morality issues (Luo *et al*., 2009). The lignocellulosic biomass represents an enormous carbon source for bioconversion, with an estimated annual production of 1 366 million tons from forest biomass and agricultural residue just in the USA (Gibbons and Hughes, 2009). The monomers retrieved after specific treatment (acid or enzymatic hydrolysis) from the lignocellulosic biomass are pentoses (C5), as xylose and arabinose (sugars from hemicelluloses, xylan and lignin fibers), and hexoses (C6), as glucose (sugar from cellulose fibers). In order to use this biomass for bioethanol, the fermentation requires four main steps: enzyme production, biomass enzymatic hydrolysis, hexose and pentose fermentations (Lynd, 1996). The main objective is the utilization of all sugar content, both C6 and C5 sugars alike. The C6 sugars are used for ethanol production at high yield, however the C5 fermentation requires specific microorganisms which have by-product formation or slow xylose conversion limiting the economical application for ethanol production (Kötter and Ciriacy, 1993). The four processes for conversion of lignocellulosic biomass into ethanol are compared in Figure 1. The methodologies are displayed from the less (Separate Hydrolysis Fermentation - SHF) to the most integrated bioprocess (Consolidated BioProcessing - CBP). The enzyme cost and production is still the bottleneck for the industrial application of these approaches.

Through SHF process, the four necessary fermentation steps occur separately. It is the simplest process. The C5 and C6 sugars fermentation may occur in sequence (Figure 1A) or be allocated in parallel (Figure 1B).

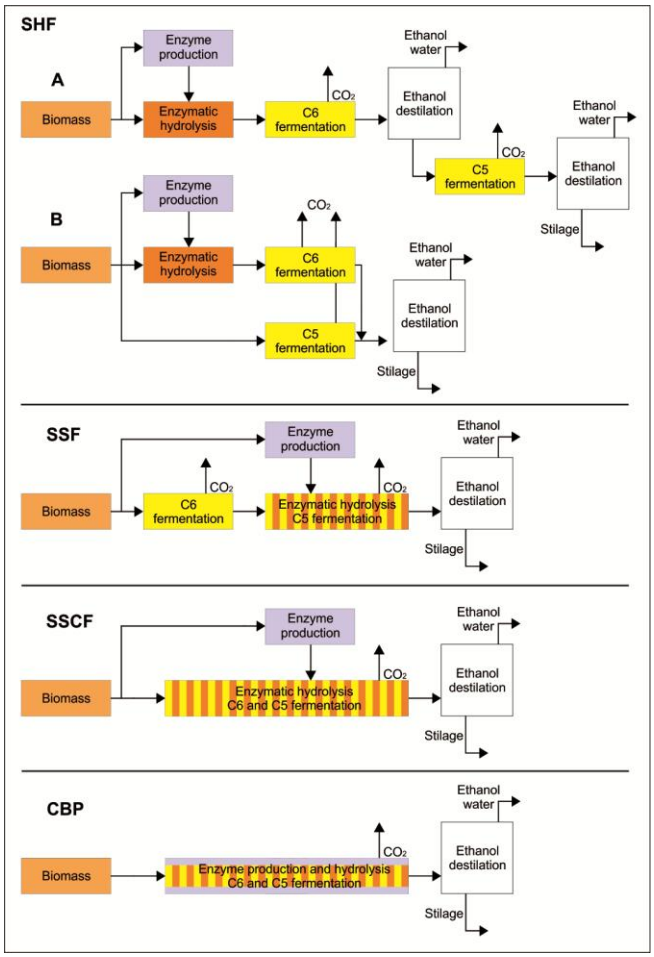



**Figure 1.** Levels of consolidation in enzyme production, enzymatic hydrolysis and fermentation to bioethanol (adapted from Hamelinck *et al.*, 2005).

For the Simultaneous Saccharification and Fermentation (SSF), the ethanol production co-occurs with the enzymatic hydrolysis, diminishing the procedure to three different fermentation batches. This avoids the problem of product inhibition associated with enzymes, given that the monomers enzymatically produced are consumed by the ethanologenic microorganism (usually *S. cerevisiae*). The Simultaneous Saccharification and Co Fermentation (SSCF) process refers to the joining of C6 and C5 fermentation step with simultaneous hydrolysis, which reduces to two fermentation batches.

The most integrated system is the Consolidated BioProcessing (CBP) which occurs in a single batch at the same time. It is the best conceptual design, although no organism or compatible combination of microorganisms is available that produces the desired enzymatic machinery and also produce ethanol at the necessary high concentration and yield (Lynd, 1996).

According to the following reactions, the ethanol theoretical maximum yield is 0.51 kg ethanol and 0.49 kg carbon dioxide per kg of sugar (Hamelinck *et al*., 2005):

$$3\ C_5H_{10}O_5 \rightarrow 5C_2H_6O + 5CO_2 \qquad (1)$$

$$C_6H_{12}O_6 \rightarrow 2C_2H_6O + 2CO_2 \qquad (2)$$

Currently, *Saccharomyces cerevisiae* is the main producer of 1G trough C6 fermentation (2), due to high ethanol concentration production from glucose and sucrose, and also due to high ethanol tolerance (Hirasawa *et al*., 2007; Abe *et al*., 2009). However, the pentose's fermentation (1) requires the utilization of C5 fermenting microorganisms, as *Zymomonas mobilis*, or recombinant *S. cerevisiae* strains (Hamelinck *et al*., 2005).

Unfortunately, several technical drawbacks still hinder industrial application of 2G, by comparison with 1G: the feedstock typically contains little protein or other nutrients (besides carbohydrates) that microbes will need; hence, nutrient supplementation may be required; improvements are needed to reduce biomass harvest and transportation costs; the lignocellulosic residue pretreatment are costly and often sufficiently intensive to generate inhibitory compounds that inhibit microbial growth; current deconstructing enzymes are more expensive and less effective than corresponding enzymes used with starch; the fermentation of both C5 and C6 reduces ethanol conversion efficiency; the currently used ethanologenic microbes lack tolerance to inhibitors generated during lignocellulose pretreatment, cannot ferment mixed sugars and cannot operate at the 50-60°C optimal temperature of the saccharification enzymes; finally, the engineered microbes that ferment mixed sugars may require regulatory approval and possess sufficient robustness to industrial conditions, high sugar and ethanol concentrations, and other variables (Gibbons and Hughes, 2009).

Given all these obstacles for implementing an industrial scale 2G production, and concerns about soil usage, environment impact and carbon cycle, a 3$^{rd}$ Generation Bioethanol (3G) was created. The 3G is based on an equilibrated carbon network that does not compete with agriculture (Goh and Lee, 2010). This 3G technology is based on algae or cyanobacteria that contain a high oil mass fraction grown in ponds. Microorganisms can convert almost all of the energy in biomass residuals and subsequently in bioethanol (Singh *et al*., 2011). The process of algal cultivation could be improved for higher yield efficiency of algal lipids through the screening and improvement of algal strains. Wastewater and flue gases are the best options for reducing the environmental burden from the cultivation of algal biomass. Further research and development are necessary to establish an economical industrial scale production of algal biofuels (Singh *et al*., 2011) due to low yield and biomass concentration, when compared with other bioethanol productions.

The availability of inexpensive sources of fermentable sugar is limited in most areas of the world, and the efficient conversion of cellulosic biomass or other inexpensive substrate sources to usable fuels will be required to increase the bioethanol production. The 3G requires further advances and an industrial scale application. The primary limitation in cellulosic biomass fermentation is the yeasts inability to directly convert cellulose, hemicelluloses and associated components into ethanol (Hahn-Hägerdal *et al*., 2006). An alternative to these options are inulin containing plants, representing a renewable, inexpensive and abundant raw material for industry and bioethanol production (Neagu and Bahrim, 2011; Chi *et al*., 2011; Bajpai and Margaritis, 1982).

### 1.2.1. Inulin and inulin rich materials

Fructans are one of the most abundant non-structural polysaccharides found in a wide range of plants. Inulin is a polydisperse fructan polymer composed by linear chains of β-2, 1-linked D-fructofuranose molecules terminated by a glucose residue through a sucrose-type linkage at the reducing

end (De Leenheer, 1996). Thus, inulin is a mixture of oligomers and polymers. The degree of polymerization (DP) in inulin varies according to plant species, weather conditions and age (Li and Chan-Halbrendt, 2009), ranging from 2 to 60 fructose monomers, or higher (Sirisansaneeyakul *et al.*, 2007; Yu *et al*., 2011). The molecular structure of inulin compounds is shown in Figure 2.

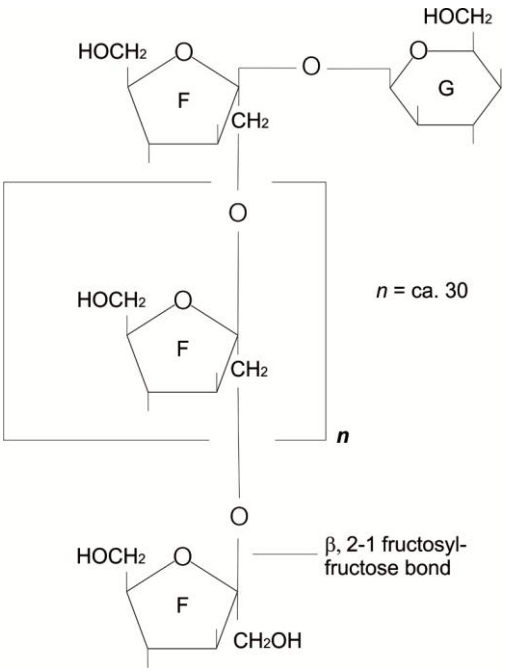



**Figure 2.** Molecular structure of inulin (from Vandamme and Derycke, 1983).

As a result, inulin is of great interest as it is a relatively inexpensive and abundant substrate for the production of fructose syrups and bioethanol (Bonciu *et al*., 2010). It is present as a reserve carbohydrate in the roots and tubers of several plants, such as Jerusalem artichoke (topinambur), chicory, dahlia and yacon (see Table 3). The inulin content depends on the plant species, being chicory, Jerusalem artichoke and dahlia the major sources of inulin for industrial scale with world production currently estimated to be about 350 000 tons (Kango and Jain, 2011).

Nowadays, inulin has gain interest for high fructose syrup, a low calorie sweetener, or inulo-oligosaccharides (IOS) synthesis, a functional food (Roberfroid, 2005), for the neutraceutical industry and for bioethanol production from renewable carbon sources. Briefly, inulin polymers have multiple industries application and potentially high relevance for our live. However, further advances are still required to assure a sustainable and profitable system.

Inulin can be actively hydrolyzed by microbial inulinases (fructofuranosyl hydrolases) to produce inulo-oligosaccharides (IOS), glucose and fructose as main products. In Figure 3 are illustrated the different inulinases activities, which can be divided into exo- and endo-acting enzymes according to the effect over the inulin. Endoinulinases (EC 3.2.1.7) are specific for inulin and can hydrolyze the internal β-2, 1-fructofuranosidic linkages to yield IOS, which are inulin chains with DP from 2 to 7 (Nguyen *et al*., 2011).

**Table 3.** Inulin content of some plants (withdraw from Kango and Jain, 2011).

| Source | Plant part | % of Inulin content (fresh weight) |
|---|---|---|
| Onion | Bulb | 2 - 6 |
| Jerusalem artichoke | Tuber | 14 - 19 |
| Dahlia | Tuber | 9 - 12.5 |
| Chicory | Root | 15 - 20 |
| Leek | Bulb | 3 - 10 |
| Garlic | Bulb | 9 - 16 |
| Artichoke | Leaves-heart | 3 - 10 |
| Banana | Fruit | 0.3 - 0.7 |
| Rye | Cereal | 0.5 - 1.0 |
| Barley | Cereal | 0.5 - 1.5 |
| Dandelion | Leaves | 12 - 15 |
| Burdock | Root | 3.5 - 4.0 |
| Camas | Bulb | 12 - 22 |
| Murnong | Tuber | 8 - 13 |
| Yacon | Tuber | 3 - 19 |
| Salsify | Tuber | 4 - 11 |

#### 1.2.1.1. Inulinases

The IOS have high applicability in the food industry, like confectionery, fruit preparations, milk desserts, yogurt and fresh cheese, as a functional food, with positive effects described as dietary fiber (Kaur and Gupta, 2002; Roberfroid, 2005). These features have increased the attention towards endoinulinase and their commercial application. Exoinulinases (EC 3.2.1.153) split off terminal fructose units from the non-reducing end of inulin, and also hydrolyze the disaccharide, sucrose, and the trisaccharide, raffinose (Yuan *et al*., 2012).

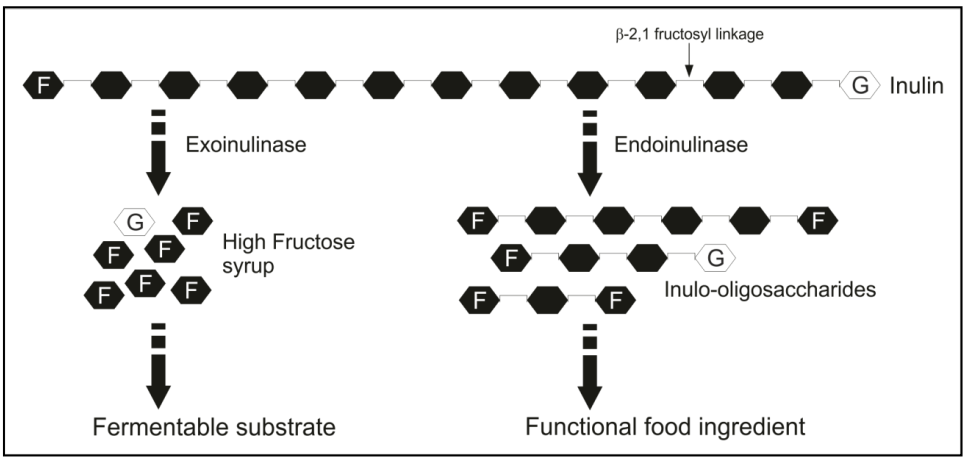



**Figure 3.** Microbial exo- and endoinulinase enzymes action. The action of exoinulinase liberates fructose from the macromolecule while endoinulinase produces IOS. (Figure adapted from Kango and Jain, 2011).

A number of microorganisms including molds, yeasts, actinomycetes and other bacteria are known to produce inulin hydrolyzing enzymes (Vandamme and Derycke, 1983; Pandey *et al*., 1999; Singh and Gill, 2006). Among the molds group, *Aspergillus* and *Penicillium* are the prominent genera, for bacteria the most promising producers include species of *Xanthomonas* and *Pseudomonas*, while *Kluyveromyces* genera is the most exploited yeast group for inulinase production, as summarized by Kango and Jain (2011). Over 35 inulinases have been characterized, from different microbial strains isolated from very different location. Several fungi and yeasts species of *Aspergillus* and *Kluyveromyces* genera have been used for inulinase production and gene characterization. Enzymes produced by most of strains are extracellular in nature (Pandey *et al*., 1999), however some authors reported as high as 50% and 75% of enzyme activity to be cell-associated (Rouwenhorst *et al*., 1991; Parekh and Margaritis, 1986).

Typically, exoinulinases present an invertase activity due to wide substrate specificity; therefore the classification of enzymatic activity depends on an activity ratio between invertase/ inulinase (I/S). Kangoo and Jain in 2011 considered the limit of 1 for the I/S ratio enough to separate inulinases from invertases, although Guiraud (1981) assumed different values: for inulinase the I/S ratio should be higher than $10^{-2}$, while for invertase the ratio values should be lower than $I0^{-4}$.

Inulinases are key enzymes for inulin based bioprocesses to occur hence described microorganisms cannot use this polymer without previous hydrolysis. Concerning the application of inulin for bioprocesses, the most desirable condition is the mix production of exo- and endo inulolytic enzymes, which results in total inulin hydrolysis into, mostly, fructose (Fru) and some glucose (Glu). The resulting ratio [Fru/Glu] depends on the initial inulin DP level. Afterwards the sugar rich hydrolysate can be used for bioprocesses as carbon source, retrieved from a natural, inexpensive and ecological source.

It is described that enzymatic inulin hydrolysis can reach yields up to 95% of fructose in high fructose syrups (HFS) (Ettalibi and Baratti, 2001; Makino *et al*., 2009). Inulin can also be hydrolyzed by acid treatment (pH=1.0 - 2.0 at 80 - 100 °C), but low pH results in degradation of fructose and the process also gives rise to difructose anhydrides, an inhibitory compound for bioprocesses. Therefore, enzymatically hydrolyzed inulin is an important substrate, with high potential for HFS production for nutritional industry and for bioethanol production.

#### 1.2.1.2. Jerusalem artichoke (*Helianthus tuberosus*)

The Jerusalem artichoke is a warm-season crop, native of North America and it has been grown to produce fructans that can be used for ethanol production, human-diet or medical and industrial applications (Meijer and Mathijssen, 1993). This interesting perennial tuberous plant belongs to the *Asteraceae* family and develops potato-like reserve tuberous with high inulin content (Table 3) when harvested at the appropriate season. Jerusalem artichoke has many desirable growing traits such as cold and drought tolerance, wind and sand resistance, saline tolerance, strong fecundity and high pest and disease resistance and does not require soil fertility (Zhang *et al*., 2010; Negro *et al*., 2006). The growth of Jerusalem artichoke with saline aquaculture wastewater (Gengmao *et al*., 2010) is a demonstration of this plant's potential as a renewable carbon source which can be produced without direct competition with arable soil and using a wastewater flow.

In the last years, many researchers and governments in the world, especially in developing countries, have been interested in using non-food grains as the materials in bioprocesses (e.g. bioethanol

production) due to the shortage of food grain, its high price and grain insecurity worldwide (Li and Chan-Halbrendt, 2009). In this context, inulin rich materials have gain importance as carbohydrates source for bioprocesses over the majority of food grains, such as starch-containing materials (Chi *et al*., 2001). Thus, inulin and inulin containing plants, such as Jerusalem artichoke, are promising substrates to use in bioprocesses, if the industrial bases are set up: development of resistant and rentable inulinase production; selection of efficient microbial strains and design of an economically viable bioprocess. Hence, Jerusalem artichoke, an inulin rich material, is one of the most interesting sources among unconventional and renewable raw materials, due to the high production of fermentable sugars (glucose and fructose) that can be used for several applications (e.g. nutritional industry, bioethanol production) and as a low cost alternative to purified inulin.

Researchers have studied the bioethanol production from Jerusalem artichoke tubers. While some consider the SSF approach as the most applicable, where a highly ethanologenic yeast strain of *S. cerevisiae* and the fungi *Aspergillus niger* are joined to ferment the raw substrate in the same fermentation batch (Nakamura *et al*., 1996; Ohta *et al*., 1993); others studied the SHF approach using a yeast strain of *S. cerevisiae* (Zhang *et al*., 2010), or the bacterium *Zymomonas mobilis* (Onsoy *et al*., 2007) or even a mix of *Z. mobilis*, *S. cerevisiae* and *Kluyveromyces fragilis* (Szambelan *et al*., 2004) for bioethanol production. The co-culture of different species for ethanol and enzyme production is difficult to be optimized due to different physiological conditions for the used species. Therefore to achieved a higher integrated bioethanol production system, yeasts capable of hydrolyzing inulin and produce ethanol like *K. marxianus* have been used directly with Jerusalem artichoke juice (Yuan *et al*., 2011; Yuan *et al*., 2008b; Bajpai and Margaritis, 1986), although with a low bioethanol yield. Strains of *S. cerevisiae* have been reported to directly utilize Jerusalem artichoke juice, again, with the production of low ethanol concentration (Lim *et al*., 2011). In addition, the Jerusalem artichoke tubers have been proposed as carbon source for different bioprocesses for production of value-added products such as: L-lactic acid, 2,3-butanediol, lipids, single-cell protein and single cell oil (Ge *et al*., 2009; Sun *et al*., 2009; Zhao *et al*., 2010; Gao *et al*., 2007; Zhao *et al*., 2011).

### 1.3. Microbial isolation and characterization

Currently, the *Saccharomyces cerevisiae* yeast is the main producer of industrial bioethanol due to its high ethanol concentration production from glucose and sucrose and its high ethanol tolerance. However, several drawbacks limit the use of this yeast in the design of high efficient industrial bioprocesses for bioethanol production from renewable and inexpensive biomass (e.g. lignocellulosic or inulin-rich materials), such as: unavailability of the necessary enzymatic machinery, inability to ferment C5 compounds (e.g. xilose) and intolerance to high temperatures. For these reasons is unfeasible to perform a consolidated bioprocessing (CBP), where the whole process occurs in one fermentation batch at the same time.

In this context, the screening and isolation of new microorganisms, mainly novel yeasts, presenting innovative characteristics, is crucial. Features such as higher thermotolerance, higher tolerance to ethanol and other inhibitors, and ability of produce the entire hydrolytic enzymes (e.g. xylanases and cellulases, inulinases, amylases, etc) are imperative to develop a CBP for bioethanol production. The CBP is the best conceptual design for biofuel production given that it is the most integrated bioprocess.

However, until now no microorganism or compatible combination of microorganisms that produce the required enzymatic machinery and produce ethanol at the necessary high concentration and yield, is available.

### 1.3.1. Yeast isolation

Yeasts are recovered from all habitats, aquatic, marine, atmospheric and terrestrial seldom occur without molds or bacteria (Kurtzman *et al*., 2011). Consequently, selective techniques must be used in order to recover yeasts. Employing selective media, with pH levels and/or water activities that reduce or inhibit the growth of bacteria or by inclusion of antibiotics/ fungistatic agents permits the isolation of one particular colony. Most yeasts are mesophilic, which means the incubation temperature should be around 20-25 ºC, although specific groups like psychrophilic require lower temperatures between 4 and 15 ºC. Several media have been designated for these purposes, with lower pH's which suppresses most bacterial growth or with addiction of antibiotics like chloramphenicol. An example is the Rose Bengal Chloramphenicol Agar, used for selective isolation and enumeration of yeasts and molds from food. Chloramphenicol is recommended as a selective agent in fungal medium with a neutral pH because of its heat stability and broad antibacterial spectrum (Mislivec *et al*., 1992). The Rose Bengal dye has also known cytotoxic effects on bacterial species (Banks *et al*., 1985), increasing the selectivity of the medium towards yeasts.

### 1.3.2. Characterization by classical biochemical tests

The classical methods used for microbial identification are culture-dependent methods based on morphological and physiological tests which require microorganisms to be isolated and cultivated prior to identification. Today, we live the era of microbial strain identification by gene sequencing, which as shown to be faster and more accurate than classical methods (Kurtzman *et al*., 2011). However, morphological and physiological properties must be reported in order to describe the general biological properties of the species. These features are of extreme importance, since they refer to the phenotypic characteristics of strains, crucial information for biotechnological purposes. The physiological tests commonly used for identification are carbohydrates fermentation, growth on various carbon and nitrogen sources, growth at different temperatures or on media with high content sugar or sodium chloride. However, these are time consuming and laborious methods and automated diagnostic systems have been developed for rapid microbial identification. Initially design for clinical purposes, these systems are applied for yeasts identification with relative success, since they support in growth reactions. The most used systems are the API (Analytical Profile Index) System, Biolog and Vitek cards. The identification capacity of these systems depends on the size of the corresponding database, which in the case of yeasts species is very incomplete.

### 1.3.3. Fluorescence *in situ* hybridization

The Fluorescence *in situ* hybridization (FISH) is a molecular method based on the hybridization of synthetic fluorescently-labeled oligonucleotide probes targeted to specific regions of the ribosomal RNA (rRNA) of a microorganism. The fluorescently-labeled FISH-probes enter inside the cells and hybridize with the rRNA target-sequence that is complementary to the cDNA sequence of the probe, emitting a fluorescent signal that allows the visualization of the intact cell by epifluorescence microscopy (Amann

and Fuchs, 2008). Since FISH is a culture-independent method that allows the identification of specific microbial species at the single-cell level, it has been considered as the most powerful method for various applications in microbiology (Amann and Fuchs, 2008).

A typical FISH protocol using rRNA-targeted oligonucleotides probes includes four steps: fixation and permeabilization, hybridization, washing, visualization and/or quantification of hybridized cells by epifluorescence microscopy or flow cytometry (see Figure 4). First, the microbial cells are permeabilized to allow penetration of the fluorescent probes into the cell and then fixed to protect RNA from degradation by endogenous RNAses. Afterwards, the fluorescently labeled FISH-probes are added to the fixed cells and allowed to hybridize with the targeted-sequence by incubating the mixture at temperatures of about 46-60 ºC during several hours, typically three hours (Moter and Gobel, 2000; Amman and Fuchs, 2008). After hybridization, a washing step is carried out to remove unbound probe.

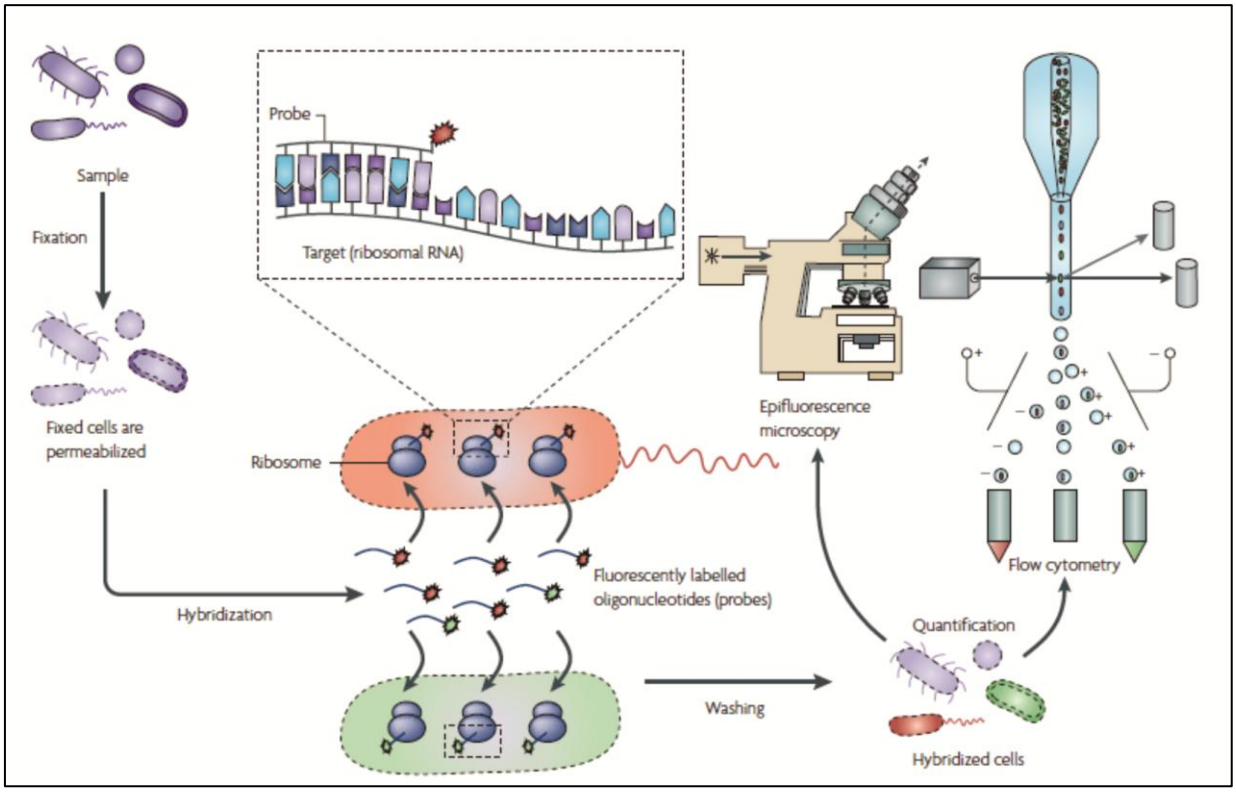



**Figure 4.** Basic steps of fluorescent in situ hybridization (withdrawn from Amann and Fuchs, 2008).

Applying the FISH procedure requires the design of species-specific probes able to hybridize with the targeted sequence. The RNA molecules that constitute the ribosome (small 16S/18S and large 23S/26S subunits) meet most of the characteristics required to choose as target-sequence: they are present in all cellular organisms; they are genetically stable containing variable and conserved regions; they exist in high numbers in metabolically active cells, which means that there are sufficient targets inside a single cell to allow its visualization; there is a high number of RNA sequences available in public databases (e.g. GenBank), which allows the design of species-specific probes through the alignment and comparison of sequences for different species. In yeasts, the D1/D2 domains of 26S rRNA exhibits a high degree of interspecies sequence variation, and consequently has been used for many phylogenetic and identification studies (Kurtzman and Robnett, 1998; Fell *et al*., 2000). The general features to consider in the designing of a FISH-probe are: specificity, taking into account dimension, the number of nucleotides

that differentiate it from phylogenetically-close species and its composition; ability to penetrate the cell and accessibility to the rRNA region where the target-sequence is located. The oligonucleotides probes used in FISH are generally 15 to 20 nucleotides long and have a fluorescent dye covalently linked to its terminal 5'-end. The most commonly used fluorescent dyes to label oligonucleotides probes are: fluorescein isothiocyanate (FITC), tetramethylrhodamine (TAMRA), carbocyanine (Cy3, Cy5 and Cy7) and tetramethylrhodamine-isotiocianate (TRICT) (Amann *et al*., 2001).

*In situ* identification of individual cells by FISH may be hindered by low cellular ribosome content, limited cell wall permeability and inaccessibility of the probe to the target sites of the rRNA, mainly due to structural issues (Bidnenko *et al*., 1998; Binder and Liu, 1998; Fuchs *et al*., 1998; Inácio *et al*., 2003). Inaccessibility of the probes to the target regions of rRNA is usually caused by molecular interactions (rRNA-rRNA and rRNA-protein) in which the target region of the ribosome is involved (Amann *et al*., 1995). To overcome the target site inaccessibility, Fuchs *et al*. (2000) evaluated the potential of several unlabeled helper oligonucleotides, which bind adjacent to the probe target site, in order to increase weak probe hybridization signals in *Escherichia coli* DSM 30083T. Their results suggested that by the joint action of multiple adjacent helper oligonucleotides, every site on the rRNA could be opened for FISH. Inácio *et al*. (2003) evaluated the accessibility of fluorescently labeled probes to the D1/D2 domains of the 26S rRNA of *S. cerevisiae* and designed an accessibility map for this species. Based on this accessibility map, Xufre (2005) designed species-specific FISH-probes that allowed the monitorization of the population evolution of several yeast species (i.e. *Hanseniaspora uvarum*, *Kluyveromyces marxianus*, *Lachancea thermotolerans*, *Metschnikowia pulcherrima*, *Saccharomyces cerevisiae*, *Torulaspora delbrueckii* and *Zygosaccharomyces bailii*) during industrial and laboratory wine fermentations.

In this work, the probes of Xufre (2005) were used in FISH assays for a preliminary molecular method for a species putative identification of the selected novel yeast isolates presenting potential for bioethanol production.

### 1.4. Scope of the thesis

The main goal of this work was to search for novel microbial strains, in special new yeasts, with high potential to produce bioethanol, due to either the presence of better ethanologenic characteristics (e. g. high tolerance to ethanol concentration, extremes pH and/or temperature; ability to perform long term fermentations) or production of relevant hydrolytic enzymatic machinery, which can be competitive in further application for industrial bioprocesses.

All the novel yeasts strains isolated which presented interesting characteristics for further application in a biofuel production process were characterized using classical biochemical methods and the fluorescence *in situ* hybridization (FISH) technique, as a preliminary molecular approach for the putative identification of the selected isolates.

In addition, novel inulinases produced by a promising selected yeast isolate, were also applied for bioethanol production in a consolidated bioprocess (CBP), and in a Simultaneous Saccharification and Fermentation (SSF) process, using inulin and inulin rich materials as carbon source.

## 2. Materials and Methods

### 2.1. Isolation of novel microorganisms

Different fruits with high levels of fermentable sugars (glucose, fructose and sucrose) were chosen as natural isolation sources, namely: carob (*Ceratonia síliqua*) pulp kibbles, cherry (*Prunus avium*), fig (*Ficus carica*), Jerusalem artichoke (*Helianthus tuberosus*) tubers, peach (*Prunus persica*), plum (*Prunus domestica*) and strawberry tree (*Arbutus unedo*) fruits. The cherries, figs, peaches and plums were collected from the Portuguese west region (Torres Vedras, Portugal), while Jerusalem artichoke tubers and strawberry tree fruits were harvest from the north region (Oleiros, Portugal) and the carob pulp kibbles were from the south region (Faro, Portugal). Each fruit was mashed into a homogeneous pulp. For carob pulp kibbles and Jerusalem artichoke tubers, a pre-enrichment step was performed, inoculating 1% (w/v) of each pulp in 100 ml shake flasks containing 25 ml of tryptone soy broth (TSB; Oxoid, Switzerland). The flasks were incubated in a rotary shaker (150 rpm) at 25 ºC for 48 h, for microbial enrichment. For the other fruits, the pulp itself was used for the isolation procedure.

#### 2.1.1. Isolation procedure

The microorganisms were isolated by decimal serial dilution of the pulps (dilution factor ranging from -4 to -7), pre-enriched or not, and plated on rose bengal chloramphenicol agar (RBC; Merck, Germany) through standard spread plate method. All plates were incubated at 25 ºC for 3 to 5 days. After incubation, individualized and morphologically different colonies (yeast or bacterium like) were selected and purified on yeast malt agar (YMA; Scharlau, Spain) plates. For purification, each isolate was transferred at least three times. The isolates were maintained on YMA slants at 4 ºC and sub-cultured monthly for laboratory routine. All isolates were also maintained at -25 ºC by addition of 30% (v/v) glicerol to previously grown cultures in yeast malt broth (YMB; Sigma, USA).

### 2.2. Alcoholic fermentation screening

The microbial isolates were previously inoculated in test tubes containing 5 ml of sterilized YMB medium and incubated for 2 - 3 days at 25 ºC without shaking**,** for inocula production. An aliquot (130 μl) of each inoculum was then transferred to a 16 cm test tube containing an invert Durham tube in 10 - 12 ml of a sterilized liquid medium with 0.5% (w/v) of yeast extract and the tested carbon source (50 mM arabinose, fructose, glucose, xylose, cellobiose, lactose or sucrose and 8.5 g/l inulin). For blank controls, an aliquot of each inoculum was transferred to a 16 cm test tube containing an invert Durham tube in 10 - 12 ml of liquid medium with 0.5% (w/v) yeast extract but without any carbon source. After 7 days of incubation at 25 ºC without shaking, all tubes were observed for growth density and gas formation inside the invert Durham tube, as an indicator of alcoholic fermentation ability.

For the strawberry tree fruits' isolates, this method was adapted to test their ability to use the strawberry tree fruit' juice (STFJ) as substrate to alcoholic fermentation. In this case, the liquid medium within the test tubes consisted on 10 - 12 ml of non-sterile pure STFJ. The test tubes were incubated at 25 ºC without shaking and the growth and gas formation were observed after 3 and 7 days.

## 2.3. Fluorescence *in situ* hybridization (FISH)

### 2.3.1. Oligonucleotide probes

In this work were used the oligonucleotides probes for *Hanseniaspora uvarum* (Huv), *Kluyveromyces marxianus* (Kma), *Lachancea thermotolerans* (Lth), *Metschnikowia pulcherrima* (Mpu), *Saccharomyces cerevisiae* (Sce), *Torulaspora delbrueckii* (Tde), *Zygosaccharomyces bailii* (Zba) and for eukaryotic cells (EUK516). These probes were those previously designated by Xufre (2005); with exception for the EUK516 probe, which was design by Amann *et al*. (1990). The specie-specific FISH probes are formed by 17-19 nucleotides and targeted the D1-D2 domain of the 26S ribosomal RNA (Table 4). The probes were synthesized and labeled with the fluorochrome Fluorescein IsoThioCyanate (FITC) at the 5'-end.

**Table 4.** Description of the probes used in FISH assay.

| Probe | D1-D2 Position (5'→3') of *S. cerevisiae* * | Sequence (5'→3') | GC (%) | Tm (ºC) | Target Specie | Reference |
|---|---|---|---|---|---|---|
| Huv | D507 | TCAATCCCGGCTAACAGTA | 47 | 56 | *H. uvarum* | Xufre, 2005 |
| Kma | D94 | AGCTACAAAGTCGCCTTC | 50 | 54 | *K. marxianus* | |
| Lth | D196 | ATAGGACTAGACTCCTCG | 50 | 54 | *L. thermotolerans* | |
| Mpu | D519 | ATTGCAGGCCTCGGGGT | 65 | 56 | *M. pulcherrima* | |
| Sce | D527 | TGACTTACGTCGCAGTCC | 56 | 56 | *S. cerevisiae* | |
| Tde | D495 | GCAGTATTTCTACAGGAT | 39 | 50 | *T. delbrueckii* | |
| Zba | D161 | GGGATCCTCGCCATACGG | 66 | 60 | *Z. bailii* | |
| EUK 516 | ** | ACCAGACTTGCCCTCC | 63 | - | Eukaryotic species | Amann *et al*., 1990 |

* Region D1-D2 of rRNA 26S subunits correspondent position to the *S. cerevisiae* gene.

** Eukaryotic probe targeting the 16S rRNA, in positions 502 to 516.

### 2.3.2. FISH procedure

#### 2.3.2.1. Silanization

The acetone resistant glass slides used (Slides for Immunofluorescence, bioMérieux, France) presented 10 circles (6 mm diameter) and were silanized to facilitate the hybridization protocol. The silanization was carried out as follows: slides were emerged in absolute ethanol for 1 h, and carefully dried after washed with Milli-Q water. Afterwards, glass slides were submerged on 2% (v/v) bis (3-trimethoxysilylpropyl) amine in acetone solution for 2 min. The glass slides were cleaned in acetone for 1 min, before emerged in Milli-Q water and carefully dried for the hybridization procedures.

#### 2.3.2.2. Fixation

The FISH procedure used in this work was adapted from Xufre *et al*. (2006) to be used in appropriate glass slides. The fixation protocol performed consisted on: 1 ml cell growth in YMB medium for approximately 24 h was centrifuged for 5 min at 4 000 × g. The pellet was then washed once with phosphate-buffered saline 1x (PBS 1x) (see Appendix) and then incubated with 4% (v/v) of paraformaldehyde solution in PBS 1x for 16 h at 4 ºC under strong agitation. Subsequently, fixed cells

were centrifuged for 2 min at 10 000 × g and resuspended in 400 μl PBS 1x and 400 μl ethanol 98% (v/v). The fixed cells samples were kept at -20 ºC until required.

2.3.2.3. Hybridization

The hybridization step involved the silanizated slides, through the following protocol: 5 μl of fixed cells sample was dried on a circle, after 15 min at 55 ºC. Subsequently, 12 μl of enzyme solution (1 mg/ml of lysozyme in PBS 1x) were add to each circle filled with fixed cells and incubated in a humidity chamber at 37 ºC for 30 min. After washing each slide in Milli-Q water and carefully dry it, a dehydration step with increasing ethanol concentration solutions was conducted: 2 min in 50% (v/v) ethanol solution, then 2 min in 80% (v/v) ethanol and finally 2 min in 98% (v/v) ethanol solution. For the hybridization step, 10 μl of the hybridization solution (hybridization buffer (Appendix) together with 5 ng/ml of FITC labeled probe) was dropped in each circle. Incubation was performed at 46 ºC for, at least, 3 h protected from light. After hybridization, the slide was emerged in Washing Buffer (Appendix) and incubated at 48 ºC for 30 min. Afterward each slide was washed in Milli-Q waters and dried at 37 ºC. For fluorescence preservation, slides were then mounted with a drop of VectaShield (Vector Laboratories, USA) for observation purposes by epifluorescence microscopy (Olympus BX-60, Tokyo, Japan) with the appropriate filter for FITC wavelength.

2.3.3. Controls

Strains well identified and routinely used at laboratory were used as positive controls for each FISH assay, namely: *Hanseniaspora uvarum* CBS 314, *Kluyveromyces marxianus* 516F, *Lachancea thermotolerans* CBS 2803, *Metschnikowia pulcherrima* NRRL Y-987, *Saccharomyces cerevisiae* CCMI 885, *Torulaspora delbrueckii* PYCC 4478 and *Zygosaccharomyces bailii* 518F.

The yeast strain designated *Saccharomyces cerevisiae* CCMI 885 was isolated from Alentejo grape musts (Albergaria *et al*., 2000) and was preserved from the previous Culture Collection of Industrial Microorganism (CCMI) of INETI, Lisbon, Portugal. The CBS 2803 strain was also isolated from grapes, in Italy. The PYCC 4478 strain was isolated from yellow and sulfurous concentrated must beverage from Azeitão, Portugal. The *K. marxianus* and the *Z. bailii* strains used were isolated from grape must produced in Borba wine cellar and identified by Xufre (2005). All strains are maintained at laboratory for routine usage.

In all FISH assays an autofluorescence control was conducted to prevent from false-positive results, where cells were subject to all the FISH procedure but without the addition of FITC probe.

## 2.4. Metabolic characterization

The metabolic characterization of some selected isolates was performed using the Analytical Profile Index (API) galleries, namely the API ZYM and API 20C AUX standard tests, according to the manufacturer's instructions (API®, bioMérieux, Switzerland). The biochemical tests included in these API galleries determine the presence of enzymatic activities (API ZYM) and the sugar assimilation profiles (API 20C AUX).

#### 2.4.1. API ZYM

For the selected isolates, a cell suspension was prepared from a fresh YMA slant (approximately 72 h) in an ampoule of NaCl 0.85% (w/v) medium (5 ml) to reach a cell density of 5 McFarland by comparison with a standard. The 20 cupules of the API ZYM strips were inoculated with 65 μl of cell suspension each. The inoculated galleries were incubated at 30 ºC for 4 h. All the reactions were evaluated by direct observation, according to the standard operational procedure supplied by the manufacturer.

#### 2.4.2. API 20 C AUX

For the selected isolates, the initial cell suspensions were prepared in 7 ml API M medium (bioMérieux, France) to obtain a cell density of 2 McFarland. The suspensions were gently homogenized with a pipette. The 20 cupules of API 20C AUX strips were then filled with each cell suspension, using a sterile Pasteur pipette, and incubated at 30 ºC. The results were registered after 48 h and 72 h of incubation, according to manufacturer's instructions.

### 2.5. Inulinase Production

#### 2.5.1. Inulinase induction

The microbial isolates positive for inulinase activity (Talf1, Talf2 and Calf2) were previously transferred from the YMA slant to 25 ml of YMB in 150 ml erlenmeyer flask and cultivated aerobically for 24 hours at 25 ºC, for the inocula production. After, 10% inoculum was used to inoculate 500 ml erlenmeyer flasks containing 100 - 150 ml of the induction medium consisting of YMB supplemented with 10% (v/v) inulin (90-95% inulin extracted from chicory roots; Fagron, Spain) or 25% (v/v) Jerusalem artichoke juice (JAJ), as inducer substrates. The JAJ was obtained by crushing the tubers in a juicer and recovering the liquid. The cultures were grown by shaking at 150 rpm, 25 ºC for 8 days. The growth samples were centrifuged at 5 000 × g, 4 ºC for 10 min, and the supernatants were sterilized by filtration through 0.2 μm membrane and kept at -20 ºC. The supernatants were used as the enzyme extracts for enzymatic activity determination. The assays were carried out in duplicates.

Moreover, for the best inulinase producer yeast strain, a new set of assays were carried out testing a wider range of inducer substrates, from different pure inulin sources to complex raw materials. Thus, in addition to inulin from Fagron, other two inulins were also tested, namely: an inulin from Sigma (extracted from dahlia tubers) and an inulin from BDH chemicals (from unknown natural source). Furthermore, besides JAJ, three new complex raw materials were also tested, namely: dahlia tubers (*Dahlia spp*), chard roots (*Beta vulgaris* L. var. cicla) and JA solid residue. The dahlia tuber is another known natural source for inulin while the chard is an edible plant close related with sugar beet (*Beta vulgaris*). The dahlia tubers and chard roots were grown in the laboratory campus (LNEG, Portugal) and milled before use. In this set of assays, the induction medium consisted of YMB supplemented with 10% (w/v) of each new inducer substrate tested. The assays were carried out in duplicates.

#### 2.5.2. Inulinase activity determination

For determination of the inulinase activity in each enzymatic extract, the amount of reducing sugars released from inulin hydrolysis in each reaction mixture was assayed using DNS method (Miller, 1959).

The reaction mixtures consisted of 25 μl of crude enzymatic extract and 975 μl of 1% inulin in 50 mM sodium citrate buffer, pH 5.5, and were incubated at 45 ºC for 15 min. Each reaction was immediately inactivated by putting the reaction mixtures on ice and adding 500 μl of DNS reagent (see Appendix). Subsequently, the reaction mixtures were put in boiling water for 5 min and then cooled on ice for 5 min. The same mixtures, but with inactivated enzyme crude extracts, were used as blank controls. All enzymatic reactions were performed in duplicates. To each reaction tube 5 ml of distilled water was added and the absorbance was determined at 540 nm in a UV/Vis microplate spectrophotometer (Multiskan GO, Thermo Fisher Scientific, Finland) in triplicate for each reaction. Quantifications of reducing sugars were carried out using a standard curve.

The standard curve for DNS method was prepared using fructose (stock solution: 2 g/l fructose in 50 mM sodium citrate buffer, pH 5.5). Several dilutions of the fructose solution were prepared in a final volume of 1 ml, as indicated in Table 5. The 50 mM sodium citrate buffer pH 5.5 was used as blank. The reactions for standard curve were also performed in duplicates. One unit of inulinase activity (U) was defined as the amount of enzyme responsible for the production of 1 μmol of reducing sugar per minute under the above conditions.

**Table 5.** DNS assay dilutions for standard curve.

| Dilutions | Fructose solution (μl) | Sodium citrate buffer (μl) | Final fructose (μmol) |
|---|---|---|---|
| Blank | 0 | 1000 | 0 |
| 1 | 100 | 900 | 1.11 |
| 2 | 200 | 800 | 2.22 |
| 3 | 300 | 700 | 3.33 |
| 4 | 400 | 600 | 4.44 |
| 5 | 500 | 500 | 5.55 |
| 6 | 600 | 400 | 6.66 |
| 7 | 700 | 300 | 7.77 |
| 8 | 800 | 200 | 8.88 |
| 9 | 900 | 100 | 9.99 |
| 10 | 1000 | 0 | 11.10 |

### 2.5.3. Characterization of inulinases

The influence of temperature and pH on inulinase activity was carried out on the sterile Talf1 crude enzymatic extract. This extract was previously obtained by growing the yeast strain Talf1 in YMB supplemented with 25% (v/v) Jerusalem artichoke juice for 8 days at 25 ºC, with shaking (150 rpm). The optimal temperature for inulin hydrolysis by Talf1 enzymatic extract was determined by incubating the crude extract (5 ml) at temperatures ranging from 20 to 65 ºC. Thermal stability data were obtained by incubating the enzyme extract (5 ml) at 30, 45 and 50 ºC, for different time intervals, from 3 hours to 24 days, after which the remaining inulinase activity was determined. The effect of pH on Talf1 enzymatic extract was studied using different buffers: 50 mM sodium citrate (pHs: 3.0 - 7.0) and 50 mM Tris/HCl buffer (pHs: 7.5, 8.0 and 8.5) (see Appendix). Data of stability to pH were also evaluated by incubating the Talf1 crude extract adjusted to different pHs (5.5; 7.5 and 10) at 30ºC for different time intervals (0 - 24 days), after which the remaining inulinase activity was determined.

## 2.6. Bioethanol production

### 2.6.1. Carbon source: inulin

A study for the ethanol production from inulin was performed by two bioprocesses: a consolidated bioprocessing (CBP) and a simultaneous saccharification and fermentation (SSF). The CBP was conducted with the yeast Talf1, a strain selected from several microbial isolates, using an Inulin Fermentative Medium (see Appendix). The SSF process was carried out with the strain *Saccharomyces cerevisiae* CCMI 885 using the same fermentative medium, but supplemented with 5% (v/v) of Talf1 enzymatic extract (previously obtained by growing the yeast strain Talf1 in YMB supplemented with 25% (v/v) Jerusalem artichoke juice, at 25 ºC with shaking (150 rpm) for 8 days). A control growth was also performed with the strain *S. cerevisiae* CCMI 885 in the same Inulin Fermentative Medium without enzymatic extract addition. For all assays, prior inocula were prepared in YMB medium and incubated at 25 ºC with shaking (150 rpm) for 24 h. Then, the assays for both bioprocesses were conducted in 150 ml of fermentative medium in 500 ml erlenmeyer flasks, inoculated with 10% (v/v) inoculum (the correspondent pellet of inoculum culture centrifuged was resuspended in the medium), and incubated at 25 ºC, without shaking, for 8 days. All the assays were carried out in triplicates except the control growth with *S. cerevisiae*. Ethanol production, sugar consumption and cell growth were monitored along the bioprocess.

### 2.6.2. Fermentative medium: Jerusalem artichoke juice

Using the same procedure as described above, the yeast strains Talf1 and *S. cerevisiae* CCMI 885 were inoculated directly in pure Jerusalem artichoke juice (JAJ) (an inulin rich raw material with approximately 130 g/l of hydrolyzable sugars), as the only nutrients source for ethanol production. The JAJ was obtained by crushing the tubers in a juicer and recovering the liquid. A CBP assay was conducted with strain Talf1 and a SSF was performed with *S. cerevisiae* CCMI 885, supplementing the JAJ with 5% (v/v) of the same Talf1 enzymatic extract described above. A control growth was also performed for the *S. cerevisiae* CCMI 885 inoculating it in the pure JAJ, but without the enzymatic extract addition. For both bioprocesses, fresh inocula were prior prepared as above described and 10% (v/v) of inocula (correspondent pellet) were used to inoculate the respective fermentative broth (150 ml) in 500 ml erlenmeyer flasks, and incubated at 25 ºC, without shaking, for 8 days. The assays were carried out in triplicates, except the control growth with *S. cerevisiae*. Ethanol production, sugar consumption and cell growth were monitored along the bioprocess.

### 2.6.3. Fermentative medium: strawberry tree fruit' juice

Five simultaneous fermentations of pure strawberry tree fruit' juice, as the only nutrients source, were conducted for the ethanol production, using 4 selected yeasts strains isolated from strawberry tree fruits (AP1, DP2, GerP3 and GluP4) and the strain *S. cerevisiae* CCMI 885. Each inoculum was prior grown in 50 ml of YMB medium in 150 ml Erlenmeyer flasks, at 25 ºC with shaking (150 rpm) for approximately 24 h. Then, the fermentation assays were carried out in 150 ml of pure strawberry tree fruit' juice in 500 ml shake flasks, inoculated respectively with 10% (v/v) of centrifuged inoculum, incubated at 25 ºC, without shaking, for 9 days. The strawberry tree fruit' juice was obtained by crushing the fruits into a

homogenous pulp and centrifuged at 4 000 × g for 15 min. The liquid phase was used as the fermentative medium without further treatment. All assays were carried out in duplicates. The ethanol production, sugar consumption and cell growth were monitored along the fermentation processes.

### 2.7. Analytical Methods

The cell growth was monitored by measurement the optical density (OD) of culture broth samples at 600 nm, using a spectrophotometer (Genesys 20, Thermo Electron Corporation, USA). The maximum growth rates ($\mu_{max}$) were calculated trought lineat regression of the 3 first ponts of exponencial phase, using Excel software (Microsoft® Office Excel®, 2007 for Windows).

Sugars and ethanol concentrations were determined by high-performance liquid chromatography (HPLC) using a Waters Sugar-Pak I column (Bio-Rad Laboratories, USA) operating at 75 ºC with Ca-EDTA at 50 mg/l as mobile phase at a flow rate of 0.5 ml/min.

In the batch fermentations for bioethanol production from inulin, the remaining amount of inulin in the fermentative broths at the end of the assays (unhydrolyzed inulin by inulinases) was quantified by performing an acid hydrolysis of the inulin present in each broth sample. These hydrolyses were carried out incubating the samples at 55 ºC and pH 2 for 96 h. The respective hydrolysates were then analyzed for sugar content by HPLC using an Aminex HPX-87H column (Bio-Rad Laboratories, USA) operating at 50 ºC with 5 mM $H_2SO_4$ as mobile phase at a flow rate of 0.4 ml/min.

## 3. Results and Discussion

### 3.1. Isolation of novel microorganisms

The isolation of novel microorganisms was performed from 7 different natural sources, namely, carob kibbles, cherries, figs, Jerusalem artichoke tubers, peaches, plums and strawberry tree fruits (STF), which were chosen due its high content in fermentable sugars (glucose, fructose and sucrose) and/or polysaccharides (inulin), a favorable habitat for ethanologenic yeasts and bacteria. The STF was also chosen by the special environment of its natural fermentation, which occurs in a very unique environment, taking much more time and with much less water content than the wine fermentation processes (Soufleros *et al*., 2005). A total of 98 new microorganisms were collected: 90 yeasts and 8 bacteria (Table 6). As the main goal was to isolate yeasts, it was used the RBC medium for the isolation procedure because it is a selective medium, i. e. the presence of chloramphenicol (a broad-spectrum antibiotic inhibitory to a wide range of Gram-negative and Gram-positive bacteria) and Rose Bengal (shown by Smith *et al*. (1944) to inhibit bacterial growth and restrict the growth of fast growing molds) in the medium decreased the possibility of collecting bacteria. RCB is also a differential medium due to the presence of the pigment Rose Bengal that can be differently assimilated by colonies, conferring them different colors. Despite the RBC influence, only bacteria (7) were isolated from Jerusalem artichoke tubers. Unlike the other materials used, the tubers are very close to the soil (a known source of bacterial species and a harsh environment for yeast species) which may explain the absence of yeasts collected and strong bacterial presence.

**Table 6.** Number of isolated yeast and bacterial strains, according to natural source.

| Isolation Source | Isolated strains | |
|---|---|---|
| | Yeast | Bacteria |
| Carob | 7 | 0 |
| Cherry | 11 | 0 |
| Fig | 13 | 0 |
| J. a. tubers* | 0 | 7 |
| Peach | 16 | 0 |
| Plum | 14 | 0 |
| STF** | 29 | 1 |
| **Total** | **98** | |

*Jerusalem artichoke tubers.

**Strawberry Tree fruit

### 3.2. Screening for alcoholic fermentation ability

To assess the potential ability of the novel 98 microbial isolates for alcoholic fermentation, simple physiological tests were conducted in test tubes using the invert Durham tube method. The potential for the ethanol production was evaluated for 9 different carbon sources: simple sugars (arabinose, fructose, glucose and xylose), disaccharides (cellobiose, lactose and sucrose), a polymer (inulin) and complex raw material (strawberry tree fruit' juice, STFJ) (see Table 15). In Table 7 are summarized the results

according to natural isolation origin. The carbon sources more fermented to ethanol were glucose (83% of the isolates), fructose (76% of isolates) and sucrose (21% of isolates), as expected. The xylose was actively converted to ethanol by 5 strains, all bacterial species (from Jerusalem artichoke tubers and STF), and the yeast strain named XilP2 (from STF) presented a positive result for lactose fermentation, but all of them achieved low ethanol concentration.

**Table 7.** Results of the screening for alcoholic fermentation potential by the several isolates, according to the isolation material and the carbon source tested.

| Source | Carob | | Cherry | | Fig | | J. a. tubers[1] | | Peach | | Plum | | STF[2] | | Total | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | G | F | G | F | G | F | G | F | G | F | G | F | G | F | G | F |
| **ARA** | 4 | 0 | 3 | 0 | 5 | 0 | 4 | 2 | 3 | 0 | 5 | 0 | 12 | 0 | 36 | 2 |
| **CEL** | 7 | 0 | 11 | 0 | 6 | 0 | 7 | 0 | 6 | 2 | 8 | 2 | 19 | 0 | 64 | 4 |
| **FRU** | 7 | 7 | 11 | 11 | 13 | 13 | 7 | 0 | 8 | 7 | 14 | 14 | 23 | 22 | 83 | 74 |
| **GLU** | 7 | 7 | 11 | 11 | 13 | 13 | 7 | 2 | 7 | 6 | 14 | 14 | 29 | 28 | 88 | 81 |
| **INU** | 7 | 3 | 4 | 0 | 5 | 0 | 6 | 0 | 0 | 0 | 2 | 0 | 16 | 0 | 40 | 3 |
| **LAC** | 4 | 0 | 0 | 0 | 4 | 0 | 5 | 0 | 5 | 0 | 4 | 0 | 14 | 1 | 36 | 1 |
| **SUC** | 7 | 7 | 5 | 1 | 6 | 0 | 5 | 4 | 2 | 0 | 6 | 2 | 22 | 7 | 53 | 21 |
| **XYL** | 4 | 0 | 0 | 0 | 4 | 0 | 7 | 4 | 1 | 0 | 3 | 0 | 10 | 1 | 29 | 5 |
| **STFJ[3]** | n. t.[4] | | | | | | | | | | | | 30 | 23 | 30 | 23 |

Note: positive results were considered by comparison with the negative control for growth and gas accumulation. Observation of growth (G) and gas accumulation inside the invert Durham tubes (F) was recorded after 7 days of incubation at 25 ºC without shaking. Carbon source tested: arabinose (ARA), cellobiose (CEL), fructose (FRU), glucose (GLU), inulin (INU), lactose (LAC), sucrose (SUC) and xylose (XYL). The assay with pure strawberry tree fruit' juice (STFJ) as complete medium is highlighted in grey.

[1]Jerusalem artichoke tubers

[2]Strawberry tree fruits

[3]Strawberry tree fruit' juice

[4]not tested

The results with more biotechnology interest concerns the inulin fermentation, where 3 yeasts were able of both strong growth and inulin fermentation. These yeast strains, named Talf1, Talf2 and Calf2, showed the ability for inulinase production, since the inulin hydrolysis is required for its consumption. Interestingly, the 3 strains were collected from the same natural source, the carob kibbles. The carob kibbles main component is sucrose, which may explain these results once inulinase activity is associated with invertase activity (Kangoo and Jain, 2011).

The strawberry tree fruit' juice (STFJ) was also tested as carbon source for alcoholic fermentation, with the 30 microorganisms (29 yeasts and 1 bacterium) isolated from STF. A similar test using invert Durham tubes permitted to distinguish 4 yeasts that were capable of a rapid and strong growth with great gas liberation, associated with ethanol production, despite all microorganisms showed growth and 23 (out of 30) showed gas liberation, after 7 days of incubation (see Table 7).

In overall, from this screening the most promising isolates, 7 yeast strains, were selected for further analysis. Four strains from STF, named AP1, DP2, GerP3 and GluP4, due to their ability of rapidly use the STFJ as only nutrient medium for alcoholic fermentation, were selected for further application in ethanol production from STFJ. Moreover, 3 strains from carob kibbles, named Talf1, Talf2 and Calf2, presenting inulinase activities, were also selected due to the great interest in develop processes using inulin rich materials, like Jerusalem artichoke, for bioethanol production.

### 3.3. Characterization of selected isolates

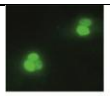
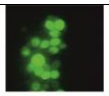
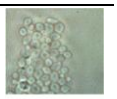
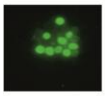
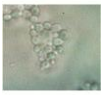
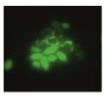
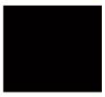
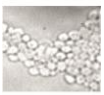
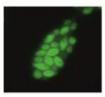
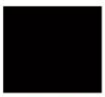
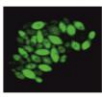
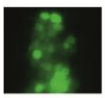
#### 3.3.1. Fluorescence *in situ* hybridization

The molecular method FISH, widely developed as laboratory routine, was employed for the putative molecular identification of the 7 selected yeast strains, namely: AP1, DP2, GerP3, GluP4, Talf1, Talf2 and Calf2. Using FITC marked oligonucleotide probes for 7 known yeast species, usually encountered in winery, all strains gave a positive result for only one probe (see Table 8). Yeast strains, well identified and routinely used were applied as positive control strains for the probes in the FISH assays. To assure the presence of rRNA molecules in the fixed cells, a positive control using a group probe for all eukaryotic cells (EUK516) was carried out, reassuring the presence and availability of rRNA molecules inside the fixed cells.

All the selected strains from carob kibbles (Talf1, Talf2 and Calf2) gave a positive result for the *Zygosaccharomyces bailii* probe, however this should be yet confirmed using more accurate molecular approaches, such as gene sequencing. If confirmed, it will be the first *Z. bailii* strain with ability of inulinase production, as far as I know. The existence of a *Z. bailii* strain with inulinase capacity opens a new focus of research, since this species, a known food-contaminant and associated with food spoilage, has interesting characteristics, such as: resistance to weak-acid preservatives, extreme osmotolerance, ability to adapt to high glucose concentrations and high temperatures, ability to vigorously ferment glucose, and growth at low pH (Martorell *et al*., 2007). In addition to the ability of inulinase production, these are important features for a strain to be used in bioprocesses for ethanol production.

The 4 strains collected from the strawberry tree fruits gave positive for 3 different yeast species: *Lachancea thermotolerans* (AP1), *Saccharomyces cerevisiae* (DP2 and GluP4) and *Zygosaccharomyces bailii* (GerP3). The *S. cerevisiae* and *Z. bailii* species have been isolated from fruits or food spoilage and are known for ethanol tolerance and vigorous sugar fermentation capacity (Kurtzman *et al*., 2011). In previous microbiological studies with strawberry tree fruit natural fermentation, yeasts from *L. thermotolerans* and *S. cerevisiae* species were also isolated (Santos *et al*., 2012). As described above, these putative identifications using the FISH technique should be confirmed using more accurate molecular methods. In addition, further morphological and physiological studies with all the selected isolates should be also carried out, as complementary characterization tests.

**Table 8.** Fluorescence *in situ* hybridization with selected strains.

| Strains | Probes | | | | | | | | Bright Field |
|---|---|---|---|---|---|---|---|---|---|
| | EUK516 | Huv | Kma | Lth | Mpu | Sce | Tde | Zba | |
| AP1 [*L. thermotolerans*] |  |  |  |  |  |  |  |  |  |
| Calf2 [*Z. bailii*] |  |  |  |  |  |  |  |  |  |
| DP2 [*S. cerevisiae*] |  |  |  |  |  |  |  |  |  |
| GerP3 [*Z. bailii*] |  |  |  |  |  |  |  |  |  |
| GluP4 [*S. cerevisiae*] |  |  |  |  |  |  |  |  |  |
| CBS 314 [*Hanseniaspora uvarum*] |  |  |  |  |  |  |  |  |  |
| *Kluyveromyces marximianus* 516F |  |  |  |  |  |  |  |  |  |
| *CBS 2803* [*Lachancea thermotolerans*] |  |  |  |  |  |  |  |  |  |
| NRRL Y-987 [*Metschnikowia pulcherrima*] |  |  |  |  |  |  |  |  |  |
| *Saccharomyces cerevisiae* CCMI 885 |  |  |  |  |  |  |  |  |  |
| Talf1 [*Z. bailii]* |  |  |  |  |  |  |  |  |  |
| Talf2 [*Z. bailii*] |  |  |  |  |  |  |  |  |  |
| PYCC 4478 [*Torulaspora delbrueckii*] |  |  |  |  |  |  |  |  |  |
| *Zygosaccharomyces bailii* 518F |  |  |  |  |  |  |  |  |  |

Fluorescence *in situ* hybridization with specie-specific probes for following yeasts: Huv - *Hanseniaspora uvarum*, Kma - *Kluyveromyces marxianus*, Lth - *Lachancea thermotolerans*, Mpu - *Metschnikowia pulcherrima*, Sce - *Saccharomyces cerevisiae*, Tde - *Torulaspora delbrueckii* and Zba - *Zygosaccharomyces bailii*. In the first column are the positive controls for the fixed cells, with the group probe EUK516 for eukaryotic cells. In the last column are the bright field photographs corresponding to the positive FISH assay for each strain. The yeast strains CBS 314, *K. marximianus* 516F, CBS 2803, NRRL Y-987, *S. cerevisiae* CCMI 885, PYCC 4478 and *Z. bailii* 518F were used as positive controls in each assay. Between brackets is the yeast specie corresponding to the positive result with FISH probe.

### 3.3.2. Metabolic Profile

A biochemical characterization was also carried out to the carob pulp kibbles isolated strains, Talf1, Talf2 and Calf2, using 2 different Analytical Profile Index (API) galleries: the API ZYM and the API 20C AUX (see Table 9). As for FISH assays, the strain *Z. bailii* 518F (Zba), previously identified by Xufre (2005), was also tested to be used as control for comparison with the results for these strains.

**Table 9.** API ZYM and API 20C AUX biochemical test results.

| API ZYM | | | | | API 20C AUX | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Enzymatic activity | Talf1 | Talf2 | Calf2 | Zba | Substrate | Talf1 | Talf2 | Calf2 | Zba |
| Control (-) | - | - | - | - | Control (-) | - | - | - | - |
| Alkaline phosphatase | + | + | + | + | Glucose | + | + | + | + |
| Esterase (C4) | - | - | - | + | Glycerol | + | + | + | + |
| Esterase lipase (C8) | + | + | + | + | 2-keto-D-gluconate | + | + | + | + |
| Lipase (C14) | - | + | + | + | Arabinose | - | - | - | - |
| Leucine arylamidase | + | + | + | + | Xylose | + | + | - | + |
| Valine arylamidase | - | - | + | + | Adonitol | - | - | - | - |
| Cystine arylamidase | - | - | - | + | Xylitol | + | + | - | - |
| Trypsin | - | - | - | - | Galactose | - | - | - | + |
| α-Chymotrypsin | - | - | - | - | Inositol | - | + | + | - |
| Acid phosphatase | + | + | + | + | Sorbitol | + | + | + | + |
| Phospho amidase | + | + | + | + | Methyl-D-glucoside | - | - | + | + |
| α-Galactosidase | - | - | - | - | *N*-acetyl-glucosamine | - | - | - | - |
| β-Galactosidase | - | - | - | - | Cellobiose | - | - | - | + |
| β-Glucuronidase | - | - | - | - | Lactose | - | - | - | - |
| α-Glucosidase | - | - | - | + | Maltose | - | - | - | + |
| β-Glucosidase | - | - | - | - | Sucrose | + | + | + | + |
| *N*-acetyl-β-glucosaminidase | - | - | - | - | Trehalose | + | + | + | + |
| α-Mannosidase | - | - | - | - | Melezitose | - | - | - | - |
| α-Fucosidase | - | - | - | - | Raffinose | + | + | + | + |

Positive result (+); negative result (-); negative control (Control (-))

Desiccated substrates for each enzymatic test: 2-naphthyl phosphate (alkaline phosphatase); 2-naphthyl butyrate [esterase (C4)]; 2-naphthyl caprylate [esterase lipase (C8)]; 2-naphthyl myristate [lipase (C14)]; L-leucyl-2-naphthylamide (leucine arylamidase); L-valyl-2-naphthylamide (valine arylamidase); L-cystyl-2-naphthylamide (cystine arylamidase); N-benzoyl-DL-arginine-2-naphthylamide (trypsin); *N*-glutaryl-phenyl-alanine-2-naphthylamide (α-chymotrypsin); 2-naphthyl phosphate (acid phosphatase); naphthol AS-BI phosphodiamide (phosphoamidase); 6-Br-2-naphthyl-α-D-galactopyranoside (α-galactosidase); 2-naphthyl-β-D-galactopyranoside (β-galactosidase); naphthol AS-B1 β-D-glucuronate (β-glucuronidase); 2-naphthyl-α-D-glucopyranoside (α-glucosidase); 6-Br-2-naphthyl-β-D-glucopyranoside (β-glucosidase); 1-naphthyl-N-acetyl-β-D glucosaminide (*N*-acetyl-β-glucosaminidase); 6-Br-2-naphthyl-α-D-mannopyranoside (α-mannosidase) and 2-naphthyl-α-L-fucopyranoside (α-fucosidase).

The results of API tests showed that the metabolic profile of the 3 isolated strains is more similar between each other than with the control *Z. bailii* strain (see Table 9). API gallery tests revealed that all strains presented alkaline phosphatase, esterase lipase (C8), leucine arylamidase, acid phosphatase and

phospho amidase and were all able to metabolize glucose, glycerol, 2-keto-D-gluconate, sorbitol, saccharose, trehalose and raffinose. No activity for trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase was detected in all tested strains. Xylose was consumed by all strains with the exception of Calf2. The Zba control strain presented the only positive result for esterase (C4), lipase (C14), cystine arylamidase, α-glucosidase and was the only strain that metabolized galactose, cellobiose and maltose. Both Calf2 and Zba strains gave positive results for valine arylamidase and metabolized methyl-D-glucoside, while the strains Talf1 and Talf2 gave positive for xylitol. The Talf2 and Calf2 both metabolized inositol.

These results are consisting with the putative molecular identification by FISH, because all strains gave consisting results for glucose, glycerol, arabinose, galactose, lactose, saccharose, melezitose and treahalose assimilation. The treahalose assimilation is an important characteristic since it is used to distinguish *Z. kombuchaensis* and *Z. lentus* from *Z. bailii*, according to Kurtzman *et al*. (2011).

Interestingly, it was for the *Z. bailii* 518F control strain where the results were less conclusive for the strain profile. Unexpected positive results for galactose, cellobiose and maltose growth created the divergences with the strain type profile in Kurtzman *et al*. (2011). The growth in xylose (Talf1 and Talf2), inositol (Talf2 and Calf2), methyl-D-glucoside (Calf2 and Z. bailii) and raffinose (for all strains tested) was also contrary to the *Z. bailii* type strain accepted profile (Kurtzman *et al*., 2011). However, replicate tests should have been used to confirm the metabolic profiles obtained by the API galleries, as well as the parallel characterization of the *Z. bailii* wild type strain for comparison. The API 20C AUX micro-method is used for identification purposes (Fenn *et al*., 1994); however fail to differentiate at strain level. Molecular methods are used to differentiate at sub-species level, such as restriction fragment length polymorphism (RFLP), chromosome karyotyping, randomly amplified polymorphic DNA (RAPD), and amplified fragment length polymorphism (AFLP) (Techera *et al*., 2001).

### 3.4. Inulinase production

#### 3.4.1. Substrates Induction

The three selected yeasts, Talf1, Talf2 and Calf2, with potential to inulinase production were further tested to produce enzymes and then evaluate their inulinase activities. So, for the production of inulinases, each of these strains were grown in YMB medium supplemented with 10% (w/v) of inulin or 25% (v/v) Jerusalem artichoke juice (JAJ), as inducer substrates. Table 10 describes the results obtained for inulinase activity (U/ml) profiles during the growth of these yeast strains in the two inducers tested. These results show that pure inulin was a weak inducer for inulinase production by all strains, reaching to a maximum inulinase activity of about 0.60 U/ml, contrary to JAJ that acted as a strong inducer, especially for Talf1 and Talf2, which attained high inulinase activities since the 5th day of growth (see Table 10). Both Talf1 and Talf2 strains are good enzyme producers, however the maximum value of inulinase activity was achieved with the strain Talf1 (8.67 U/ml).

An important factor for inulinases production is the carbon source that is used as inducer, since it influences the production level of extracellular inulinases (Vijayaraghavan *et al*., 2009). Commercially available inulin has been reported as a good inducer for inulinase producing microorganisms

(Vijayaraghavan *et al*., 2009), but inulin rich raw materials can sometime outstand the pure polymer induction capacity (Mazutti *et al*., 2006). The JAJ was neither treated nor purified and could have brought a mix of ions and other compounds, which may act as a nutrient supplement allowing, in this way, the achievement of higher levels of extracellular inulinase activity by these strains. Further analysis can be carried on JAJ to search for the presence of important metallic ions, which may contribute to improve the inulinase activity, such as $Fe^{3+}$ and $Mg^{2+}$ (Azhari *et al*., 1989). In addition, studies on the influence of ions in inulinase activity can also be performed.

**Table 10.** Inulinase activity (U/ml) profile during the growth of the yeast strains Talf1, Talf2 and Calf2 in YMB supplemented with 10 % (w/v) inulin or 25 % (v/v) Jerusalem artichoke juice, as inducer substrates. Data are given as activity means ± standard deviation (n=2).

| Time (days) | Inulinase Activity (U/ml) | | | | | |
|---|---|---|---|---|---|---|
| | Inulin induction | | | Jerusalem artichoke juice induction | | |
| | Talf1 | Talf2 | Calf2 | Talf1 | Talf2 | Calf2 |
| 5 | 0.60 ± 0.18 | 0.46 ± 0.23 | 0.16 ± 0.22 | 8.04 ± 0.90 | 6.39 ± 0.79 | 0.16 ± 0.07 |
| 7 | 0.60 ± 0.30 | 0.54 ± 0.16 | 0.57 ± 0.06 | 8.49 ± 0.55 | 7.30 ± 0.02 | 2.94 ± 0.47 |
| 8 | 0.48 ± 0.12 | 0.61 ± 0.11 | 0.61 ± 0.63 | 8.67 ± 0.08 | 7.81 ± 0.08 | 4.08 ± 0.20 |

In overall, the highest enzymatic activity was obtained in the crude extract after 8 days of incubation at 25ºC with agitation on 25% (v/v) JAJ, being Talf1 the best producer of inulinases. Hence, strain Talf1 was selected for the inulinase production for further characterization and application to bioethanol production.

Moreover, for Talf1, the best yeast strain producer of inulinases, a new set of assays were carried out testing a wider range of inducer substrates, from different pure inulin sources to complex raw materials, viewing the improvement of the inulinase production. Inulin purified from different natural sources has different characteristics, such as different DP levels in the polymer, which can influence the induction ability for the production of inulinases by the yeast strain selected. Thus, in addition to inulin from Fagron, other two inulins were also tested, namely: an inulin from Sigma (extracted from dahlia tubers) and an inulin from BDH chemicals (from unknown natural source). Furthermore, besides JAJ, three new complex raw materials were also tested, namely: dahlia tubers, chard roots and JA solid residue. The results obtained in these assays are described in Figure 5. All the purified inulins tested were weak inducers regarding the enzymatic induction. The highest value achieved in pure inulin was 0.6 U/ml with inulin from Sigma. As in the prior assays, the best inducers are the complex natural raw materials. All of them induced better enzyme activities than the purified forms of inulin. Regarding the inulin-rich materials tested as inducer substrates, these assays confirmed that JA is the best inducer substrate, inducing the highest inulinase activities even when used in the form of solid residue. The inulinase activities by Talf1 observed with JA ranged from 5.9 U/ml (JA solid residue) to 8.7 U/ml (JAJ). So, JAJ was selected as the best inducer substrate for the production of Talf1 inulinases.

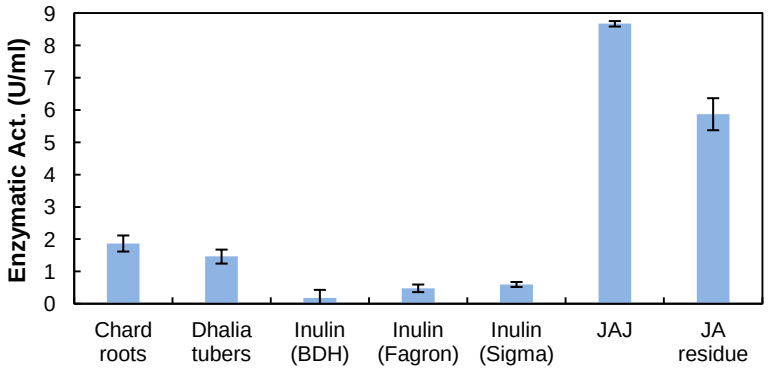



**Figure 5.** Induction of inulinase production by Talf1 strain using different substrates. Data are presented as inulinase activity means ± standard deviation (n = 2).

### 3.4.2. Inulinase characterization

The characterization of Talf1 enzymatic extract (obtained from Talf1 growth in YMB medium supplemented with 25% (v/v) JAJ for 8 days) was performed to verify the optimum temperature and pH of its activity for inulin, as substrate, as well as to evaluate its thermal and pH stability. Figure 6 shows the results of Talf1 inulinase characterization. Figure 6A shows the influence of temperature (25 ºC - 65 ºC) on Talf1 inulinase activity. The maximal inulinase activity observed was obtained at 45 ºC. At 65 ºC almost all inulinase activity was inactivated (1.7%), which was caused by enzymatic extract denaturation. In Figure 6B can be observed the pH profile of Talf1 inulinase, where the optimum pH was 5.5. Thus, for further assays to determine the inulinase activity on Talf1 crude extracts, the best buffer for the reactions was considered the sodium citrate 50 mM buffer at pH 5.5.

In Figure 6C is shown the effect of temperature on enzyme denaturation, which causes enzyme inactivation. The thermal stability of Talf1 enzymatic extract was performed at 30, 45 and 50 ºC (Figure 6C). When the enzymatic extract was kept at 50 and 45 ºC, a complete inactivation occurred after 3 h and 6 h, respectively. In contrast, when kept at 30 ºC, the enzymatic extract maintains 91% of the original activity after 3 days. The effect of pH (5.5, 7.5 and 10) on the enzyme activity stability was also studied at a temperature of 30 ºC (Figure 6D). The enzymatic extract showed reasonable stability at the original pH (5.5) retaining 57% of its original activity after 24 days when incubated at 30 ºC. However, the pH increase of the Talf1 crude extract to 7.5 and 10 influenced drastically the enzyme activity stability, remaining, after 3 days, only 10 and 2% of its original activity, respectively.

Enzymes are influenced by many factors, such as temperature, pH and metal ions or co-factors affinity (Chi *et al*., 2009). From all potentially deactivating factors, temperature is the best studied. At elevated temperature many enzymes become (partly) unfolded and/or inactivated, meaning that they are no longer able to perform the desired tasks (Eijsink *et al*., 2005). The medium pH may also cause denaturation of the enzymatic structure, affecting the enzyme interaction with substrate and altering the activity levels. Therefore, is very important to determine the optimal conditions for enzymatic activity determination assay, by obtaining the activity profiles for temperature and pH.

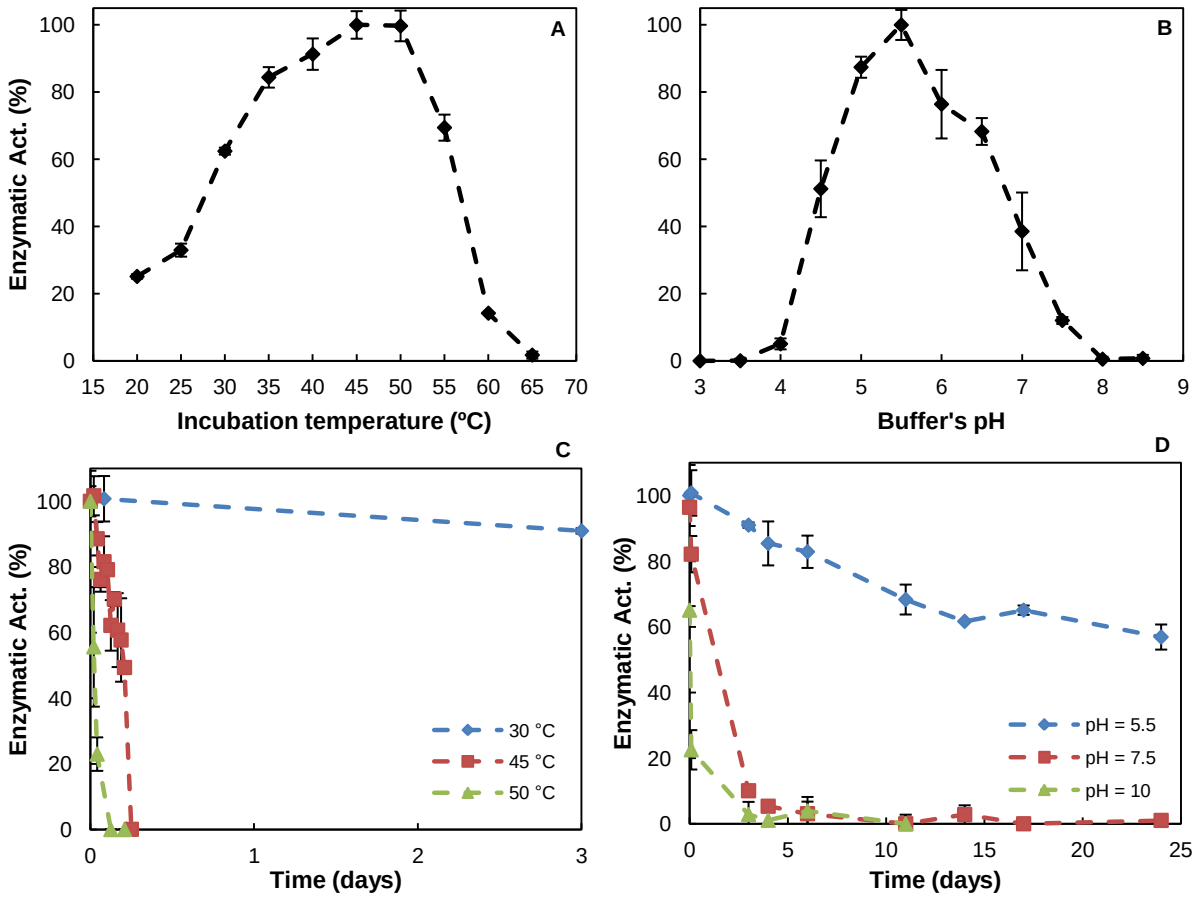



**Figure 6.** Characterization of Talf1 enzymatic extract. (**A**) Effect of temperature on Talf1 inulinase activity (reactions were carried out at pH 5.5). (**B**) Effect of pH on Talf1 inulinase activity (reactions were carried out at 45 ºC). (**C**) Thermal stability of enzyme assayed at 30, 45 and 50 ºC (extract at natural pH of 5.5). (**D**) Effect of pH (5.5, 7.5 and 10) on enzyme activity stability, kept at 30ºC.

A review by Kango and Jain (2011) describes several inulinases from molds, yeasts and bacteria with very similar features. In the molds' group, the inulinases optimum pH and temperature ranges from 4 to 6.5 and 25 - 40 ºC, respectively. The bacterial inulinases showed a higher pH and temperature range, from 6.5 to 7.5 and 37 - 50 ºC. The yeasts are the most studied group, and their inulinases optimum pH and temperature ranges from 3.5 to 8 and 28 - 36 ºC, respectively.

The results of enzymatic activity are difficult to compare due to differences in the units used. Some authors described inulinases in Units per grams of dry substrate (U $gds^{-1}$) and others in specific activity (U.$mg^{-1}$). A high disparity is notice along different works: some reports suggest inulinase activities as high as 1 139 U/ml for a *K. marxianus* strain (Sguarezi *et al*., 2009), while others referred levels of 0.463 U/ml for a different strain of the same specie (Selvakumar and Pandey, 1999).

Enzymatic stability, both to temperature and pH, is the most necessary feature for application of enzymes in bioprocesses (Mateo *et al*., 2007; Iyer and Ananthanarayan, 2008). In this context, the characteristics of Talf1 inulinases: optimum pH and temperature of 5.5 and 45 ºC, respectively, and the high stability at 30 ºC (maintaining 57% activity after 24 days at 30 ºC) are favorable for its further application in a SSF process of bioethanol production from inulin or inulin-rich materials.

## 3.5. Bioprocesses for bioethanol production

### 3.5.1. Application of inulin or inulin-rich materials in bioprocesses

Inulin is present in several plants as a reserve polymer and can be used as a carbon source for microbial growth if hydrolyzed into fermentable sugars. By the action of microbial inulinases a fructose rich syrup can be obtained from inulin or inulin-rich materials and further used for bioethanol production.

#### 3.5.1.1. Inulin fermentation in CBP and SSF

Bioethanol production assays from inulin were performed by two bioprocesses: in a CBP and in a SSF approach. The results of these batch fermentations containing 200 g/l inulin, in the Inulin Fermentative Medium (IFM, see Appendix), are presented in Figure 7, while in Table 11 are summarized the most important metabolic parameters.

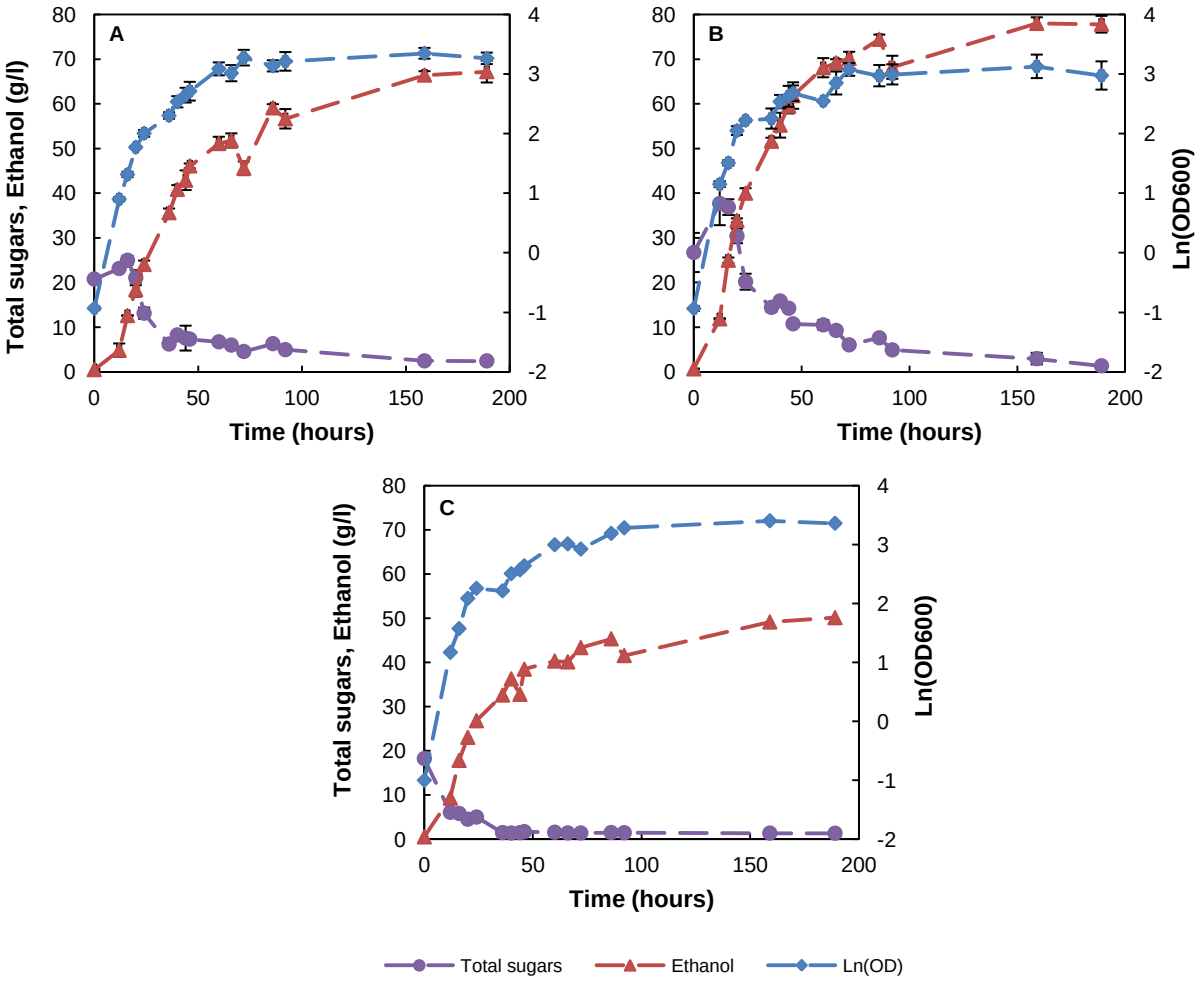



**Figure 7.** Inulin application in CBP and SSF process. Yeast growth, sugar consumption and ethanol production profiles during batch fermentation of 200 g/l inulin (Inulin Fermentative Medium), as carbon source, by: yeast strain Talf1 in a CBP (**A**), *S. cerevisiae* CCMI 885 in a SSF process with the addition of 5 % (v/v) Talf1 enzymatic extract (**B**), and *S. cerevisiae* CCMI 885 in IFM without inulinases addition (**C**).

**Table 11.** Metabolic parameters from inulin fermentation assay.

| Fermentation substrate | Strain | Enzyme addition | $\mu_{max}$ ($h^{-1}$) | Ethanol Productivity ($g.l^{-1}.h^{-1}$) | Maximum Ethanol (g/l) | Ethanol Yield (%) |
|---|---|---|---|---|---|---|
| IFM[1] | Talf1 | - | 0.14 | 1.70 | 67.2 | 45 |
| | *S. cerevisiae* CCMI 885 | - | 0.17 | 1.72 | 50.1 | |
| | | Inulinases[2] | 0.15 | 2.75 | 78.0 | 47 |

[1]Inulin Fermentative Medium (described in Appendix)

[2]Talf1 crude enzymatic extract growth

The CBP was carried out with the selected yeast strain Talf1, producer of inulinases, which had given positive results for inulin fermentation ability in the alcoholic fermentation screen. In Figure 7A is shown the growth, sugar consumption and ethanol production profiles during the batch fermentation of 200 g/l inulin, as sole carbon source. In this assay, strain Talf1 presented a growth rate ($\mu_{max}$) of 0.14 $h^{-1}$ and a maximum ethanol productivity of 1.70 $g.l^{-1}.h^{-1}$, achieving 67 g/l of ethanol directly from the inulin since it produced all the necessary enzymes (Table 11). An acid hydrolysis carried on the end samples of the process revealed a remaining 50 g/l of inulin (as glucose and fructose) in the CBP fermentative medium, which could be explained by the presence of long-chain inulin harder to enzymatic hydrolysis or due to low enzymatic efficiency. Therefore the ethanol yield obtained was 45%, quite close to the 51% of the theoretical ethanol yield (Dien *et al*., 2003).

The SSF bioprocess for bioethanol production from 200 g/l of inulin (Figure 7B) was carried out using the yeast *Saccharomyces cerevisiae* CCMI 885 and supplementing the IFM with 5% (v/v) Talf1 crude enzymatic extract (with 8.7 U/ml of inulinase activity). In the presence of inulinases, the *S. cerevisiae* strain presented a similar growth rate to the isolated strain Talf1 ($\mu_{max}$ = 0.15 $h^{-1}$), however achieved a higher ethanol production, 78 g/l (see Table 11), remaining yet 34 g/l of non hydrolyzed inulin in the fermentative medium. In these conditions, the maximum ethanol productivity was 2.75 $g.l^{-1}.h^{-1}$ and the ethanol yield was 47%. The ethanol production by *S. cerevisiae* corresponds to 92% of theoretical ethanol yield, which is 4% higher in comparison with that obtained by the yeast strain Talf1 in CBP. The yeast *S. cerevisiae*, contrary to strain Talf1, did not have to produce the enzymatic complex necessary to inulin hydrolysis because the inulinases were added to the fermentative medium, and thus achieved higher ethanol productivity. The quantity of enzyme added to the SSF process (5% v/v) was sufficient to prevent sugar limitation, because at the first 24 h of fermentation there was an accumulation of sugars in the broth, ready to be used (see Figure 7B).

In order to access the influence of the Talf1 inulinases addition in the inulin fermentation ability by *S. cerevisiae* CCMI 885, a control growth was conducted without inulinases addition. In this assay, the *S. cerevisiae* strain was only able to produce 50 g/l of ethanol from the 200 g/l of inulin (Figure 7C). These results suggest that this strain was able to partially consume inulin, but not all, which has been previously reported to other *Saccharomyces* strains (Lim *et al*., 2011; Schorr-Galindo *et al*., 2000). This ability as been connected to the consumption of small size inulin molecules, with DP under 6, probably by action of an invertase with affinity for small D-fructo-furanosides. So, in SSF process, the Talf1 inulinases

hydrolyzed fibers which were not naturally used by the *S. cerevisiae* strain, leading to a bioethanol production increase, from 50 to 78 g/l (see Table 11).

3.5.1.2. Jerusalem artichoke juice fermentation in CBP and SSF

Jerusalem artichoke juice (JAJ), an inulin-rich complex material containing about 130 g/l of sugars (quantified by acid hydrolyses), was used as the only nutrients medium for bioethanol production in a CBP and a SSF process. The results of batch fermentations of JAJ are presented in Figure 8, and Table 12 summarizes the most important metabolic parameters.

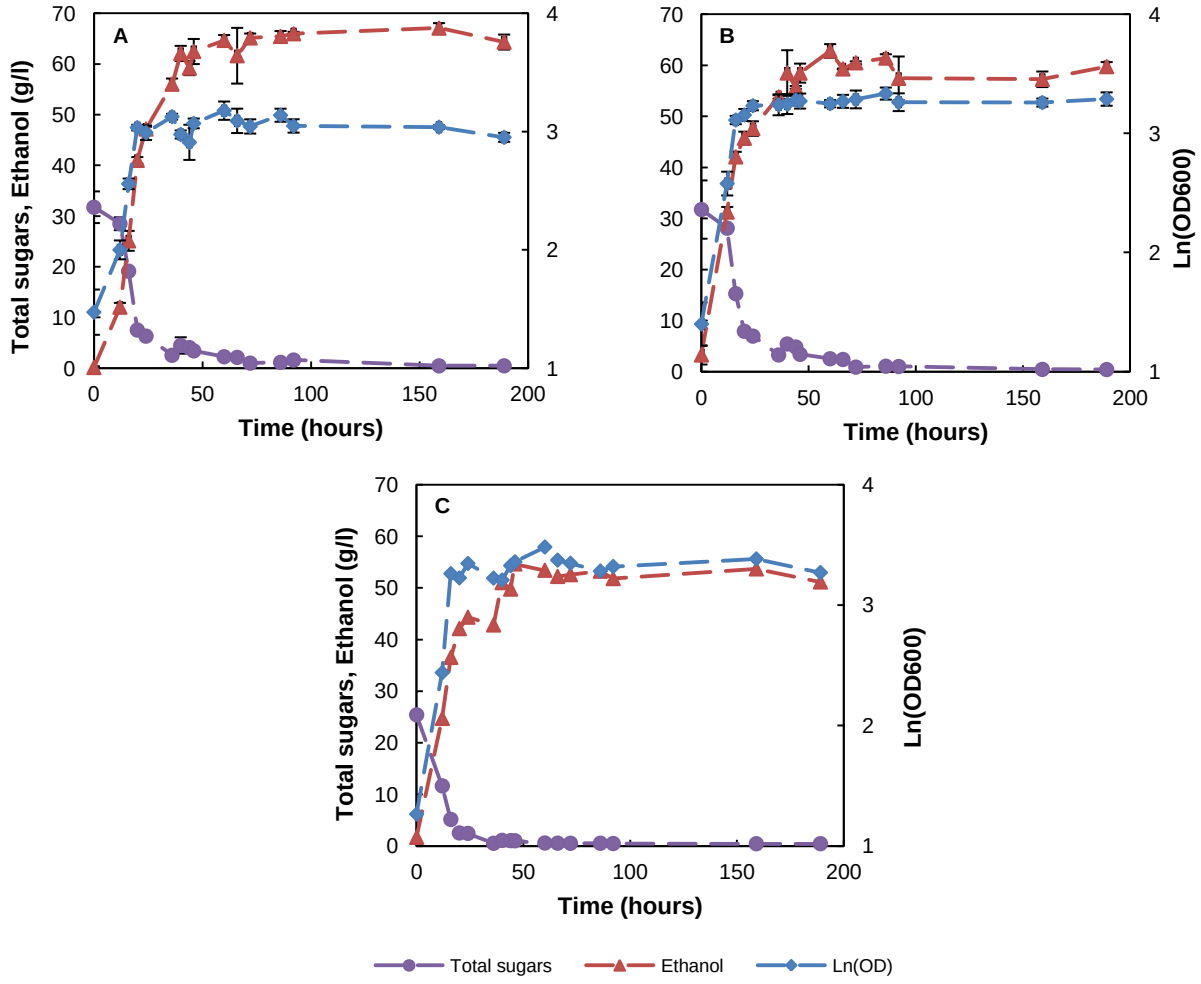



**Figure 8.** Jerusalem artichoke juice application in CBP and SSF process. Time-course profiles displaying the growth, sugar consumption and ethanol production during batch fermentation of Jerusalem artichoke juice (130 g/l inulin), as sole nutrient source, by: strain Talf1 in a CBP (**A**), *S. cerevisiae* CCMI 885 in a SSF process with the addition of 5 % (v/v) Talf1 enzymatic extract (**B**), and *S. cerevisiae* CCMI 885 in Jerusalem artichoke juice without inulinases addition (**C**).

For the CBP, the fermentation assays were conducted with the isolated yeast Talf1, and the results are shown in Figure 8A. The maximum growth rate achieved was 0.13 $h^{-1}$ and the highest productivity measured was 3.62 $g.l^{-1}.h^{-1}$ (see Table 12). The maximum value of ethanol achieved by Talf1 strain was about 67 g/l of ethanol, and the acid hydrolysis of the end samples revealed no remaining sugars. Therefore, the calculated ethanol yield was 51% considering only the measurable sugars quantified, which corresponds to the theoretical ethanol yield.

**Table 12.** Metabolic parameters from Jerusalem artichoke juice fermentation assay.

| Fermentation substrate | Strain | Enzyme addition | $\mu_{max}$ ($h^{-1}$) | Ethanol Productivity ($g.l^{-1}.h^{-1}$) | Maximum Ethanol (g/l) | Ethanol Yield (%) |
|---|---|---|---|---|---|---|
| JAJ[1] | Talf1 | - | 0.13 | 3.62 | 67.1 | 51 |
| | *S. cerevisiae* CCMI 885 | - | 0.12 | 2.07 | 54.6 | 42 |
| | | Inulinases[2] | 0.11 | 2.40 | 62.8 | 48 |

[1]Jerusalem artichoke juice has complete medium.

[2] Talf1 crude enzymatic extract growth

In SSF process, the fermentations of JAJ were conducted with *S. cerevisiae* CCMI 885 in the presence of 5% (v/v) Talf1 crude enzymatic extract (with 8.7 U/ml of inulinase activity), and the results obtained are presented in Figure 8B. In these conditions, the yeast *S. cerevisiae* strain presented a weaker growth rate (0.11 $h^{-1}$) and a lower productivity (2.40 $g.l.^{-1}h^{-1}$) in comparison to Talf1 in the CBP (see Table 12). The maximum ethanol concentration attained was about 63 g/l, with no residual sugar. In overall, the *S. cerevisiae* strain was less efficient than the strain Talf1 for the bioethanol production from pure JAJ, achieving a maximum ethanol yield of 48%.

In addition, to verify the ability of *S. cerevisiae* CCMI 885 to directly ferment the JAJ, a control fermentation assay with this *S. cerevisiae* strain in pure JAJ was conducted (see Figure 8C). The *S. cerevisiae* strain was able to produce 55 g/l of ethanol directly from this natural raw material. The maximum growth rate and ethanol productivity attained were 0.12 $h^{-1}$ and 2.07 $g.l^{-1}.h^{-1}$, respectively (see Table 12). These results are consistent with those obtained in pure inulin fermentation, indicating once again the inability of *S. cerevisiae* CCMI 885 to utilize most carbon fibers present in JAJ as inulin, since the ethanol yield reached was 42%, substantially lower when compared to that obtained in the SSF process (see Table 12).

The Talf1 enzymes supplementation to a SSF process with a known good ethanologenic strain (*S. cerevisiae*), even though it improved the bioethanol production yield from JAJ, it did not attained the same ethanol yield of the CBP approach with Talf1 (see Table 12).

From the comparison between the two SSF processes conducted with Talf1 inulinases and the *S. cerevisiae* strain for bioethanol production from inulin (Figure 7B) and JAJ (Figure 8B), it can be observed that the bioprocess with pure inulin was more successful in terms of maximum ethanol produced (78.0 g/l versus 62.8 g/l for JAJ), however in terms of ethanol yield the value obtained was slightly lower (47% versus 48% for JAJ), due to the remaining non hydrolyzed inulin (34 g/l) in the fermentative broth at the end of the assay. The ethanol yields attained in both these SSF processes are higher than those previous described by other authors (Ohta *et al*., 1992).

The CBP bioprocess with Talf1 strain to obtain bioethanol was improved for ethanol yield when applied directly to the Jerusalem artichoke (JA) which attained the theoretical ethanol yield (51%). The best results obtained for JA fermentation were described using a *S. cerevisiae* strain in a SHF (Zhang *et al*., 2010) and a SSF (Nakamura *et al*., 1996), however, in this work, the CBP with the novel Talf1 yeast strain achieved higher ethanol yields. Other researchers also studied a CBP method using inulinase producing yeast like *K. marxianus* (Yuan *et al*., 2008b; Bajpai and Margaritis, 1986), but achieving lower ethanol concentrations or yields.

The direct fermentation of JA with a *S. cerevisiae* strain was assayed by Lim and colleagues (2011), but only 36 g/l of ethanol were produced. The *S. cerevisiae* CCMI 885 strain, used in this work, was able to produce 55 g/l of ethanol, which may be explained by raw material differences in nutrients content and/or indicates the ability of this strain to efficiently use some inulin molecules.

### 3.5.2. Strawberry tree fruit' juice as Fermentation Medium

Batch fermentation assays, using strawberry tree fruit' juice (STFJ) as sole nutrients source, were conducted using four novel yeast strains isolated from strawberry tree fruit (STF), namely AP1, DP2, GerP3 and GluP4, and a known highly ethanologenic yeast strain, *S. cerevisiae* CCMI 885. The results of the batch fermentations of STFJ are presented in Figure 9, and in Table 13 are summarized the most important metabolic parameters.

These results indicate that the best ethanol producing yeast was the *S. cerevisiae* strain CCMI 885 (see Figure 9E), which achieved the highest ethanol concentration (about 108 g/l), productivity (1.29 $g.l^{-1}.h^{-1}$) and yield (51%, the theoretical yield for ethanol production) (see Table 13). The isolate GerP3, a putative *Z. bailii* strain, achieved the second best series of results. It was able to produce 100 g/l of bioethanol (see Figure 9C), with a productivity of 1.11 $g.l.^{-1}.h^{-1}$ and an ethanol yield of 50%, due to the remaining 7 g/l of free sugars in the fermentation broth at the end of the assay. The STFJ fermentations with the yeasts DP2 and GluP4 (Figure 9B and 9D), two putative *S. cerevisiae* strains, resulted in lower ethanol yield, caused by lower ethanol concentration produced, about 95 and 92 g/l, respectively (see Table 13), with a remaining sugar content of 13 g/l on the fermentation broth for both strains**.**

**Table 13.** Metabolic parameters from strawberry tree fruit' juice fermentation assay.

| Strain | $\mu_{max}$ ($h^{-1}$) | Ethanol Productivity ($g.l^{-1}.h^{-1}$) | Maximum Ethanol (g/l) | Ethanol Yield (%) |
|---|---|---|---|---|
| *S. cerevisiae* CCMI 885 | 0.09 | 1.29 | 107.5 | 51 |
| AP1 | 0.11 | 1.01 | 86.0 | 47 |
| DP2 | 0.10 | 0.95 | 95.4 | 49 |
| GerP3 | 0.10 | 1.11 | 100.2 | 50 |
| GluP4 | 0.11 | 1.15 | 92.4 | 47 |

With the DP2 strain was obtained the lowest productivity (0.95 $g.l.^{-1}.h^{-1}$), while with the GluP4 isolate was obtained the highest productivity (1.15 $g.l.^{-1}.h^{-1}$) between the new isolates tested. The less effective fermentation was the one using AP1, a putative *L. thermotolerans* strain. The maximum ethanol concentration produced by this strain was 86 g/l with a remaining sugar content of 25 g/l (see Figure 9A), resulting in an ethanol yield of 47%.

The STFJ was characterized by HPLC for its sugar content, showing a high soluble sugar concentration, 209 g/l, most of which fructose (157 g/l) and the rest glucose (52 g/l). STFJ is a complex raw material, presenting most of the necessary nutrients for microbial growth, contributing for a total sugar conversion into ethanol within a fermentation process.

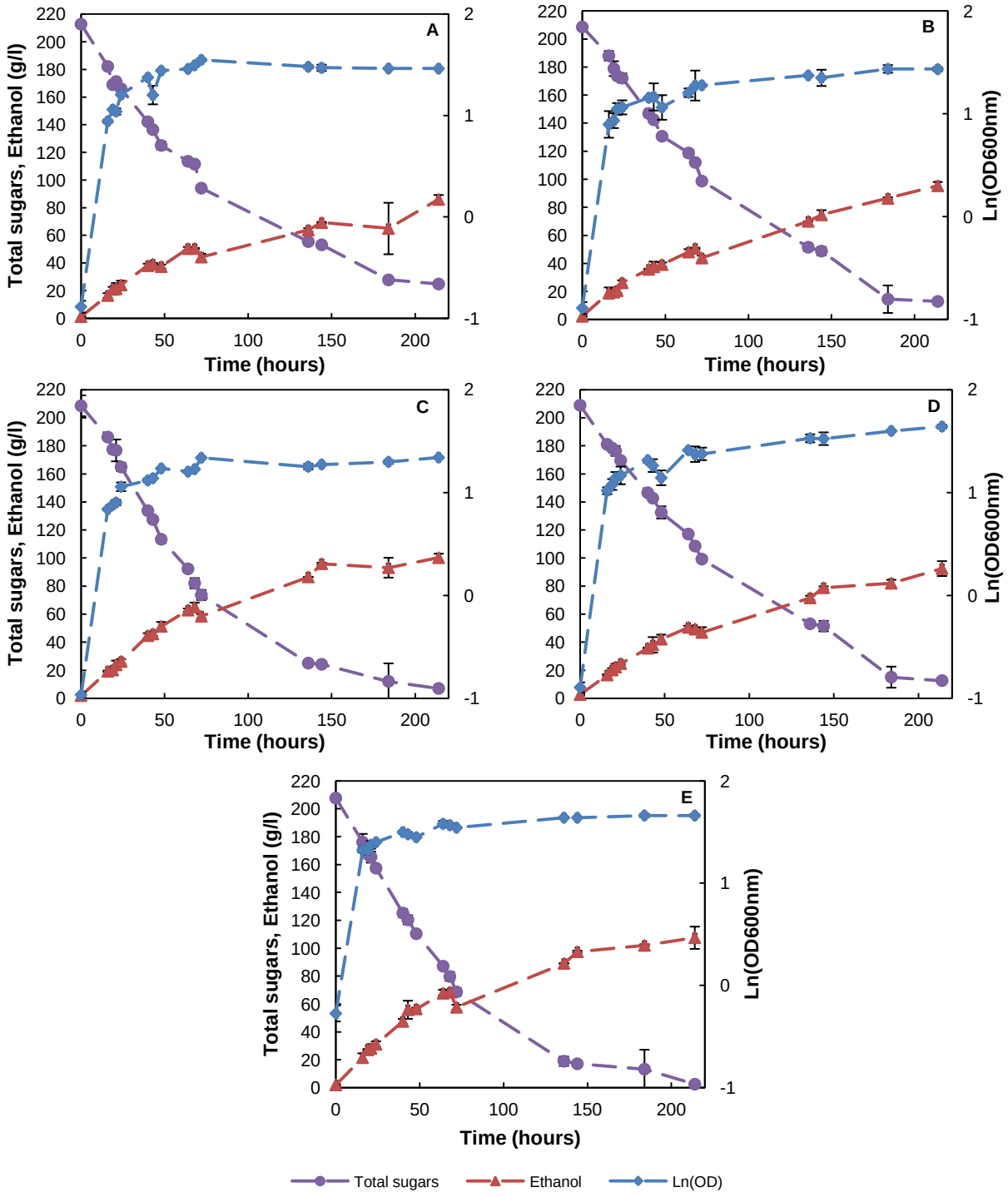



**Figure 9.** Strawberry tree fruit' juice fermentation assay. Time-course profiles displaying the growth, sugar consumption and ethanol production during batch fermentation of strawberry tree fruit' juice (209 g/l total sugar), as sole nutrient source, by: *L. thermotolerans* AP1 strain (**A**), *S. cerevisiae* DP2 strain (**B**), *Z. bailii* GerP3 strain (**C**), *S. cerevisiae* GluP4 strain (**D**) and *S. cerevisiae* CCMI 885 strain (**E**).

## 4. Conclusions

The main goal of this work was to search for novel microbial strains, in special new yeasts, with high potential to produce bioethanol, due to either the presence of better ethanologenic characteristics (e. g. high tolerance to ethanol, and extremes pH and/or temperature) or the production of relevant hydrolytic enzymatic machinery, which can be competitive in further application for industrial bioprocesses. In this context, an isolation of novel microorganisms was carried out from different materials rich in fermentable sugars or polysaccharides (inulin), followed by a screening for their ability to produce relevant enzymes and/or alcoholic fermentation.

A total of 98 microbial isolates were collected, from which 7 yeasts strains were selected based on their promising features observed: all strains had potential to bioethanol production and three present the ability to produce inulinases, important enzymes to apply in bioprocesses using inulin or inulin-rich materials.

A preliminary molecular identification, using oligonucleotide FITC marked probes by FISH, gave a putative identification for the selected isolates, separating them in three species: *L. thermotolerans* (AP1), *S. cerevisiae* (GluP4 and DP2) and *Z. bailii* (GerP3, Calf2, Talf1 and Talf2). These results must be further concluded with more classical and molecular standard identification methods, as morphological description, carbon and nitrogen fermentation and assimilation capacity and genetic sequencing for the identification validation.

In order to use inulin or inulin-rich materials as a carbon source for bioprocesses, they must be hydrolyzed either chemically (acid pre-treatment) or enzymatically (through inulinases). The pre-treatment of raw substrates in an industrial scale represents a substantial cost increase, which means that the application of inulinases or the use of an ethanologenic and inulinase producer to the process to obtain bioethanol could be an advantage, contributing to a less expensive bioprocess. Hence, in this work the Talf1 strain was selected as the best inulinase producer, and both the optimization of the inulinase production as well as its characterization were carried out. Talf1 crude extract activity attained the maximum values (8.7 U/ml) at the optimal pH and temperature, 5.5 and 45 ºC, respectively, and its stability is high at 30ºC (the enzyme presents > 50% activity after 24 days at 30 ºC), which are promising characteristics for its further application for bioethanol bioprocesses.

The assays carried out with inulin (IFM) in a SSF process, using *S. cerevisiae* strain CCMI 885 and Talf1 inulinases, attained higher ethanol production (78 g/l) than the CBP assay (67 g/l), while the fermentation assays with the Jerusalem artichoke juice attained better results in CBP, using the Talf1 strain. These results show the enormous potential of inulin and inulin rich raw materials, as Jerusalem artichoke, as substrates for bioethanol production. Further optimization and scale-up analysis is required using these novel isolated yeast strains.

Moreover, batch fermentations directly from strawberry tree fruit' juice (a complex raw material composed by high sugar content and the nutrients required for microbial growth) were carried out using the 4 selected strains isolated from strawberry tree fruits. In overall, the 4 yeast strains tested gave satisfactory results for bioethanol production from strawberry tree fruit' juice fermentation, with yields ranging from 47 to 50%. However, the GerP3 strain, a putative *Z. bailii*, achieved the most promising results for its further application in a solid state fermentation directly from strawberry tree fruit, as

performed at industrial scale for the production of an economically important distilled drink from this material.

The development of efficient and economically viable bioethanol production processes is crucial in today's society, due to worldwide fuel depletion and increasing energetic requirements. The utilization of raw materials, such as Jerusalem artichoke and strawberry tree fruit, for bioethanol production could help to reduce the energy dependence of fossil fuel. Optimization studies of the processes with these natural raw materials must be carried out for further application at industrial scale.

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## 6. Appendix

### Solutions

PBS 10x:

- $Na_2HPO_4$ 8 mM
- $KH_2PO_4$ 1.5 mM
- NaCl 137 mM
- KCl 2.7 mM

Adjust the pH to 7.2

Washing Buffer:

- Tris/HCl 25 mM
- NaCl 0.5 M

Hybridization Buffer:

- Formamide 20 % (v/v)
- NaCl 0.9 M
- Tris/HCl pH 8 20 mM
- SDS 0.01 % (w/v)

All solutions were prepared with Milli-Q water and filtrated by 0.2 μm Millipore filters.

DNS Reagent:

- 3,5-Dinitrosalicylic acid 1 % (w/v)
- NaOH 1.2 % (w/v)
- Potassium sodium tartrate 30 % (w/v)

Tris/HCl 50 mM buffer (pHs: 7.5, 8.0 and 8.5)

- Trizma Base solution 0.05 M
- pH adjustment by 1 M of HCl solution addition.

Sodium Citrate 50 mM buffer:

**Table 14.** Preparation of Sodium Citrate 50 mM buffer at several pHs. The mixture of solutions A and B volumes were adjusted to 1 L in volumetric flask with Milli-Q Water.

| pH | Solution A (ml) | Solution B (ml) | $H_2O$ (ml) |
|---|---|---|---|
| 3.0 | 422.2 | 77.8 | |
| 3.5 | 361.6 | 138.4 | |
| 4.0 | 302.5 | 197.5 | |
| 4.5 | 234.6 | 265.4 | |
| 5.0 | 167.1 | 332.9 | 500 |
| 5.5 | 100.1 | 399.9 | |
| 6.0 | 43.9 | 456.1 | |
| 6.5 | 15.6 | 484.4 | |
| 7.0 | 5.1 | 494.9 | |

- Solution A:
    - Acid Citrate 0.1 M
- Solution B:
    - Trisodium Citrate 0.1 M

**Culture Medium**

Inulin Fermentative Medium:

- Yeast extract 3 g/l
- Peptone 5 g/l
- Malt extract 3 g/l
- Inulin 20% (w/v)

Note: All commercial and remaining culture medium used were previously sterilized by autoclave at 121 ºC for 15 min prior to utilization.

**Table 15. Fermentation test with invert Durham tube.**

| Source | Strains | ARA | | FRU | | GLU | | XYL | | CEL | | LAC | | SUC | | INU | | Control | | STF Juice | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | G | F | G | F | G | F | G | F | G | F | G | F | G | F | G | F | G | F | G | F |
| **Identified strains** | Kth | + | - | +++ | +++ | +++ | +++ | ++ | - | ++ | - | + | - | +++ | +++ | + | - | +++ | - | | |
| | Sce | ++ | - | +++ | +++ | +++ | +++ | + | - | + | - | ++ | - | +++ | +++ | ++ | - | ++ | - | +++ | +++ |
| | Zba | +++ | - | +++ | +++ | +++ | +++ | ++ | - | + | - | + | - | +++ | +++ | ++ | - | ++ | - | | |
| **Carob kibbles** | Calf1 | ++ | - | +++ | +++ | +++ | +++ | - | - | +++ | - | + | - | +++ | +++ | +++ | - | - | - | | |
| | Calf2 | + | - | +++ | +++ | +++ | +++ | + | - | ++ | - | ++ | - | +++ | +++ | +++ | ++ | - | - | | |
| | Calf3 | + | - | +++ | +++ | +++ | +++ | + | - | ++ | - | + | - | +++ | +++ | +++ | - | + | - | | |
| | Calf4 | + | - | +++ | +++ | +++ | +++ | - | - | ++ | - | + | - | +++ | +++ | +++ | - | + | - | | |
| | Calf5 | ++ | - | +++ | +++ | +++ | +++ | ++ | - | ++ | - | ++ | - | +++ | +++ | +++ | - | + | - | | |
| | Talf1 | - | - | +++ | +++ | +++ | +++ | + | - | + | - | - | - | +++ | +++ | +++ | +++ | - | - | | |
| | Talf2 | + | - | +++ | +++ | +++ | ++ | + | - | + | - | + | - | +++ | +++ | +++ | +++ | - | - | | |
| **Cherry** | H1 | +++ | - | +++ | +++ | +++ | +++ | +++ | - | +++ | - | +++ | - | +++ | - | +++ | - | +++ | - | | |
| | H2 | +++ | - | +++ | +++ | +++ | +++ | +++ | - | +++ | - | +++ | - | +++ | - | +++ | - | +++ | - | | |
| | H3 | - | - | +++ | +++ | +++ | +++ | - | - | +++ | - | - | - | + | - | - | - | - | - | | |
| | H4 | + | - | +++ | +++ | +++ | +++ | - | - | +++ | - | - | - | ++ | - | + | - | - | - | | |
| | H5 | +++ | - | +++ | +++ | +++ | +++ | +++ | - | +++ | - | +++ | - | +++ | - | +++ | - | +++ | - | | |
| | H6 | +++ | - | +++ | +++ | +++ | +++ | +++ | - | +++ | - | +++ | - | +++ | - | +++ | - | +++ | - | | |
| | H8 | +++ | - | +++ | +++ | +++ | +++ | +++ | - | +++ | - | +++ | - | +++ | - | +++ | - | +++ | - | | |
| | H9 | ++ | - | +++ | +++ | +++ | +++ | - | - | +++ | - | - | - | + | - | ++ | - | - | - | | |
| | H10 | +++ | - | +++ | +++ | +++ | +++ | +++ | - | +++ | - | +++ | - | +++ | - | +++ | - | +++ | - | | |
| | H11 | ++ | - | +++ | ++ | +++ | ++ | ++ | - | +++ | - | ++ | - | +++ | +++ | +++ | - | ++ | - | | |
| | H12 | + | - | +++ | +++ | +++ | +++ | - | - | +++ | - | - | - | ++ | - | + | - | - | - | | |
| **Fig** | I1 | +++ | - | +++ | +++ | +++ | +++ | +++ | - | +++ | - | +++ | - | +++ | - | +++ | - | +++ | - | | |
| | I2 | +++ | - | +++ | ++ | +++ | +++ | - | - | +++ | - | +++ | - | +++ | - | +++ | - | +++ | - | | |
| | I3 | + | - | +++ | +++ | +++ | +++ | + | - | +++ | - | - | - | +++ | - | + | - | - | - | | |
| | I4 | + | - | +++ | +++ | +++ | +++ | + | - | +++ | - | + | - | +++ | - | + | - | - | - | | |
| | I5 | +++ | - | +++ | +++ | +++ | +++ | +++ | - | +++ | - | +++ | - | +++ | - | +++ | - | +++ | - | | |
| | I6 | +++ | - | +++ | ++ | +++ | ++ | + | - | +++ | - | +++ | - | +++ | - | - | - | +++ | - | | |

**Table 15.** (Continued)

| Source | Strains | ARA | | FRU | | GLU | | XYL | | CEL | | LAC | | SUC | | INU | | Control | | STF Juice | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | G | F | G | F | G | F | G | F | G | F | G | F | G | F | G | F | G | F | G | F |
| Fig | I7 | +++ | - | +++ | ++ | +++ | ++ | + | - | +++ | - | +++ | - | +++ | - | - | - | +++ | - | | |
| | I8 | + | - | +++ | +++ | +++ | +++ | + | - | +++ | - | + | - | + | - | + | - | - | - | | |
| | I9 | + | - | +++ | + | +++ | + | + | - | +++ | - | - | - | +++ | - | + | - | +++ | - | | |
| | I10 | ++ | - | +++ | +++ | +++ | +++ | +++ | - | +++ | - | +++ | - | +++ | - | +++ | - | ++ | - | | |
| | I11 | +++ | - | +++ | +++ | +++ | +++ | +++ | - | +++ | - | +++ | - | +++ | - | +++ | - | +++ | - | | |
| | I12 | ++ | - | +++ | +++ | +++ | +++ | - | - | +++ | - | + | - | +++ | - | +++ | - | - | - | | |
| | I13 | +++ | - | +++ | ++ | +++ | + | + | - | +++ | - | +++ | - | +++ | - | +++ | - | - | - | | |
| J. artichoke tubers | Ctup1 | +++ | + | +++ | + | +++ | + | +++ | ++ | +++ | + | +++ | + | +++ | ++ | +++ | + | ++ | + | | |
| | Ctup2 | +++ | ++ | +++ | + | +++ | + | +++ | ++ | +++ | + | +++ | + | +++ | ++ | +++ | + | ++ | + | | |
| | Ctup3 | +++ | + | +++ | + | +++ | + | +++ | ++ | +++ | + | +++ | + | +++ | ++ | +++ | ++ | ++ | + | | |
| | Ttup1 | ++ | - | +++ | - | +++ | - | +++ | - | +++ | - | +++ | - | ++ | - | +++ | - | ++ | - | | |
| | Ttup3 | ++ | - | +++ | - | +++ | + | +++ | - | +++ | - | +++ | - | +++ | - | +++ | - | ++ | - | | |
| | Ttup4 | +++ | ++ | +++ | + | +++ | ++ | +++ | ++ | +++ | + | +++ | + | +++ | ++ | +++ | ++ | ++ | + | | |
| | Ttup5 | ++ | - | +++ | - | +++ | - | +++ | - | +++ | - | - | - | ++ | - | ++ | - | ++ | - | | |
| Peach | L1 | + | - | - | - | - | - | - | - | - | - | ++ | - | - | - | - | - | - | - | | |
| | L2 | + | - | +++ | - | ++ | - | ++ | - | + | - | ++ | - | + | - | ++ | - | ++ | - | | |
| | L3 | + | - | ++ | - | ++ | - | ++ | - | + | - | ++ | - | ++ | - | ++ | - | ++ | - | | |
| | L4 | + | - | ++ | ++ | +++ | ++ | + | - | ++ | + | - | - | +++ | - | + | - | + | - | | |
| | L5 | - | - | - | - | - | - | - | - | - | - | ++ | - | ++ | - | - | - | - | - | | |
| | L6 | ++ | - | - | - | - | - | - | - | - | - | +++ | - | - | - | - | - | - | - | | |
| | L7 | ++ | - | ++ | - | ++ | - | ++ | - | ++ | - | +++ | - | ++ | - | ++ | - | ++ | - | | |
| | L8 | ++ | - | - | - | - | - | ++ | - | - | - | ++ | - | - | - | ++ | - | ++ | - | | |
| | L9 | ++ | - | - | - | - | - | - | - | - | - | ++ | - | - | - | - | - | - | - | | |
| | L10 | +++ | - | ++ | - | +++ | - | +++ | - | +++ | - | +++ | - | ++ | - | ++ | - | +++ | - | | |
| | L11 | - | - | +++ | ++ | +++ | ++ | + | - | +++ | - | + | - | + | - | + | - | ++ | - | | |
| | L12 | ++ | - | +++ | ++ | +++ | ++ | ++ | - | ++ | - | + | - | ++ | - | ++ | - | +++ | - | | |
| | L13 | ++ | - | +++ | ++ | +++ | ++ | +++ | - | +++ | - | ++ | - | ++ | - | ++ | - | ++ | - | | |
| | L14 | - | - | +++ | ++ | ++ | - | + | - | +++ | - | + | - | + | - | - | - | + | - | | |
| | L15 | +++ | - | +++ | ++ | +++ | ++ | +++ | - | +++ | ++ | +++ | - | +++ | - | +++ | - | +++ | - | | |
| | L16 | + | - | +++ | ++ | +++ | ++ | + | - | +++ | - | ++ | - | + | - | + | - | + | - | | |

**Table 15.** (Continued)

| Source | Strains | ARA | | FRU | | GLU | | XYL | | CEL | | LAC | | SUC | | INU | | Control | | STF Juice | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | G | F | G | F | G | F | G | F | G | F | G | F | G | F | G | F | G | F | G | F |
| STF | AP1 | ++ | - | - | - | +++ | ++ | + | - | ++ | - | ++ | - | +++ | +++ | ++ | - | - | - | +++ | +++ |
| | AP2 | ++ | - | +++ | +++ | +++ | +++ | ++ | - | +++ | - | ++ | - | +++ | - | ++ | - | ++ | - | + | ++ |
| | AP3 | ++ | - | +++ | +++ | +++ | +++ | +++ | - | +++ | - | +++ | - | +++ | - | +++ | - | +++ | - | + | ++ |
| | BP2 | ++ | - | +++ | +++ | +++ | +++ | + | - | +++ | - | ++ | - | +++ | - | ++ | - | - | - | + | ++ |
| | BP4 | ++ | - | +++ | +++ | +++ | +++ | +++ | - | +++ | - | +++ | - | +++ | - | +++ | - | +++ | - | + | ++ |
| | CRB1 | +++ | - | - | - | +++ | + | ++ | - | +++ | - | +++ | - | +++ | - | +++ | - | ++ | - | + | - |
| | DP1 | - | - | +++ | +++ | +++ | ++ | - | - | - | - | - | - | + | - | - | - | - | - | + | ++ |
| | DP2 | - | - | +++ | +++ | +++ | ++ | - | - | + | - | + | - | +++ | +++ | + | - | - | - | +++ | +++ |
| | DP3 | - | - | +++ | +++ | +++ | +++ | - | - | ++ | - | - | - | + | - | - | - | - | - | + | - |
| | EP1 | + | - | - | - | +++ | +++ | - | - | ++ | - | - | - | + | - | + | - | - | - | + | ++ |
| | EP2 | + | - | +++ | ++ | +++ | ++ | - | - | ++ | - | - | - | + | - | + | - | - | - | + | ++ |
| | FP1 | + | - | +++ | ++ | +++ | +++ | - | - | ++ | - | - | - | + | - | - | - | - | - | + | ++ |
| | FruP1 | - | - | +++ | ++ | +++ | ++ | + | - | ++ | - | ++ | - | ++ | - | ++ | - | - | - | + | ++ |
| | FruP2 | ++ | - | +++ | +++ | +++ | +++ | ++ | - | +++ | - | +++ | - | +++ | +++ | +++ | - | + | - | + | ++ |
| | FruP3 | ++ | - | +++ | ++ | +++ | ++ | + | - | ++ | - | ++ | - | ++ | - | - | - | + | - | + | - |
| | FruP4 | - | - | +++ | ++ | +++ | ++ | + | - | - | - | ++ | - | ++ | - | ++ | - | - | - | + | ++ |
| | FruP5 | +++ | - | +++ | ++ | +++ | +++ | + | - | +++ | - | ++ | - | +++ | +++ | +++ | - | + | - | + | ++ |
| | GerP3 | - | - | +++ | ++ | ++ | - | + | - | ++ | - | - | - | + | - | - | - | - | - | +++ | +++ |
| | GerP4 | - | - | - | - | - | - | - | - | - | - | ++ | - | - | - | + | - | - | - | + | - |
| | GerP5 | ++ | - | +++ | +++ | +++ | +++ | +++ | - | +++ | - | +++ | - | +++ | - | +++ | - | +++ | - | + | ++ |
| | GluP1 | +++ | - | +++ | +++ | +++ | +++ | ++ | - | +++ | - | ++ | - | +++ | +++ | +++ | - | ++ | - | + | ++ |
| | GluP2 | - | - | - | - | +++ | ++ | + | - | - | - | ++ | - | ++ | - | - | - | + | - | + | ++ |
| | GluP3 | ++ | - | +++ | ++ | +++ | ++ | - | - | - | - | - | - | ++ | - | ++ | - | ++ | - | + | ++ |
| | GluP4 | + | - | ++ | ++ | +++ | +++ | + | - | + | - | ++ | - | +++ | +++ | - | - | + | - | +++ | +++ |
| | SMP1 | - | - | +++ | ++ | +++ | +++ | - | - | ++ | - | - | - | - | - | +++ | - | + | - | + | - |
| | SMP2 | ++ | - | +++ | +++ | +++ | +++ | +++ | - | +++ | - | +++ | - | +++ | - | +++ | - | +++ | - | + | ++ |
| | SMP5 | ++ | + | +++ | + | +++ | ++ | +++ | ++ | +++ | + | ++ | + | +++ | ++ | +++ | + | ++ | + | + | - |
| | SMP6 | - | - | +++ | ++ | +++ | +++ | + | - | ++ | - | - | - | + | - | - | - | - | - | + | ++ |
| | XilP1 | ++ | - | - | - | +++ | ++ | + | - | ++ | - | ++ | - | - | - | ++ | - | - | - | + | ++ |
| | XilP2 | ++ | - | - | - | +++ | ++ | + | - | - | - | ++ | + | ++ | - | ++ | - | - | - | + | - |

**Table 15.** (Continued)

| Source | Strains | ARA | | FRU | | GLU | | XYL | | CEL | | LAC | | SUC | | INU | | Control | | STF Juice | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | G | F | G | F | G | F | G | F | G | F | G | F | G | F | G | F | G | F | G | F |
| Plum | J1 | + | - | ++ | ++ | +++ | ++ | + | - | +++ | + | + | - | +++ | ++ | + | - | + | - | | |
| | J2 | +++ | - | +++ | ++ | +++ | ++ | +++ | - | +++ | - | +++ | - | +++ | - | +++ | - | +++ | - | | |
| | J3 | +++ | - | +++ | ++ | +++ | ++ | ++ | - | +++ | - | +++ | - | ++ | - | +++ | - | +++ | - | | |
| | J4 | +++ | - | +++ | ++ | +++ | ++ | +++ | - | ++ | - | +++ | - | +++ | - | - | - | - | - | | |
| | J5 | + | - | +++ | ++ | +++ | ++ | + | - | +++ | - | + | - | + | - | + | - | + | - | | |
| | J6 | - | - | +++ | ++ | +++ | ++ | + | - | +++ | - | + | - | + | - | + | - | - | - | | |
| | J7 | +++ | - | +++ | ++ | +++ | ++ | +++ | - | +++ | - | +++ | - | +++ | - | - | - | +++ | - | | |
| | J8 | + | - | +++ | ++ | +++ | ++ | +++ | - | +++ | + | + | - | + | - | + | - | - | - | | |
| | J9 | +++ | - | +++ | ++ | +++ | ++ | +++ | - | +++ | - | +++ | - | +++ | ++ | +++ | - | +++ | - | | |
| | J10 | ++ | - | +++ | ++ | ++ | ++ | + | - | +++ | - | - | - | + | - | + | - | + | - | | |
| | J11 | +++ | - | +++ | ++ | +++ | ++ | +++ | - | +++ | - | ++ | - | +++ | - | +++ | - | +++ | - | | |
| | J12 | +++ | - | +++ | +++ | +++ | +++ | ++ | - | +++ | - | +++ | - | +++ | - | ++ | - | ++ | - | | |
| | J13 | +++ | - | +++ | ++ | +++ | ++ | ++ | - | +++ | - | ++ | - | ++ | - | - | - | ++ | - | | |
| | J14 | +++ | - | +++ | ++ | +++ | ++ | +++ | - | +++ | - | +++ | - | +++ | - | - | - | +++ | - | | |

Tests: negative control (Control), arabinose (ARA), fructose (FRU), glucose (GLU), xylose (XYL), Cellobiose (CEL), lactose (LAC), sucrose (SUC), inulin (INU) and Strawberry Tree Fruit' Juice (STF Juice). All positive results were confirmed by HPLC

Growth observation (G): (-) absent growth, (+) partial turbidity, (++) strong turbidity, (+++) strong turbidity which unable to see the invert Durham tube.

Gas accumulation inside Durham tube observation (F): (-) absent gas, (+) small gas bubble, (++) gas bubble filling half Durham tube, (+++) gas bubble filling full Durham tube.