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ISOLAMENTO, IDENTIFICAÇÃO E CARACTERIZAÇÃO DE MICRORGANISMOS PRODUTORES DE OLIGOSSACARÍDEOS A PARTIR DE COLETAS EM DIFERENTES REGIÕES BRASILEIRAS

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"Não importa o tempo, a ausência, os adiamentos,
as distâncias, as impossibilidades.
Quando há afinidade, qualquer reencontro retoma
a relação, o diálogo, a conversa, o afeto
no exato ponto em que foi interrompido."

(Arthur da Távola)

A meus pais

A meus irmãos

Ao Lucas

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RESUMO

Devido ao aumento da demanda por alimentos saudáveis e ao aumento da aplicação, de oligossacarídeos prebióticos, na indústria cosmética, agro-química, farmacêutica e na indústria de alimentos, a pesquisa visando a utilização de diferentes enzimas na produção dos oligossacarídeos se tornou algo necessário. O objetivo deste trabalho foi obter linhagens de leveduras, produtoras de frutooligossacarídeos a partir da sacarose. As linhagens foram isoladas de flores e frutos de diversas eco-regiões do Brasil. A partir da gama de microrganismos isolados foram selecionadas 4 cepas potencialmente aplicáveis na produção de frutooligossacarídeos: *Candida* sp. LEB-I3; *Rhodotorula* sp. LEB-U5; *Cryptococcus* sp. LEB-V2 e *Rhodotorula* sp. LEB-V10. Os quatro microrganismos estudados produzem enzimas semelhantes em relação a algumas características bioquímicas. As condições ótimas para a atividade de transferência de frutose foram pH entre 4,0 e 5,0 e temperaturas entre 65 e 70ºC, enquanto que para a atividade de frutofuranosidase o pH ótimo foi de 3,0 a 4,0 e a temperatura ótima entre 55 e 75ºC. A cinética enzimática (em relação à atividade de transferência de frutose) da enzima I3 seguiu o modelo de Michaelis-Menten, enquanto que U5 e V2 seguiram o modelo de inibição pelo substrato e a enzima V10 apresentou uma cinética de "cooperatividade". O estudo da síntese de FOS mostrou que o microrganismo *Rhodotorula* sp. LEB-V10 foi o único cuja enzima promoveu uma síntese constante de FOS, não apresentando nenhum indício de hidrólise dos frutooligossacarídeos, sendo devido a esta característica que tanto a produção da enzima quanto a síntese de frutooligossacarídeos foram otimizadas. Neste caso a produção de enzima foi máxima nas seguintes condições: 9% ± 1% de AMM e 7,5% ± 0,7% de açúcares redutores totais (melaço), com uma agitação de 250 rpm, a 30-35ºC, pH inicial do meio de 4,5. A produção de frutooligossacarídeos por esta enzima também foi otimizada chegando-se a 55-65% de rendimento nas seguintes condições: 50% de sacarose (P.A, comercial ou melaço), 6,5 (± 0,5) $UTF.ml^{-1}$, temperatura entre 50 ºC (± 1ºC) e pH 5,0 (± 0,5). Desta forma foi alcançado o rendimento dos processos comerciais, com a vantagem de estarmos trabalhando com enzimas e não células. Além disso, a produção da enzima utilizando meios industriais, e o uso de açúcar cristal e mesmo melaço na síntese enzimática resultam em uma diminuição dos custos de produção. Desta forma há uma chance de que a continuação destes estudos resulte em um processo economicamente viável.

ABSTRACT

In response to the increasing demand for healthier foods and as a result of the expanding applications of oligosaccharides in the cosmetic, agrochemical, pharmaceutical and food industries, the search for "new" enzymes concerning the oligosaccharides production, became necessary. The present study reports on the screening for high transfructosylating enzymes in yeasts strains isolated from fruits and flowers obtained from tropical Brazilian biomass. The efforts made to screen for high extra-cellular transfructosylating enzyme producing yeasts provided very promising results. Although the enzymes from the strains *Candida* sp. LEB-I3, *Rhodotorula* sp. LEB-U5 and *Cryptococcus* sp. LEB-V2 showed high hydrolytic activity, the production of fructooligosaccharides (FOS) by the *Rhodotorula* sp. LEB-V10 enzyme was successful, showing a continuous increase in FOS concentration up to the end of the synthesis reaction. The best operational conditions for these enzymes, considering the transfructosylating activities, were determined to be in the pH range from 4.0 to 5.0 and temperatures from 65 to 70°C. While the fructofuranosidase activities had shown the optimum activity on pH values from 3.0 to 4.0 and temperatures between 55 and 75°C. The enzymatic kinetic (fructosyl transferase activity) of the *Candida* sp. LEB-I3 showed a Michaellis-Mentem behavior, while the *Rhodotorula* sp. LEB-U5 and *Cryptococcus* sp. LEB-V2 showed a substrate inhibitory kinetic and the *Rhodotorula* sp. LEB-V10 showed a sigmoid shape, similar to that of allosteric enzymes. Considering that the *Rhodotorula* sp. LEB-V10 process was the only one that may be regarded as economically possible, the response surface methodology was employed to study the fermentation and the synthesis condition aiming the process optimization. On basis of the experimental results, the optimum conditions to obtain high fructosyl transferase activity were: 250 rpm, 30-35°C, 9% ± 1% (w/v) corn steep liquor and 7.5% ± 0.7% (w/v) of total reducing sugar from sugar cane molasses. The synthesis of FOS was also optimized (55 to 65% of yield), being the optimum conditions: 50% sucrose (P.A., commercial or from sugar cane molasses), 50°C (± 1°C), pH 5.0 (± 0.5) and 6.5 FTA.ml-1 (± 0.5). This data is very similar to those from the commercial process, and the use of commercial sucrose and sugar cane molasses led to a reduction on the production cost, consequently, further studies on the enzyme and fructooligosaccharides production conditions may show its potential for commercial application.

PARTE I

1. INTRODUÇÃO GERAL

Inicialmente grande parte dos estudos sobre alimentos funcionais visava o emprego de alimentos probióticos, alimentos que contêm microrganismos vivos capazes de colonizar o cólon. Mas esses microrganismos dificilmente conseguiam colonizar o cólon, já que a taxa de sobrevivência nas condições do sistema digestivo era baixa. Na década de 80 foi observado que muitos oligossacarídeos alcançavam o cólon humano e animal sem ter sofrido degradação (oligossacarídeos não digeríveis), tornando-se fonte de carbono para bactérias presentes no cólon, como as bifidobactérias. Neste caso esses alimentos são chamados de prebióticos.

Os oligossacarídeos são carboidratos com grau de polimerização de 2 a 10, e estão presentes como componentes majoritários em muitos produtos naturais, mas também podem ser produzidas química ou enzimaticamente. Hoje em dia, são produzidos enzimaticamente a partir de açúcares simples, por transglicosilação, ou por degradação de polissacarídeos de origem vegetal, animal ou microbiana (Playne & Crittenden, 1996). São conhecidos vários tipos de oligossacarídeos não digeríveis, e dentre esses os frutooligossacarídeos, galactooligossacarídeos, oligossacarídeos da soja e alguns glucooligossacarídeos são reconhecidamente prebióticos (O`Sullivan, 1996).

Os oligossacarídeos são de grande interesse para a indústria de alimentos, farmacêutica e de cosméticos, onde são usados como adoçantes, estabilizantes, agentes de corpo e agentes prebióticos, capazes de estimular o desenvolvimento de flora benéfica ao ser humano.

Atualmente há um grande interesse de se estudar não somente o efeito prebiótico de oligossacarídeos conhecidos, mas também o de novos oligossacarídeos, produzidos por diferentes enzimas e microrganismos. Considerando a grande biodiversidade brasileira, torna-se interessante o estudo de novos microrganismos capazes de produzir oligossacarídeos prebióticos a partir de um substrato simples, como a sacarose.

2. OBJETIVOS GERAIS

O objetivo deste trabalho foi obter as linhagens de leveduras produtoras de oligossacarídeos prebióticos a partir da sacarose, além da caracterização e purificação das enzimas escolhidas, o estudo da produção de enzimas em frascos agitados e finalmente, a síntese dos frutooligossacarídeos.

As linhagens foram isoladas de diversas fontes (solo, polens de flores, frutos, etc.) em diferentes regiões do Brasil (Figura 1) e testadas quanto à produção de oligossacarídeos. Além disso, foram realizados testes para a verificação do potencial prebiótico destes oligossacarídeos.

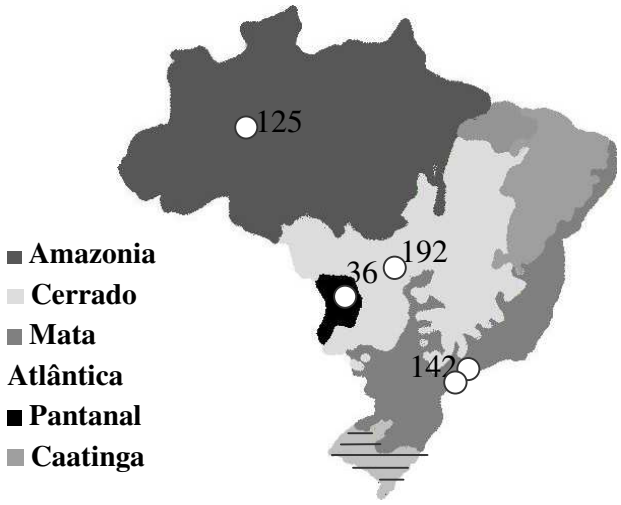

Figura 1: Regiões de coleta das amostras e número de leveduras isoladas (○)

3. REVISÃO BIBLIOGRÁFICA GERAL

3.1. Alimentos Funcionais

A relação entre alimentação e saúde é inequívoca, sendo atualmente estudada visando o conhecimento mais aprofundado de como os componentes dos alimentos agem no organismo humano e animal. Na década de 80, no Japão, houve a criação do conceito "FOSHU" (Food for Specified Health Use), fruto da colaboração entre órgãos públicos, privados e científicos. Este grupo de alimentos foi definido como alimentos convencionais que apresentem benefícios à saúde se consumido regularmente como parte de uma dieta saudável. Fibras alimentares, oligossacarídeos, polióis, ácidos graxos polinsaturados,

peptídeos e proteínas, glicosídeos, vitaminas, minerais, entre outros, foram englobados neste novo conceito que veio a gerar a definição de alimentos funcionais (Hartemink, 1997).

A primeira geração de alimentos funcionais foi composta por vitaminas e sais como cálcio, mas logo se passou a direcionar esta classe de alimentos aos que proporcionavam efeito positivo na composição da microbiota intestinal. Ainda no início houve grande concentração dos estudos em alimentos probióticos, alimentos que contêm microrganismos vivos capazes de colonizar o cólon, como os *Lactobacillus* spp., *Enterococcus faecalis* e *Bifidodacteria* spp. (Hartemink, 1997).

A partir da introdução deste novo conceito, foi observado um interesse crescente por alimentos funcionais. No entanto a definição de alimentos funcionais depende de cada pesquisador e de cada país. No Brasil, há a divisão entre alimentos funcionais (alegação de propriedade funcional) e nutracêuticos (alegação de propriedade de saúde), ocorrendo a associação de alimentos nutricionais com a nutrição e não com a farmacologia. A definição legal de alimento funcional é: "todo aquele alimento ou ingrediente que, além das funções nutricionais básicas, quando consumido como parte da dieta usual, produz efeitos metabólicos e/ou fisiológicos e/ou efeitos benéficos à saúde, devendo ser seguro para consumo sem supervisão médica“(Portaria n 398 de 30/04/99, Secretaria de Vigilância Sanitária do Ministério da Saúde).

Há diferentes definições de alimentos funcionais, entre elas: alimentos e ingredientes que são capazes de proporcionar um benefício à saúde, à performance física ou à capacidade mental, além dos nutrientes tradicionais que contém; alimentos que promovem a saúde além de sua propriedade nutricional básica; alimentos ou produtos alimentares comercializados com a alegação de serem benéficos à saúde; alimentos que causam efeitos potencialmente benéficos à saúde quando consumidos regularmente como parte de uma dieta variada e em níveis efetivos; alimentos ou bebidas derivados de substâncias naturais, consumidos como parte da dieta que causam algum efeito fisiológico benéfico ao consumidor, ou que regulam ou afetam algum processo do corpo humano, ou ainda que reduzem o risco de doenças crônicas. Independentemente de qual definição utilizada, “alimentos funcionais” apresentam um conceito único, não possuem efeito terapêutico já que são alimentos e não remédios, mas podem reduzir o risco de algumas doenças e prevenindo-as (Berner e O’Donnell, 1998).

Entre os diversos alimentos funcionais reconhecidos, temos os prebióticos, alimentos não digeríveis que beneficiam o consumidor por estimular seletivamente o crescimento ou a atividade das bactérias residentes no cólon intestinal. Para serem efetivos os prebióticos não devem ser hidrolisados ou absorvidos no trato gastrintestinal "superior" e devem ser fermentados (fontes de carboidrato) somente pela microbiota benéfica, os microrganismos chamados probióticos, como os pertencentes ao gênero *Lactobacillus* e *Bifidobacterium* (Gibson e Roberfroid, 1995).

3.2. Oligossacarídeos Prebióticos

Para que os oligossacarídeos tenham efeito prebiótico, e portanto sejam resistentes à digestão e suas enzimas (α-amilase, pancreatina, etc.), eles devem possuir ligações glicosídicas específicas, responsáveis por esta resistência. Entre os oligossacarídeos reconhecidamente prebióticos, tem-se os frutooligossacarídeos (líderes no mercado europeu), galactooligossacarídeos e xilooligossacarídeos entre outros.

O efeito prebiótico da inulina e dos frutooligossacarídeos foi largamente estudado, no entanto foi observado que o tamanho da cadeia é um fator muito importante na promoção da microbiota intestinal benéfica à saúde. Desta forma são considerados prebióticos os frutooligossacarídeos que possuem de 2 a 3 unidades de frutose ligadas a uma sacarose por ligação β-(2,1) (Hidaka *et al*., 1986).

Galactooligossacarídeos são outra classe de prebióticos, já produzidos e comercializados na Europa e Japão, sendo aplicados também em produtos infantis, já que ocorrem naturalmente no leite materno. Outros tipos de oligossacarídeos são os oligossacarídeos da soja, também prebióticos, já que estimulam as bactérias benéficas e não são digeridos pelo homem. Para a degradação destes oligossacarídeos é necessária a enzima α-galactosidase, que não é produzida pelo organismo humano. Uma outra classe destes oligossacarídeos é formada pelos oligossacarídeos transgalactosilados, que devido às ligações específicas, também não sofrem digestão (O`Sullivan, 1996).

Glicooligossacarídeos também são prebióticos, como isomalto-oligossacarídeos, mas na verdade, eles são parcialmente prebióticos. Estes oligossacarídeos são metabolisados pelo corpo humano de uma forma muito lenta e foi observado que somente 20% dos glucooligossacarídeos com ligações α-(1,2) são realmente digeridos pelo

homem, enquanto os microrganismos da flora intestinal probiótica são capazes de metabolisá-los (Remaud-Simeon *et al.*, 1994).

Xilooligossacarídeos possuem a propriedade de serem muito mais estáveis em meios ácidos que os outros prebióticos listados, tendo uma aplicação muito interessante em refrigerantes, que tendem a ser acidificados (O`Sullivan, 1996).

Outro prebiótico interessante é a lactulose, um açúcar não redutor, híbrido de sacarose e lactose, cuja aplicação está crescendo rapidamente, principalmente no Japão. Atualmente a maioria dos oligossacarídeos é produzida comercialmente a partir de reações enzimáticas, ou pela transglicosilação ou pela hidrólise de polissacarídeos, a não ser a lactulose, que é produzida quimicamente por isomerização (álcalis) (O`Sullivan, 1996).

3.3. Efeitos Prebióticos

Até a metade do século XX, acreditava-se que todos os alimentos não digeridos eram excretados nas fezes, mas após alguns anos de estudos, observou-se que alguns destes alimentos, apesar de não serem passíveis de digestão, eram fermentados por certos microrganismos da microbiota intestinal (Playne & Crittenden, 1996). Apesar de alguns peptídeos, proteínas e lipídeos serem prebióticos, foram os oligossacarídeos não digeríveis (principalmente os frutooligossacarídeos) que receberam a atenção dos pesquisadores (Ziemer & Gibson, 1998).

Devido às dificuldades encontradas na administração oral de alimentos probióticos, microrganismos vivos capazes de melhorar o equilíbrio microbiano intestinal produzindo efeitos benéficos à saúde do individuo (baixas taxas de sobrevivência), os alimentos prebióticos se tornaram uma alternativa atraente de alimentos funcionais. O comportamento das culturas em um ecossistema competitivo, como o cólon, mostra que os microrganismos probióticos se desenvolvem muito melhor na presença destes alimentos que os patógenos, gerando assim um efeito prebiótico (Ziemer & Gibson, 1998).

Os microrganismos probióticos são capazes de melhorar a saúde humana da seguinte forma: melhoram o metabolismo de proteínas e vitaminas; previnem constipação; têm ação antibiótica no intestino, prevenindo a colonização por patógenos; reduzem os riscos de câncer de cólon. (Arunachalan, 1999; Modler, McKellar & Yaguchi, 1990).

A pesquisa na área dos probióticos, prebióticos e simbióticos (alimentos funcionais que em geral contem um componente prebiótico que favoreça o efeito do probiótico associado) é muito atual e há ainda muitas possibilidades de estudos, desde o estudo do incremento da sobrevivência dos microrganismos probióticos em diferentes meios ou condições de processo e o aumento de sua disponibilidade em diferentes mercados, e consumidores, até o isolamento, identificação e caracterização de microrganismos produtores de oligossacarídeos.

Até o presente ainda não se tem conhecimento suficiente em relação à função da estrutura dos oligossacarídeos no potencial prebiótico, e ainda é necessário o desenvolvimento de tecnologias de produção destes oligossacarídeos para a redução dos custos e sua aplicação mais generalizada aos alimentos.

3.4. Microrganismos Produtores de Oligossacarídeos Prebióticos

Os carboidratos não digeríveis mais estudados são os frutooligossacarídeos. Na década de 50 foi observado que *Aspergillus orysae* e *Claviceps purpurea* eram capazes de produzir tri e tetra sacarídeos ligados por ligações glicosídicas β-(2,1). Na década de 80 as pesquisas foram intensificadas e o microrganismo *Fusarium oxysporum* foi estudado por diversos centros para a produção de glicofrutanas (Yun, 1996).

Somente no fim da década de 80 encontraram-se enzimas com potencial de aplicação comercial (Hidaka *et al*., 1988; Hayashi *et al*., 1989). A produção de frutooligossacarídeos, em escala industrial, é realizada com células imobilizadas de *Aspergillus niger* e *Aureobasidium pullulans*, a partir da sacarose, obtendo-se um produto final com 55 a 60% de frutooligossacarídeos (Yun, 1996).

Bactérias como *Leuconostoc mesenteroides*, *Leuconostoc dextranicum*, *Streptococcus* e *Lactobacillus* são capazes de produzir glucosiltransferases, que catalisam a formação de glucooligossacarídeos prebióticos, pela transferência de moléculas de glicose da sacarose (doador) para a maltose (aceptor) (Shwengers, 1987; O`Sullivan, 1996).

Desde o início do século XX, quando se isolou a primeira levedura, tem-se observado o interesse na descoberta de novas linhagens, capazes de metabolizar e sintetizar diferentes substâncias. Apesar de já haver produção satisfatória de

frutooligossacarídeos, ainda há substancial interesse de pesquisadores, principalmente pela substituição da produção por fungos filamentosos (bolores) pela produção por leveduras, como as do gênero *Kluyveromyces,* resultando em vantagens de processamento (Santos, 1998).

Entre as leveduras, a *Sterigmatomyces elviae*, *Rhodothorula minuta* e *Sirobasidium magnum* têm alta atividade de transgalactosilação produzindo glucooligossacarídeos em meio contendo celobiose, e galactooligossacarídeos em meio contendo lactose (Onishi & Yokozeki, 1996).

Em estudos mais recentes, as leveduras *Sporobolomyces singularis* e *Schwanniomyces occidentalis* foram descritas como produtoras de oligossacarídeos, como glicosil-lactose e galactosil-lactose (Spences *et al*., 2001). Outro microrganismo capaz de produzir oligossacarídeos prebióticos (neokestose – 6^{G}-β-D-fructofuranosylsucrose) a partir da sacarose é a levedura *Xanthophyllomyces dendrorhaus* (*Phafia rhodozyma*), conhecida produtora de astaxantina (Kritzinger *et al*., 2003).

3.5. Perspectiva do Mercado de Produtos Prebióticos

Tomomatsu (1996) projetou um mercado de alimentos funcionais no valor de U$ 4,5 bilhões, com crescimento anual de 8% em 1995. Entretanto, as estimativas da magnitude deste mercado variam significativamente porque não há consenso mundial no que constitui um alimento funcional. O mercado dos alimentos funcionais foi estimado pelo *Institute of Food Technologists* (www.ift.org) em torno de 30 bilhões de dólares em 1999 e em 2004 acredita-se que o mercado mundial de alimentos funcionais movimentou mais de 50 bilhões de dólares. Somente nos Estados Unidos, o mercado de alimentos funcionais com alegação de propriedade de saúde (considerados nutracêuticos no Brasil) movimenta US$ 0,5 bilhões e os sem esta alegação (considerados funcionais no Brasil), US$15 bilhões por ano. O Japão e a Europa são também grandes consumidores de alimentos funcionais, movimentando 14 e 8 bilhões de dólares por ano, respectivamente (Sangeetha *et al*, 2005).

Há um grande número de alimentos funcionais no mercado mundial, incluindo fibras, oligossacarídeos, peptídeos, proteínas, prebióticos, probióticos, fitoquímicos, antioxidantes e ácidos graxos. Mas devido às características e ao efeito prebiótico dos frutooligossacarídeos, a demanda por estes açúcares vem aumentando tanto como

ingrediente de alimentos para consumo humano ou animal, quanto como adoçante alternativo para formulações visando o público diabético.

Recentemente os avanços da enzimologia estão sendo aplicados na síntese de novos oligossacarídeos, fazendo-se grande uso das técnicas de mutação para a obtenção de linhagens com alta produtividade. Embora nem todos esse oligossacarídeos sejam prebióticos, os estudos realizados são de grande importância para o entendimento da viabilidade das enzimas e sua forma de ação (Ziemer & Gibson, 1998).

3.6. Produção Enzimática de Oligossacarídeos

Na literatura é observada uma grande divergência em termos de denominação da enzima, quando se trata da produção de frutooligossacarídeos. Alguns autores se referem às enzimas como fructofuranosidases (E.C.3.2.1.26) e outros como fructosiltransferases (E.C.2.4.1.9), mesmo quando tratam da enzima produzida pelo mesmo microrganismo, como *Aspergillus niger*. A razão para esta divergência é que enquanto alguns autores utilizam a denominação de fructosiltransferase para diferenciá-la das enzimas hidrolíticas, outros consideram a atividade de transfrutosilação como uma reação que ocorre na utilização de invertase em altas concentrações de sacarose (Yun, 1996).

Teoricamente os dois tipos de enzimas microbianas podem ser responsáveis pela síntese de oligossacarídeos: as hidrolases (glicosidases, EC 3.2.) e as transferases (glicosil-transferase, EC 2.4.) (Monsan & Paul, 1995).

3.6.1. Hidrolases

Essas enzimas hidrolíticas são capazes de catalisar a ligação direta de unidades glicosídicas por reversão da reação de hidrólise ou transferir uma unidade glicosídica de um doador para um aceptor.

No caso das hidrólises, a direção da reação é determinada pelas condições do sistema. Com o aumento da concentração de substrato, diminuição da atividade de água e/ou remoção do produto, estimula-se a reversão da reação, podendo-se sintetizar glico, fruto, mano e galactooligossacarídeos.

Entretanto a formação de um composto intermediário enzima-substrato é freqüentemente observado nos mecanismos de reação, sendo possível, portanto, que

esta enzima catalise a transferência de uma unidade glicosídica de um doador para uma molécula aceptora (que contém grupo hidroxila). As velocidades de reação deste caso são maiores que a da hidrólise reversa, mas para isso deve-se escolher um aceptor eficiente, além de se promover as condições necessárias para a hidrólise reversa (Monsan & Paul, 1995).

3.6.2. Transferases

Neste caso temos duas formas de transferência: intermolecular e a intramolecular (quando o próprio doador age como aceptor). As enzimas deste tipo são capazes de catalisar reações de transferência mesmo em soluções diluídas, obtendo a energia necessária da quebra da ligação osídica e armazenando-a na forma de um intermediário, enzima-substrato, covalente (Monsan & Paul, 1995).

Isomalto-oligossacarídeos são produzidos por enzima α-transglucosidase a partir de hidrolisados de amido com alto teor de maltose, enquanto que ciclodextrina pode ser produzida pela ação da enzima ciclodextrina glucanotransferase (Monsan & Paul, 1995).

Para a produção de frutooligossacarídeos é comum a aplicação da enzima frutosiltransferase, produzida pelos fungos *Aspergillus* e *Aureobasidium*, utilizando a sacarose como substrato (Yun, 1996).

As glicosiltransferases também são amplamente aplicadas na obtenção de oligossacarídeos a partir da sacarose, sendo necessária a presença de maltose ou isomaltose como aceptor (Remaud-Simeon *et al.*, 1994).

3.7. Enzimas: considerações teóricas

As enzimas são biocatalisadores, aumentam a taxa da reação química sem sofrer transformação química permanente e sem afetar o equilíbrio da reação. Sua ação depende das forças eletrostáticas presentes na molécula enzimática. Geralmente apresentam alta especificidade como conseqüência de sua conformação tridimensional provocando a formação de “sítios ativos”, sendo muitas vezes necessário a presença de cofatores para formar um complexo cataliticamente ativo.

3.7.1. Cinética de Michaelis-Menten

Michaelis e Menten desenvolveram um modelo cinético para uma reação enzimática simples, considerando a velocidade inicial de reação, quando a concentração de substrato é constante e a concentração de produto desprezível, e considerando também a existência de um complexo Enzima-Substrato (ES). A dependência da velocidade inicial de reação (v) em função da concentração de substrato (S), desenvolvida por Michaellis-Menten é apresentada na Equação 1.

$$v = v_{max} \frac{[S]}{Km + [S]} \qquad \text{(eq. 1)}$$

A determinação das constantes, K_m e v_{max} da Equação de Michaelis-Menten, podem ser realizadas pela técnica de linearização da equação como descrita por Lineweaver-Burk , Eadie-Hanes e Hofstee. No entanto esta técnica acaba resultando em valores pouco precisos, sendo mais adequada a estimativa das constantes pela utilização de recursos computacionais e softwares (Doran, 1995). Além disso, nem todas as reações enzimáticas, seguem a teoria clássica descrita por Michaelis-Menten. Via de regra, encontra-se problemas de inibições, seja por um dado composto presente na solução, seja pelo próprio substrato ou produto formado. Assim correções nesta equação podem ser utilizadas (Schmidell, 2001).

3.7.2. Cinética alostérica

Para Michaelis-Menten, a baixas concentrações de substrato, as flutuações na concentração causam uma grande diferença na velocidade de reação enzimática, no entanto em altas concentrações de substrato, o aumento da velocidade de reação se torna difícil sendo necessário uma grande variação desta.

Algumas enzimas agem de forma diferenciada, como as enzimas alostéricas, que conseguem manter a velocidade de reação mesmo havendo pequenas flutuações na concentração de substrato, ao mesmo tempo em que respondem de maneira mais uniforme à variação desta concentração (Cornish-Bowden, 1995).

Enzimas alostéricas possuem mais de um sítio ativo e a ligação de uma substância a um dos sítios é influenciada pela ligação de outra substância em outro sítio. A

substância que controla a ligação da outra é chamada de modulador, e no caso de ambas serem iguais, têm-se as interações homotrópicas (Voet e Voet, 1990).

As iterações homotrópicas podem ser explicadas por dois modelos: Modelo de Simetria e Modelo Seqüêncial. O modelo de simetria considera que a proteína alostérica é um oligômero de protômeros simetricamente ligados, sendo que cada protômero tem dois estados conformacionais em equilíbrio, e a ligação da substância ao sítio é considerada como no modelo de chave e fechadura. O modelo seqüencial ("induced fit") considera que a presença de um ligante provoca uma alteração conformacional na proteína facilitando a ligação da outra substância, ocorrendo "cooperação" (Cornish-Bowden, 1995; Voet e Voet, 1990).

A cooperatividade considera a existência de cooperação entre os sítios ativos de enzimas poliméricas, modificando a cinética enzimática. Este fenômeno pode ser modelado utilizando a equação de Hill (eq. 2) (Cornish-Bowden, 1995).

$$v = v'_{max} \cdot \frac{S^h}{K_{0,5}^h + S^h} \qquad \text{(eq. 2)}$$

Sendo:

v = velocidade de reação;

v'_{max}= velocidade limitante da reação);

h = coeficiente de Hill (índice de cooperatividade);

$K_{0,5}$ = concentração de substrato para $v = 0{,}5\ v'_{máx;.}$

3.7.3. Efeito da temperatura na atividade enzimática

A velocidade de uma reação enzimática depende da concentração da enzima ativa, conforme a Equação 3, por sua vez, a constante "k" é influenciada pela temperatura, seguindo Arrhenius (Eq. 4). Logo, a dependência da velocidade de reação com a temperatura pode ser expressa pela Equação 5 (Shuler e Kargi, 2002).

$$v = k \cdot [E] \qquad \text{(Eq.3)}$$

$$k = k_o^* \cdot e^{-\frac{Ea}{RT}} \qquad \text{(Eq. 4)}$$

$$v = v_o \cdot e^{-\frac{Ea}{RT}} \qquad \text{(Eq. 5)}$$

Onde:

k: constante cinética; k^*_o: constante cinética hipotética para uma T infinitamente alta

E_a: energia de ativação; R: constante universal dos gases; T: temperatura absoluta

Por outro lado, a desativação enzimática a temperaturas mais altas pode não ser desprezível. A enzima, sendo uma proteína, esta sujeita à desnaturação, o que implica na perda da atividade. Geralmente esta desnaturação é irreversível, significando a perda definitiva do poder catalisador, sendo ideal o conhecimento das condições ótimas de estabilidade não só para o armazenamento do produto, como também para minimizar a perda durante um processo (Shuler e Kargi, 2002).

A cinética de desnaturação é normalmente descrita como uma equação de 1ª ordem (Eq. 6), muito embora casos mais complexos sejam freqüentes. Considerando-se k_d a constante cinética de desnaturação numa dada temperatura, dependente da temperatura conforme a equação de Arrhenius (Eq. 7) e E_d a energia de desnaturação da enzima, pode-se calcular o tempo de meia vida da enzima ($t_{1/2}$) pela Equação 8.

$$E = E_o \cdot e^{-k_d \cdot t} \qquad \text{(Eq. 6)}$$

$$k_d = k_d^* \cdot e^{-\frac{Ed}{RT}} \qquad \text{(Eq. 7)}$$

$$\frac{E}{E_o} = 0{,}5 = e^{-k_d \cdot t_{1/2}} \qquad \text{(Eq. 8)}$$

Assim sendo, considerando-se a dependência da velocidade de reação com a concentração da enzima ativa e com a temperatura (Eq. 3 a 5), e considerando-se também os efeitos da desnaturação térmica (Eq. 6 a 8), obtém-se uma equação global da dependência da velocidade de reação em relação à temperatura (Eq. 9 e 10) (Shuler e Kargi, 2002).

$$v = k_o^* \cdot E_o \cdot \exp\left[-\frac{E_a}{RT} + t \cdot k_d^* \cdot \exp\left(-\frac{E_d}{RT} \right) \right] \qquad \text{(Eq. 9)}$$

$$v = k_o^* \cdot E_o \cdot \exp\left(-\frac{E_a}{RT} + 0{,}69 \cdot \frac{t}{t_{1/2}} \right) \qquad \text{(Eq. 10)}$$

3.7.4. Outros fatores que influenciam a atividade enzimática

Em geral, enzimas são ativas numa faixa limitada de pH, isto porque a enzima possui vários grupos ionizáveis e mudanças no pH afetam o sítio catalítico e a conformação da enzima, essenciais para a manutenção da capacidade catalítica (Shuler e Kargi, 2002).

Muitos outros fatores podem afetar a atividade enzimática como a força iônica do meio, pressão, o tampão empregado, a pureza dos reagentes e da enzima. Todos estes fatores devem ser experimentalmente determinados ou no mínimo, escolhidos arbitrariamente e mantidos constantes durante estudos (Shuler e Kargi, 2002).

3.8. Identificação de leveduras

Considerando-se a biologia tradicional, as leveduras são caracterizadas, classificadas e identificadas através de características morfológicas e fisiológicas. Para a sua identificação os estudos bioquímicos e de exigências nutricionais são mais relevantes que suas características morfológicas e sexuais. Há diversos métodos/chaves para identificação de leveduras utilizando-se somente testes bioquímicos e algumas poucas características morfológicas.

O método mais simples, que requer o menor número de ensaios, é a "taxonomia numérica" (Numericlature) proposta por Griffths (1981). Nesta forma de identificação fazem-se os testes de: assimilação e fermentação de açúcares, assimilação de nitrato, crescimento em alta concentração osmótica, hidrólise de amido, crescimento a 37^{o}C e teste de forma celular. É atribuído um valor numérico específico a cada um dos 21 testes, de acordo com o resultado observado. Estes 21 testes são agrupados de três em três, somando-se o resultado de cada trinca gerando um código numérico final de identificação da levedura, composto de 7 números. No entanto este método se mostra eficiente para um grupo de 496 espécies, sendo facilmente empregado no controle de fermentações alcoólicas, por exemplo. Mas se o intuito é estudar a gama de leveduras presentes em um certo ambiente natural, ele se mostra restrito e pouco eficaz.

Barnett e Pankhurst (1974) apresentam uma chave de identificação de leveduras, analisando-se a assimilação e fermentação de carboidratos e a assimilação de nitrato.

Além das chaves apresentadas, há também algumas obsevações que visam à facilitação do procedimento, como:

- Se há fermentação de um determinado carboidrato também ocorre a sua assimilação, no entanto o inverso não é verdadeiro;
- Se a levedura não fermenta a glicose, também não fermenta os outros carboidratos;
- Leveduras de cores róseas pertencem aos gêneros *Rhodotorula*, *Rhodosporidium*, *Cryptococcus*, *Sporobolomyces* e *Sporodioboulus*, não fermentam carboidratos.
- Se a levedura assimilar inositol, não fermentar glicose e não produzir hifas/filamentos em agar, ela pertence ao grupo *Cryptococci*;

Outro método de identificação que não requer muitas observações microscópicas é o proposto por Kreger-van Rij (1984): uma chave para espécie e gênero que não leva em consideração as características de reprodução sexual. Nesta chave além dos testes de assimilação e fermentação de carboidratos e assimilação de nitrato, há também observações em relação à reprodução vegetal e à formação de micélios/pseudo-micélios.

No entanto, a forma mais segura é a identificação por métodos moleculares (biologia molecular), apesar de requerer pessoal treinado e equipamentos específicos, resultando em custos elevados. A classificação ideal deve ser baseada na filogenia dos microrganismos e é a interação entre a filogenia e a classificação que nos permite predizer similaridades genéticas, resultando na maior compreensão das leveduras (Kreger-van Rij,1984; Kurtzman, 1988).

3.9. Biodiversidade e microrganismos

O Brasil tem uma área de 8,5 milhões de kilômetros quadrados, possuindo várias zonas climáticas que incluem o trópico úmido, o semi-árido e áreas temperadas. As diferenças climáticas contribuem para as diferenças ecológicas formando zonas biogeográficas distintas (biomas). A variedade de biomas reflete a riqueza da flora e fauna brasileira, tornando-as as mais diversas do mundo. Diversidade biológica, ou biodiversidade inclui a variedade genética dentro das populações e espécies, a variedade de espécies de flora, fauna e de microrganismos, a variedade de funções ecológicas

desempenhadas pelos organismos nos ecossistemas, a variedade de comunidades, habitats e ecossistemas formados pelos organismos.

A composição total da biodiversidade brasileira não é conhecida e talvez nunca venha a ser, sabendo-se, entretanto que o número de espécies ainda não identificadas pode alcançar a ordem de dezena de milhões. Neste contexto é fundamental a implementação de programas na busca de um melhor aproveitamento da biodiversidade brasileira. Mas essa diversidade, ao mesmo tempo em que representa uma excepcional riqueza de patrimônio genético e paisagístico, torna o ecossistema extremamente frágil. A destruição de parcelas, ainda que pequenas, destes ecossistemas pode significar a perda irreversível de inúmeras espécies, por vezes sequer estudadas pela ciência (*Geo Brasil 2002* - IBAMA).

Apesar da grande importância na manutenção da biosfera, estima-se que menos de 5% dos microrganismos existentes na Terra, tenham sido descritos. Isso porque os esforços dos centros de pesquisa estão focados principalmente nos macro-organismos (mamíferos, répteis, anfíbios, aves, peixes e plantas), resultando no conhecimento de 80 a 90% destes seres. No entanto os microrganismos, mesmo exercendo funções vitais nos ecossistemas e na biosfera em geral foram pouco estudados, tanto devido à falta de interesse quanto à dificuldade na pesquisa destes seres invisíveis a olho nu (*Primeiro Relatório Nacional para a Convenção sobre Diversidade Biológica* - Ministério do Meio Ambiente, 1998).

É importante notar também os imensos avanços científicos e econômicos obtidos a partir de estudos de microrganismos, resultando não somente na biotecnologia moderna, como em melhorias na qualidade de vida dos seres humanos em geral.

Com o advento da biologia molecular e outras técnicas avançadas de estudo de microrganismos, a atenção dos pesquisadores está se voltando a estes organismos. Estas pesquisas estão começando a dar uma idéia da grandeza deste mundo desconhecido, sabendo-se que sua diversidade fisiológica, metabólica e genética não encontra paralelo em nenhuma outra classe de seres vivos (Hawksworth, 2002).

3.10. Substratos alternativos (Meios industriais)

Na produção de enzimas por processos biotecnológicos, após a fermentação, dependendo da aplicação, são necessárias operações de purificação que devem ser incluídas no custo final do produto. Para minimizar esses custos, a escolha de métodos de separação e purificação adequados é importante, além do estudo da otimização destes processos, de modo a se obter um bioproduto comercialmente viável.

Melaço é um subproduto da indústria de açúcar e é utilizado como fonte de carbono para alimentação de animais, biofertilizantes e matéria-prima para a indústria de fermentações. Para o uso industrial, o melaço é uma das matérias-primas de menor custo e mais apropriada para processos de fermentação. Mas a grande concentração de metais pesados é a causa de um problema crítico na utilização do melaço em fermentações. A presença desses metais pesados inibe o crescimento de microrganismos, influencia o pH do substrato e está envolvida na inativação de enzimas associadas a biossíntese do produto. Neste contexto, observa-se a importância do estudo de um pré-tratamento de meios de cultura contendo melaço de forma a clarificar o meio sem provocar prejuízos na fermentação, garantindo maior facilidade na recuperação e purificação da enzima (Çalik *et al*., 2001).

O pré-tratamento de melaço de beterraba, com diferentes técnicas a fim de remover metais pesados (Roukas,1998), foi realizado com resinas catiônicas, tratamento com ácido sulfúrico, tratamento com fosfato tricálcico, ferrocianeto de potássio e EDTA, e os resultados mostraram que o tratamento com ácido sulfúrico foi o que gerou a maior produção de pululanas (polímeros de α-D-glucose unidos por ligações 1→4 e 1→6) por *Aureobasidium pullulans*.

Treichel (2004) estudando a produção de inulinase por *Kluyveromyces marxianus* em meio de cultura contendo melaço e água de maceração de milho (AMM), determinou uma metodologia de pré-tratamento do meio de cultura utilizando o carvão ativo Carvonite-ANF, sendo que o uso da metodologia de planejamento experimental permitiu a determinação das melhores condições de tratamento: 8% (p/p) de carvão no tratamento de melaço ou AMM, a 70ºC e 150 rpm por 1 hora.

Na continuação deste estudo utilizando-se um outro tipo de carvão, Carvonite-ANFC, obteve-se uma melhora do processo (em termos econômicos) com a diminuição do tempo, da temperatura e da quantidade de carvão necessária: 4%(p/p) de carvão ativo tipo ANFC, sob agitação de 150 rpm a 60ºC durante 10 minutos (Mendes *et al.*, 2005).

O pré-tratamento do meio industrial, contendo melaço, além de resultar em uma maior produção de lipase de *Geotrichum candidum* NRRL-Y 552, alterou algumas características da enzima, conferindo, por exemplo, uma maior estabilidade térmica (Pozza *et al.*, 2004).

4. CONCLUSÕES GERAIS

A metodologia empregada no isolamento e seleção de leveduras produtoras de frutooligossacarídeos resultou na obtenção de quatro cepas com grande potencial de aplicação: *Candida* sp. LEB-I3; *Rhodotorula* sp. LEB-U5, *Cryptococcus* sp. LEB-V2 e *Rhodotorula* sp. LEB-V10.

As características bioquímicas das enzimas foram determinadas tanto para a atividade de transferência de frutose, quanto para a atividade de hidrolise de sacarose, sendo marcante que a temperatura ótima em todos os casos foi superior que 60ºC (60 – 70ºC) e o pH ótimo menor que 5,0.

Considerando-se a atividade de transferência de frutose, a enzima de *Candida* sp. LEB-I3 seguiu a cinética de Michaellis-Mentem, enquanto que as enzimas de *Rhodotorula* sp. LEB-U5 e *Cryptococcus* sp. LEB-V2 sofreram inibição pelo substrato (sacarose). A enzima de *Rhodotorula* sp. LEB-V10 apresentou uma cinética diferenciada, com um comportamento de “cooperatividade”, normalmente associado às enzimas alostéricas.

A purificação das enzimas utilizando a resina de troca iônica Q-sepharose, resultou em um processo de alto rendimento (60-70%), sendo necessário, entretanto, um estudo mais aprofundado para promover uma maior pureza das amostras.

A síntese de FOS a partir de sacarose mostrou que o microrganismo *Rhodotorula* sp. LEB-V10 foi o único cuja enzima promoveu uma síntese constante de FOS (não sendo notada a hidrólise dos frutooligossacarídeos), sendo observado somente uma diminuição da concentração de GF2, quando há um incremento na produção de GF3 e GF4. Devido

a esta observação, este foi o único microrganismo cujos processos de produção de enzima e de síntese de FOS foram otimizados.

Utilizando-se a metodologia de planejamento experimental foi possível a determinação das melhores condições de fermentação em frascos agitados para *Rhodotorula* sp. LEB-V10: 85-95 $g.l^{-1}$ de água de maceração de milho, 55-70 $g.l^{-1}$ de melaço, 250rpm, a 35°C, pH inicial do meio ajustado a 4,5. Nestas condições obteve-se aproximadamente 100 $FTA.ml^{-1}$ (Unidades de transferência de frutose por mililitro), após 36 horas de fermentação.

A produção de FOS por enzima de *Rhodotorula* sp. LEB-V10 também foi otimizada chegando-se a um rendimento em torno de 55-65%, utilizando-se 6 - 7 $FTA.ml^{-1}$, 47 - 50°C, pH 5,0 e de 30 a 60% de sacarose, utilizando- se como substrato tanto sacarose P.A., quanto açúcar cristal ou melaço.

O alto rendimento da síntese (até 60%) similar ao de processos comerciais e a possibilidade de uso de meios industriais (na produção da enzima) e de açúcar cristal e mesmo melaço (na síntese enzimática) resultam em uma diminuição dos custos de produção. Apesar de todo este trabalho representar uma primeira fase no desenvolvimento de um processo de produção de frutooligossacarídeos por enzimas extracelulares de leveduras e de ainda ser necessário estudos mais aprofundados visando a otimização do processo, os dados indicam a possibilidade de desenvolvimento de um processo economicamente viável.

5. SUGESTÕES PARA TRABALHOS FUTUROS

Apesar dos ótimos resultados obtidos, para podermos determinar a viabilidade de aplicação desta enzima (*Rhodotorula* sp. LEB-V10) em um processo em grande escala, a produção de enzima deve ser mais eficiente, sendo necessário estudos sobre a otimização da produção em fermentadores.

Outro fator que deve ser estudado é a imobilização enzimática, de modo que se possa desenvolver um processo contínuo, com alta estabilidade operacional.

Além disso é necessário um estudo mais aprofundado da recuperação e purificação enzimática, visando a identificação da fração responsável pela síntese dos frutooligossacarídeos e sua caracterização bioquímica.

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PARTE II

ARTIGOS

ARTIGO 1 :

SCREENING OF YEASTS STRAINS FOR TRANSFRUCTOSYLATING ACTIVITY

Seleção de leveduras com alta atividade de transfrutosilação

(Journal of Catalysis B: Enzymatic)

SCREENING OF YEAST STRAINS FOR TRANSFRUCTOSYLATING ACTIVITY

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Abstract

Fructooligosaccharides (FOSs) are functional food ingredients with prebiotic properties, and a recent increase in the use of oligosaccharides in the food industry has led to the search for "new" microorganisms and enzymes for the production of oligosaccharides. This paper focuses on the screening of yeasts obtained from fruits and flowers (from Brazilian tropical forests), and capable of secreting extra-cellular enzymes with high fructosyl transferase activity (FTA). The screening and isolation procedures resulted in 4 potentially interesting yeast strains: *Candida* sp. (LEB-I3), *Rhodotorula* sp. (LEB-U5.), *Cryptococcus* sp. (LEB-V2) and *Rhodotorula* sp. LEB-V10. All were able to produce more then 100 $g.L^{-1}$ of FOS from a 500 $g.L^{-1}$ sucrose solution, but only the last one, (*Rhodotorula* sp. LEB-V10), showed no hydrolytic activity with respect to the FOS produced, giving a continuous increase in FOS content up to the end of the reaction, when it was about 50% of the total carbohydrates.

Key words: Fructooligosaccharides, prebiotics, fructosyl transferase, yeasts, screening

1 Introduction

There is general agreement on the importance of "functional foods" in human health. Fructooligosaccharides (FOSs) are functional food ingredients, showing prebiotic properties. Prebiotic oligosaccharides are defined as "non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of a limited number of bacteria in the colon, such as *Bifidobacterium* and *Lactobacillus* [1].

FOSs can be produced by either invertase (β-fructofuranosidase) or transferases (β-D-fructosyltransferase) [2], and many reports can be found in the literature on the production of FOS by fungi such as *Aspergillus niger [3]*, *Aspergillus japonicus [4]* and *Aureobasidium sp*.[5]. However, few reports can be found on its production by extra cellular yeast enzymes, although studies do exist on *Kluyveromyces* sp. and its inulinase enzyme [6] and on *Rhodotorula* sp., referring to the production of gluco- and galactooligosaccharides [7]. Vranešić at al. [8], Risso [9] and Treichel [10] have done a great number of studies on inulinase and FOS production by *Kluyveromyces* sp, but despite all their efforts and the high enzyme production achieved (1300 $UTF.mL^{-1}$), the maximum yield in FOS was about 12% in aqueous media and 18% in organic solvents.

The search for "new" enzymes for oligosaccharide production, using either microbial screening or molecular engineering, became necessary as a result of the increasing number of applications of oligosaccharides in the cosmetic, agrochemical, pharmaceutical and food industries.

The present study reports on the screening for high transfructosilating enzymes in yeasts strains isolated from fruits and flowers obtained from tropical Brazilian biomass. Tropical environments (forests) are one of the greatest sources of all kinds of living organisms, including yeasts, unicellular fungi with a ubiquitous distribution throughout almost all ecosystems. Brazil shows four regions of great biodiversity: the Amazon Forest; the Atlantic Forest (stretches along the Brazilian coast, showing an extremely diverse and unique mixture of vegetation); the Cerrado (tropical savanna eco-region) and the Pantanal (the world's largest wetland). Since Brazil is also a sucrose producing country (sugar-cane), and considering the increasing demand for FOS as a functional food, it would be interesting to find alternative processes for the production of fructooligosaccharides.

2 Experimental

2.1 Sample collection and yeast isolation

Fresh fruits and flowers were collected from the Amazon and Atlantic Forests and the Cerrado and Pantanal areas of Brazil. The samples collected were inoculated into 125 ml Erlenmeyer flasks containing 25 ml of a standard medium consisting of 2% yeast extract, 5% sucrose, 1% $NaNO_3$, 0.01% $MgSO_4.7H_2O$, 0.1% K_2HPO_4 (pH 5.5) and incubated at 25ºC in a reciprocal shaker (150 rpm) for 72 hours [4].

The cultures were streaked onto plate media (PDA, WLN and Inulin agar), supplemented with nalidixic acid and ampicilin (50 ppm, pharmaceutical grades) for bacterial inhibition.

The WLN agar (Wallerstein Laboratory nutrient agar) is a complex medium that provides easy differentiation of colonies by yeast morphology. It consists of (per litre): 4 g yeast extract; 5 g bactocasitone; 50 g dextrose; 550 mg KH_2PO_4; 125 mg KCl; 250 mg $MgSO_4.7H_2O$; 2.5 mg $FeCl_3$; 2.5 mg $MnSO_4$; 22 mg Bromocresol green and 20 g agar.

Inulin agar medium was used to screen for inulinase producing yeasts, and showed the following composition (per litre): 20 g inulin (Raftline – Orafti); 1 g yeast extract; 3 g $NaNO_3$; 500 mg $MgSO_4.7H_2O$; 200 mg $MnSO_4$; 1 g K_2HPO_4 and 20 g agar.

The pH values of the media were 5.0, and the cultures were grown on the agar plates at 25ºC for 48 to 72 hours. Individual colonies were transferred to PDA plates to avoid contamination and the strains were maintained at 4ºC in GYMP-agar slants (2% glucose; 0.5% yeast extract; 1% malt extract; 0.2% KH_2PO_4 and 2% agar).

2.2 Primary Screening - Enzyme activity determination

Loopfuls of cells (from each strain), sub cultured on GYMP agar slants, were inoculated into 10 mL of standard medium in test tubes and cultivated at 25 ℃ for 24 hours. After adequate development of the cultures, they were transferred into 500 mL flasks (with baffles) containing a further 100 mL of the same medium, and cultivated aerobically at 25 ℃ for 72 hours in a reciprocal s haker (150 rpm).

Samples were collected after 24, 48 and 72 hours, the cells harvested by centrifugation at 6.000 G (10 minutes, 10 ºC), and the cell-free supernatants used to screen for transfructosylating activity.

2.3 Secondary Screening- Oligosaccharide production

Strains shown to be potential producers of fructofuranosidase activity (FA) and transfructosylating activity (FTA) underwent a secondary screening procedure, with monitoring of the oligosaccharide production.

The reaction was performed at 50ºC in 50 mM sodium acetate buffer (pH 5.0) containing 60% (w/v) sucrose with an enzyme: reaction medium (v/v) ratio of 1:10.

Samples were taken at appropriate times during the reaction, inactivated in boiling water and the oligosaccharide composition analysed by ion chromatography (HPLC-PAD)

2.4 Strain genus identification procedures

The genera were identified by the methodology described in "Key to specimen and genera" by Kregger-van-Rij [11].

2.5 Prebiotic effect

The growth of *Bifidobacterium longum* (BL-04, Rhodia) using the FOS produced as the carbon source, was studied by preparing a modified MRS medium containing 0.05% L-cysteine and about 0.20% carbohydrate (Glucose or FOS). Strain development was monitored from the optical density at 595 nm (OD) and by carbohydrate consumption (HPLC-PAD). Growth on glucose was considered as the positive control and growth on MRS with no carbohydrate source (basal MRS) as the negative control.

2.6 Enzyme properties

As a guide for future studies on the enzyme kinetics and mechanisms some properties of the extra-cellular enzymes were studied. Thus, the enzyme activities (initial reaction rate) with substrates of 2% and 50 % sucrose and 2% inulin in 50 mM sodium acetate buffer (pH 4.5) at 50ºC were measured. Oligosaccharides were produced by incubating the enzyme (1 FTA.ml^{-1}) in 0.5%, 5% and 50% sucrose (sodium acetate buffer 50 mM, pH 4.5), at 50ºC for 72 hours.

The enzyme was concentrated by precipitation from a cell free culture broth, using ethanol at 4ºC to the required concentration. Precipitation with 70% ethanol and a two-step fractional precipitation (first adding ethanol to 50% saturation, centrifuging, and then adding more ethanol up to 70%) were tested. The cell-free supernatant and the enzyme rich precipitate were collected by centrifugation (6.000 G, 4ºC, 10 min.). Both the enzyme activities and protein concentration were analysed (Lowry, 1951).

2.7 Enzyme activity assay

The reaction medium to determine enzyme activity consisted of 50% sucrose in 50 mM sodium acetate buffer (pH 4.5) and a 10% adequately diluted enzyme suspension, incubated at 50 ºC. Samples were collected at constant time intervals for 30 minutes and assayed for glucose (glucose-oxidase commercial kit) and reducing sugars (Somogi-Nelson method).

Sucrose conversion by fructofuranosidase yields glucose and fructose. However in the presence of fructosyl transferase activity, part of the fructose is built into a fructan polymer, so it is possible to measure both the hydrolytic and transfructosylating activities from the amounts of glucose and reducing sugars released into the reaction media [4]. The activities can be determined from the equations below (eq.1) after measuring the amounts of glucose (G) and reducing sugars (R) in the reaction media (F = fructose, F' = transferred fructose):

$$R = G + F \Leftrightarrow F = R - G$$

$$F' = G - F \Leftrightarrow F' = 2\,G - R \qquad \text{(eq.1)}$$

One unit of fructofuranosidase activity (FA) is defined as the amount of enzyme required to hydrolyse 1 µmol of sucrose per minute. One unit of transfructosylating activity (FTA) is defined as the amount of enzyme required to transfer one µmol of fructose per minute.

2.8 Chromatographic analysis for FOS

Identification and quantification of the oligosaccharides was achieved by ion exchange chromatography with a pulsed amperometric detector (HPLC-PAD). Chromatography was performed on a Carbopac PA-100 column at 22 - 24 ºC, using a GP50 gradient pump, ED40 electrochemical detector and the software PEAKNET, all from DIONEX (USA). The sugars were eluted in 50 mM sodium hydroxide with a linear gradient of sodium acetate (0 to 200 mM), at a flow rate of 1 $ml.min^{-1}$.

3 Results and Discussion

3.1 Yeast selection

The isolation procedure resulted in 495 yeast strains. The first step in the selection of the interesting yeast strains was the determination of their extra-cellular fructofuranosidase and transferase activities (on sucrose). About 25% of the isolated strains had some kind of activity on sucrose (*Primary screening*), resulting in 130 strains for further testing. Figure 1 shows the distribution of the yeast strains within the collection areas and the results of the primary screening procedure.

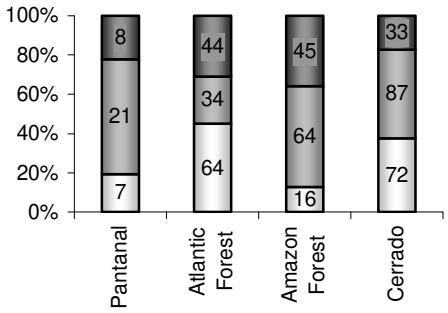

Figure 1: Data from the isolation and primary screening procedures
■ Strains with FTA activity; ■ Strains with FA activity; □ Strains without activity over sucrose

After the quantitative analysis of the enzyme activity and the qualitative analysis of the oligosaccharide production (*Secondary screening*), 4 strains showing potential for FOS production were selected. After the qualitative analysis of oligosaccharide production, a synthesis reaction was carried out to determine the FOS content (Figure 2). All the enzymes studied produced the fructo-oligosaccharide 1-kestose (GF2). On the other hand, the nystose (GF3) and fructofuranosyl-nystose (GF4) contents were only important in the syntheses performed by the LEB-V10 and LEB-U5 enzymes.

The genera of these microorganisms were, in sequence, *Candida* (LEB-I3 strain), *Rhodotorula* (LEB-U5 and LEB-V10 strains) and *Cryptococcus a* (LEB-V2 strain).

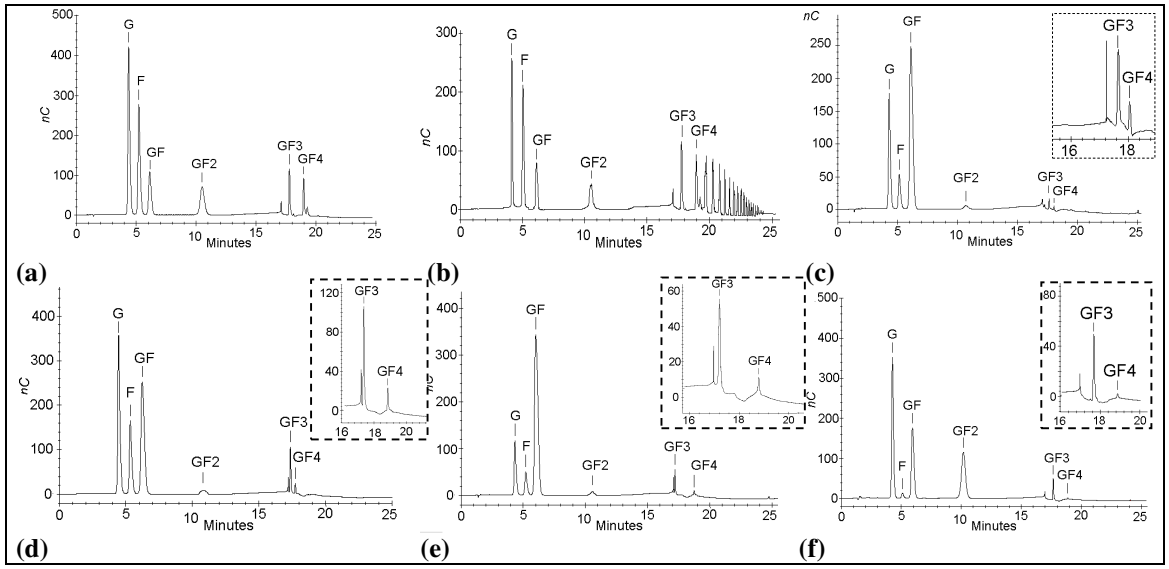

Figure 2: FOS analysis by HPLC-PAD after 10 hours of reaction (Secondary screening)
(a) FOS standard sample; (b) Inulin sample; (c) LEB-I3; (d) LEB-U5; (e) LEB-V2; (f) LEB-V10

3.2 Enzyme production and concentration

The production assays were performed in shaken flasks and the results obtained for the FA and FTA activities of the selected strains during fermentation shown in Figure 3. The strains I3 and V10 showed remarkable FTA activities and, interestingly, low FA activities.

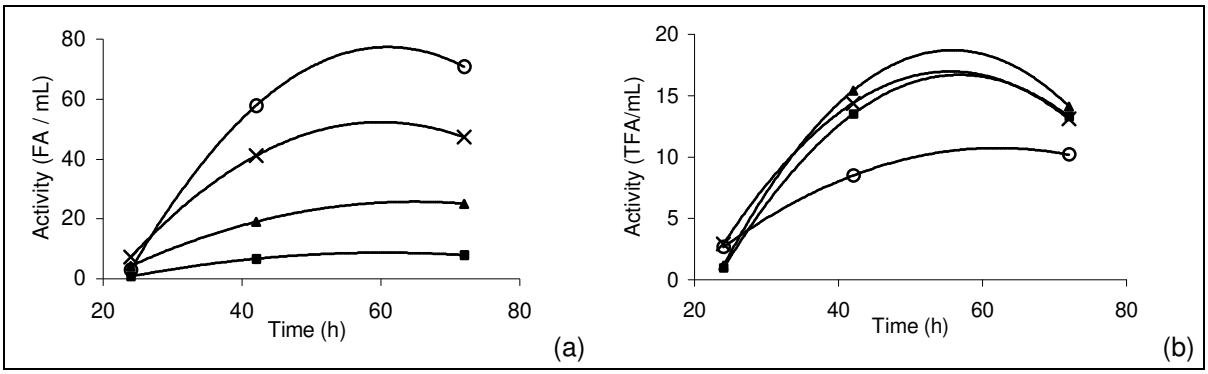

Figure 3: Enzyme production by different strains during fermentation:
(a) FA and (b) FTA activity (■ LEB-I3; × LEB-U5; O LEB-V2; ▲ LEB-V10)

The enzymes were recovered from the culture broth and concentrated by adding ethanol to the required concentration, as shown on Table 1. Attempts at fractional precipitation gave poor results, since the loss of activity was considerable, although low protein concentrations were achieved. In the case of single step precipitation with 70% ethanol saturation, the protein content was almost the same as in the crude broth, but the loss of enzyme activity was significantly decreased, except for the enzyme from I3, where yields of only about 30% were recovered.

Table 1: Enzyme recovery from the cell-free broth

Enzyme	Concentration step	Enzyme (FTA)	Protein (mg)	Specific Activity (FTA/mg)	Purification factor	Yield (%)
I3	Crude broth	480	1178	0.4	1.0	100
	one step*	140±4	927±3	0.2	0.4	29
	two steps**	55±5	48±2	1.1	2.8	11
U5	Crude broth	510	654	0.8	1.0	100
	one step*	484±5	614±4	0.8	1.0	95
	two steps**	91±8	26±1	3.5	4.6	18
V2	Crude broth	450	1354	0.3	1.0	100
	one step*	414±4	1298±4	0.3	1.0	92
	two steps**	45±6	105±5	0.4	1.3	10
V10	Crude broth	910	1561	0.6	1.0	100
	one step*	546±6	1445±3	0.4	0.6	60
	two steps**	119±7	214±5	0.6	1.0	13

*final ethanol concentration of 70%

**first step: final ethanol concentration of 40% and second step: ethanol up to 70%.

3.3 Enzyme properties

In order to assess what type of enzyme we were dealing with, fructofuranosidase or fructosyl transferase, a series of experiments were carried out involving sucrose conversion and FOS production. Considering the enzyme activities with 2% and 50% sucrose and with 2% inulin 2 (Table 2), and analysing for oligosaccharide production (Figure 4), it was possible to determine important enzyme characteristics. As can be seen in Table 2, all the enzymes showed higher fructofuranosidase activities with 2% sucrose and some inhibitory effects could be noticed with 50% sucrose.

Hidaka *et al*. [12] showed that the greater the ratio between the fructosyl transferase and hydrolytic activities, the greater the production of FOS, and only enzymes with high ratios between the activities were able to produce FOS at low sucrose concentration (5.0 and 0.5%). They also showed that enzymes with lower ratios between the activities, had hydrolytic activity for the FOS, and consequently all the fructooligosaccharide produced during the first 20 hours of reaction was hydrolysed by the end of the reaction period. At

low sucrose concentrations, transfructosylating activity is more common in the transferase type enzymes, although fructofuranosidase enzymes can also produce FOS under these conditions if there is an adequate transglycosilation mechanism present (requiring an efficient accepting molecule).

Table 2: Enzyme activities with different substrates

Enzymes	50% Sucrose			2% Sucrose			2% Inulin
	FTA	FA	$^{FTA}/_{FA}$	FTA	FA	$^{FTA}/_{FA}$	U_F
I3	16.5±0.8	2.6±0.5	6.3	3.0±0.5	4.7±0.4	0.6	0.6±0.2
U5	6.4±0.7	2.8±1.2	2.3	2.6±1.4	2.9±1.5	0.9	0.5±0.3
V2	13.6±1.0	3.8±1.4	3.6	7.8±1.7	8.2±1.4	1.0	0.3±0.2
V10	6.9±0.5	0.9±0.4	7.7	1.7±0.2	2.9±0.2	0.6	0.3±0.1

The U5 enzyme maintained almost the same fructofuranosidase activity at both sucrose concentrations, but showed higher transfructosylating activity at 50% sucrose. On the other hand, the V10 enzyme showed low FTA activity on 2% sucrose, but at 50% sucrose concentrations the FTA activity was almost 5 times the FA activity, so although this enzyme shows some fructofuranosidase characteristics, it may have a very particular kinetic behaviour.

Other valuable information was that no enzyme showed remarkable inulin hydrolysing activity, so they could not be characterized as inulinase enzymes. At 50% sucrose concentration, the V2 enzyme showed high transfructosylating activity, but at low sucrose concentration, the two kinds of activity were equilibrated.

During the synthesis of fructooligosaccharides from 0.5%, 5% and 50% sucrose solutions, the changes in carbohydrate composition were followed for 72 hours of reaction (Figure 4, 5, 6 and 7). As the reaction progressed, and sucrose was converted into FOS, the glucose and fructose concentrations increased.

All four enzymes produced FOS (from 5% and 50% sucrose solution), but the enzymes I3, U5 and V2 showed a remarkable degree of hydrolysis of the FOS produced, after 24 hours of reaction. The FOS content, considering the synthesis from 50% sucrose, reached almost 40% of the total carbohydrates within 48 hours for enzyme I3, and the

enzymes U5 and V2 showed the same behaviour with respect to FOS production, reaching maximum concentrations of 30 and 22%, respectively, after 24 hours. Observing the oligosaccharide contents during the course of the reactions, the enzyme V10 was the only one that showed a continuous production of FOS, with considerable amounts of GF3 and GF4.

At 0.5% sucrose solutions, all experiments led to the complete hydrolysis of sucrose, producing only glucose and fructose. The use of reaction media containing 5% sucrose yielded 14-25% FOS, showing the high fructosyl transferase activity. On basis of the analysis of the enzyme activities and the FOS production with different sucrose concentration, and considering that β-D-fructosyltransferase only possesses transfructosylating activity [4], the results suggest that although the high transfructosylating activity, all four enzymes should therefore be classified as β-fructofuranosidase.

At commercial scale, FOS production is made with immobilized cells from *Aspergillus niger* and *Aureobasidium pullulans* on 60-70% sucrose solution, resulting in 55-60% FOS yield after 24 hours of reaction [13]. The comparison of our data and the literature best results, shows that although the process was not optimised and up-scaled, the FOS production and yield from these fours yeasts strains, despite the long reaction time needed to reach it, are very similar to the commercial production.

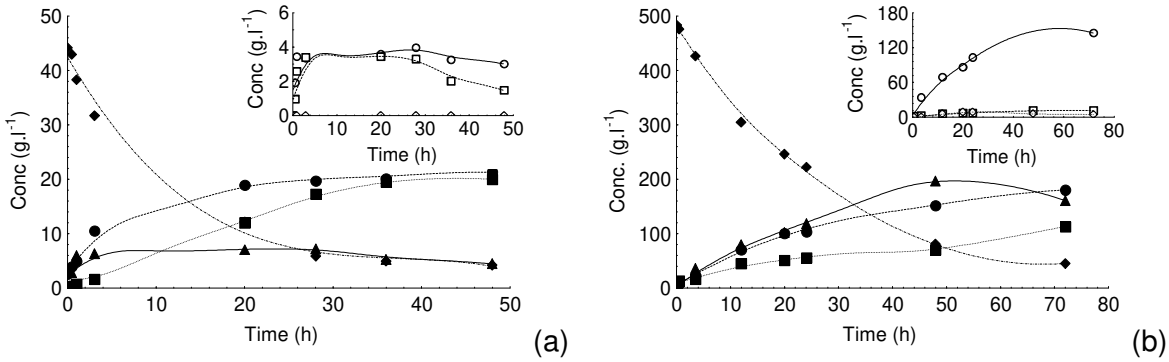

Figure 4: FOS production by the LEB-I3 enzyme (a) 5 % sucrose; (b) 50 % sucrose
◆ sucrose, ■ fructose, ● glucose, ▲FOS, ○ GF2, □ GF3, ◇ GF4

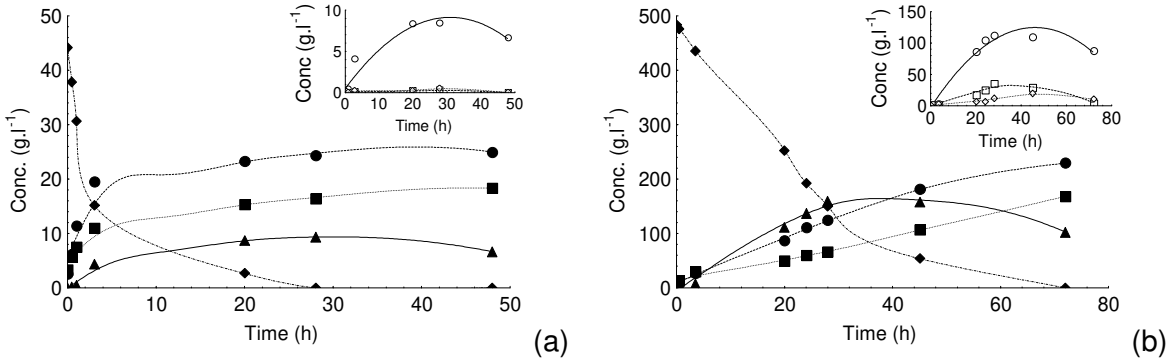

Figure 5: FOS production by the LEB-U5 enzyme (a) 5 % sucrose; (b) 50 % sucrose
◆ sucrose, ■ fructose, ● glucose, ▲FOS, ○ GF2, □ GF3, ◇ GF4

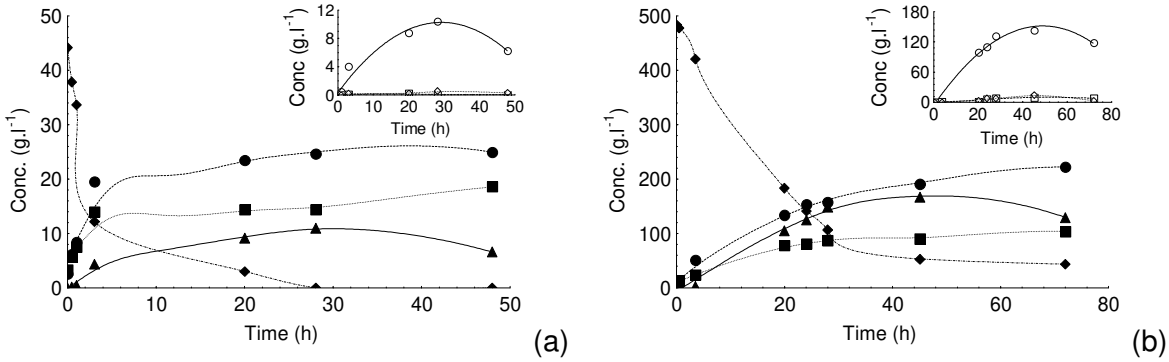

Figure 6: FOS production by the LEB-V2 enzyme (a) 5 % sucrose; (b) 50 % sucrose
◆ sucrose, ■ fructose, ● glucose, ▲FOS, ○ GF2, □ GF3, ◇ GF4

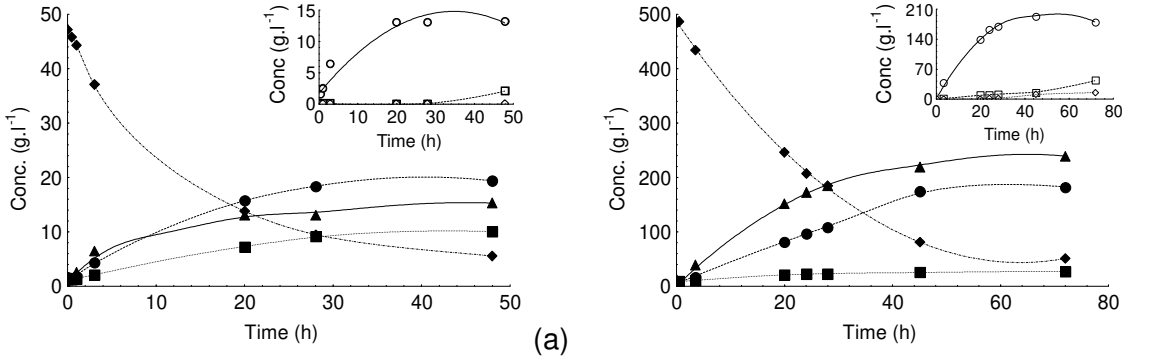

Figure 7: FOS production by the LEB-V10 enzyme (a) 5 % sucrose; (b) 50 % sucrose
◆ sucrose, ■ fructose, ● glucose, ▲FOS, ○ GF2, □ GF3, ◇ GF4

3.4 Prebiotic effect

To study the prebiotic effect of the oligosaccharides synthesized, the growth of a probiotic bacterium, *Bifidobacterium longum* was tested (in duplicate). Figure 8 shows how the microorganism grew on the MRS-FOS and MRS-G media and how much of the substrate was consumed. After sterilization and adequate dilution, the carbohydrate composition of the FOS culture medium was (per litre): LEB *I3* – 11.8 g sucrose; 1 g glucose; 1 g fructose; 11 g FOS; LEB *U5* – 4 g glucose; 3.6 g fructose; 15.3 g FOS; LEB *V2* – 8.3 g sucrose; 3.3 g glucose; 2.5 g fructose; 14.2 g FOS; LEB *V10* – 4.9 g glucose; 4.3 g fructose; 16 g FOS.

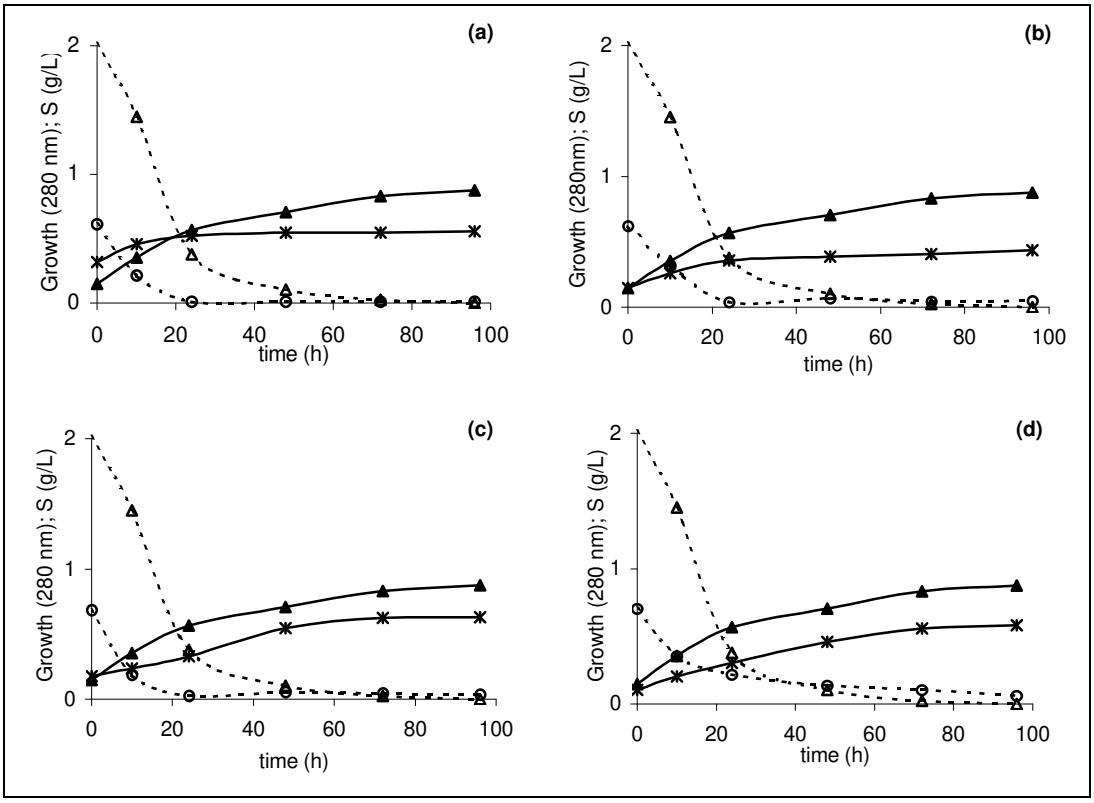

Figure 8: Time course of growth and substrate (S) consumption by *Bifidobacterium longum*
(a): MRS-FOS-I3; (b) : MRS-FOS-U5; (c): MRS-FOS-V2; (d): MRS-FOS-V10
▲ - Growth on MRS-glucose; △ - Glucose Consumption;
∗- Growth on MRS-FOS; ○ - FOS Consumption

Development of the probiotic bacterium on the glucose medium resulted in total consumption of the substrate after 60 hours of fermentation. For the medium containing fructooligosaccharides from the strains LEB-I3, LEB-U5 and LEB-V2, the oligosaccharides were consumed before 30 hours of fermentation. The LEB-V10 FOS consumption rate

was slower than that of the other strains, however it had been completely vanished after 80 hours of fermentation.

FOS has been demonstrated as prebiotic ingredient trough in vivo and in vitro assessments, studying the development of probiotic bacteria and the metabolism effect of both pure and commercial FOS [14,15]. Showing that glucose is the preferred substrate for growth, but FOS is equally a good substrate in supporting growth, although the use of each fructooligosaccharide is strain sensitive.

4 Conclusions

The results of this study showed that a great source of microorganisms is present in our environment, with the possibility of screening for several biotechnological uses. The screening and isolation procedures for extra cellular transfructosylating yeast activity (FTA) resulted in 4 yeast strains with great potentials for application. Although the enzymes from the strains LEB-I3 (*Candida sp.*), LEB-U5 (*Rhodotorula sp.*) and LEB-V2 (*Cryptococcus sp.*) showed high hydrolytic activity as well, a study of their biochemical characteristics might determine one condition where the FOS content was optimum. The efforts made to screen for high extra-cellular transfructosylating enzyme producing yeasts gave very promising results. The production of fructooligosaccharides by the LEB-V10 enzyme (another *Rhodotorula sp.*) was the most successful of the four, showing a continuous increase in FOS concentration up to the end of the synthesis reaction, when it was about 50% of the total carbohydrate content.

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Acknowledgments

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ARTIGO 2 :

SYNTHESIS OF PREBIOTIC FRUCTOOLIGOSACCHARIDES USING EXTRACELLULAR ENZYMES FROM RHODOTORULA SP

Síntese de frutooligossacarídeos prebioticos por enzima extracelular de *Rhodotorula* sp.

(Applied Microbiology and Biotechnology)

SYNTHESIS OF PREBIOTIC FRUCTOOLIGOSACCHARIDES USING EXTRACELLULAR ENZYMES FROM *RHODOTORULA* SP

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Abstract

Considering the great biodiversity of the still unexploited Brazilian environment, efforts were made to select microorganisms capable of producing fructooligosaccharide (FOS). Amongst a small number of interesting selected yeast strains, one in particular, identified as a Rhodotorula red yeast strain, isolated from flowers from the Brazilian tropical biomass, was able to produce an effective thermo-stable extra cellular enzyme, with both sucrose hydrolytic and transfructosylating activities. The best operational conditions for this enzyme were determined to be in a pH range from 3.8 to 4.3 and at temperatures of around 65°C. Under these conditions, the transfructosylating activity reached its maximum, being more then 10 times higher then the hydrolytic activity. The synthesis of fructooligosaccharides from sucrose resulted in total conversion of the substrate into FOS (48%), glucose (31.3%) and fructose (20.7%). In addition it was shown that the synthesized FOS had a prebiotic effect, promoting the growth of probiotic bacteria, such as Lactobacillus sp and Bifidobacterium sp.

1 Introduction

Since the 1980s there has been general agreement about the importance of gastro-intestinal microflora in the health status, for both humans and animals. In response to the increasing demand for healthier foods, a great number of functional foods have been developed, mainly in the probiotic category - “mono or mixed cultures of live microorganisms which, when consumed by animal or man, beneficially affect the host by

improving the properties of the indigenous microflora" (Havenaar et al., 1992) or prebiotic category – "a non digestible food ingredient that beneficially affects the host by selectively stimulating the growth and / or activity of one or a limited number of bacteria in the colon, thus improving host health" (Gibson and Roberfroid, 1995).

Due to the poor survival of probiotics taken by oral administration and processing difficulties such as low concentrations in the final product and undesirable tastes, the addition of prebiotics to a variety of foods (dairy, bakery, etc.) is much more common. Most prebiotics are carbohydrates with a taste similar to that of sucrose, but less sweet, the best known being fructooligosaccharides (FOS), glucooligosaccharides (GOS), galactooligosaccharides and oligosaccharides from soybean (Tuohy et al., 2003). Both FOS (polymers having one to three fructose units bound to the β-2,1 position of sucrose) and fructan polymers (inulin), occur in many plants as carbohydrate reserve materials, such as in oat, agave, onion, garlic, banana, asparagus, dahlia tubers, Jerusalem artichokes and chicory, the latter being used in the commercial production of inulin.

Fructooligosaccharides with a low degree of polymerization (D.P. 2 ~ 4) can be produced by the action of enzymes with transfructosylating activity (fructofuranosidase or transferases) in concentrated sucrose solutions, or by the enzymatic hydrolysis of inulin, using inulinase from *Kluyveromyces* sp. or *Aspergillus* sp. (Roberfroid, 2000).

A great number of organisms have been studied since the 50s for the production of FOS, such as *Aspergillus oryzae*, *Claviceps purpurea* and *Fusarium oxysporium*. However, it was not until the late 80s and early 90s that these microorganisms or their enzymes were applied in large-scale productions, as in the case of *Aspergillus niger*, *A. phoenicus* and *Aureobasidium pullulans* (Yun, 1996).

Although the process with fungal enzymes has been well studied and already scaled-up, these microorganisms mainly show high intracellular activity, and the process is carried out using immobilized cells, which is a complex industrial process. Despite the superiority of immobilized enzyme columns, it was found that the stability of these enzymes was less than half that of the cells (Shin et al., 2004, Yun, 1996).

From an economic point of view, yeasts are the most important of the microorganisms exploited by men, with many technical advantages over filamentous fungi, such as shorter fermentations, high cell concentration cultures and cell recycling, amongst

others. In this work, an evaluation of the production of FOS by an extra cellular fructosyltransferase from *Rhodotorula* sp. yeast, isolated from flowers found in the tropical Brazilian biomass, was carried out.

2 Material and Methods

2.1 Microorganism and maintenance

Rhodotula sp. was isolated from flowers from the Brazilian biota. The working culture was maintained at 4℃ in GYMP agar slants containin g (per liter): 20 g glucose, 5 g yeast extract, 10 g malt extract, 2 g KH_2PO_4 and 20 g agar (pH 5.5).

2.2 Enzyme production

The medium for the inoculation and fermentation of *Rhodotorula* sp., contained (per litre): 50 g sucrose, 20 g yeast extract, 10 g $NaNO_3$, 0.5 g $MgSO_4 \cdot 7H_2O$ and 5 g K_2HPO_4 (pH 5.0). The submerged cultures were grown in 500 mL flasks containing 100 ml standard medium (10% inoculum) at 30 ℃ for 24 - 48 hours and 150 rpm.

2.3 Enzyme recovery

The culture broth was harvested by centrifugation (6000 G, 10 min, 5℃). The clear supernatant was used as the extra cellular enzyme source, and concentrated by adding 95% ethanol to 70% saturation (0 to 4 ℃). The enzy me-rich precipitate was collected by centrifugation (6000G, 15 min., 2℃), re-suspended in sodium acetate buffer (50 mM, pH 4.5) and maintained in the freezer (-18℃).

2.4 Enzyme assay

The reaction media used to determine enzyme activity consisted of 50% (w/v) sucrose (in 50 mM sodium acetate buffer, pH 4.5) and 10% (v/v) of an adequately diluted enzyme suspension at 50 ºC. Samples were collected at constant time intervals up to 30 minutes of reaction time. Glucose was determined using commercial glucose-oxidase kits and reducing sugars by the Somogi-Nelson method (Chen & Liu, 1996). The following equation (eq.1) was used for the sugar determinations, where G, R, F and F´ are the glucose, reducing sugar, fructose and transferred fructose concentrations, respectively.

$$R = G + F \Leftrightarrow F = R - G \quad \Rightarrow \quad F' = G - F \Leftrightarrow F' = 2\,G - R \qquad \text{(eq.1)}$$

One unit of fructofuranosidase activity (FA) was defined as the amount of enzyme required to hydrolyse 1 µmol of sucrose per minute. One unit of transfructosylating activity

(FTA) was defined as the amount of enzyme required to transfer one µmol of fructose per minute.

2.5 Determination of optimum pH and temperature for enzyme activity

A central composite rotatable design (CCRD) of experiments was used to obtain the optimal pH and temperature for enzyme activity. Table 1 shows the values of the coded levels used in the design. The reaction media used to determine enzyme activity consisted of 50% (w/v) sucrose (in 50 mM sodium acetate buffer) and 10% (v/v) of an adequately diluted enzyme.

Factorial design and response surface analysis are important tools to determine the optimal process conditions. Factorial design is advantageous compared to the conventional method, which handles a single parameter per trial. The conventional method does not consider the effect of possible interactions between factors, very important in biological and biochemical process and when it is used substrates as agro-industrial by-products and surpluses (Kalil et al., 2000).

Table 1: Definition and levels of the independent factors in the CCD

Independent factors	Coded Levels				
	-1.41 (-α)	-1	0	+1	+ 1.41 (+α)
pH	3.3	3.5	4.0	4.5	4.7
Temperature - T (℃)	58	60	65	70	72

2.6 Fructooligosaccharide production

The oligosaccharides were produced by incubating 1 FTA ml^{-1} of the enzyme with a 50% sucrose solution in 50 mM sodium acetate buffer (pH 4.5), at 50℃, for 72 hours. The samples were analysed for their carbohydrate composition by high performance liquid chromatography with pulsed amperometric detection (HPLC-PAD), using a Dionex equipment (DIONEX – USA) and a Carbopac PA-100 column, equilibrated with 50 mM NaOH and eluted with 500 mM sodium acetate in 50 mM NaOH (1 ml min^{-1}).

2.7 Assay for the consumption of the synthesized FOS by probiotic bacteria

Modified MRS media containing 0.05% L-cysteine and 0.25% carbohydrates (glucose, glucose + fructose + sucrose or FOS) were prepared. A total of 11 probiotic strains were studied, each one previously reactivated in Glucose-MRS. The development

of each strain was monitored from the optical density at 595 nm (OD), pH values and the carbohydrate concentration (chromatographically). Growth on glucose was considered as the positive control, and growth on MRS with no carbohydrate source (basal MRS) as the negative control. The pH of the culture media was adjusted to 6.2 prior to sterilization at 121°C for 15 min. Cysteine was sterilized by filtration and added to the media just before inoculation. All assays were run at least twice.

2.8 *In vitro* digestibility of oligosaccharides

All digestion assays were run in triplicate and performed for each of the following oligosaccharides: synthesized FOS, dextran (4~6 KDa – Sigma), inulin ("Raftline"–Orafti - Belgium) and commercial FOS (Wako Pure Chemical Industries – Japan).

For all digestion experiments, 1% oligosaccharide solution was incubated at 37°C with solutions simulating the main digestion enzyme solutions: saliva, gastric and pancreatic "juice". The samples were heated in boiling water for 5 minutes to stop the digestion reactions. The solutions were centrifuged at 6000 x G, the pH of the supernatant adjusted to 7.0 and then filtered (0.45 μm).

The oligosaccharide solutions were first incubated in an artificial saliva solution containing a final concentration of 3.25% (w/v) fungal α-amylase (*Aspergillus niger*), 0.25% invertase (Baker's yeast) and 0.25% lysozyme (egg white – Boehringer Mannheim). HCL was added after 30 minutes of reaction in order to reach pH 1.2, and the solution was subsequently incubated with the artificial gastric juice containing (per mL): 0.15 mg cellulase (*Aspergillus niger*), 3.25 mg bile salts (sodium cholate and sodium deoxycholate) and 8.25 mg porcine pancreatin. After 2 hours, the pH was adjusted to 8.0 and the last digestion procedure carried out by incubating the FOS solution with a complex digestive juice containing (per mL): 0.71 mg dimetocone, 0.60 mg dehydrocholic acid, 0.47 mg amylase (*Bacillus* sp.), 0.24 mg pepsin (cattle stomach), 0.47 mg pancreatic porcine lipase, 0.71 mg cellulase (*Aspergillus niger*) and 0.95 mg porcine pancreatin. After two hours of reaction, the pH was adjusted to 6.0 and the reaction stopped by heating the samples in boiling water for 5 minutes.

3 Results

3.1 Enzyme production

Enzyme production was followed during 72 hours of incubation, although the fermentation time required to reach maximum enzyme production was 48 hours for both the hydrolytic (fructofuranosidase) and transfructosylating (fructosyl transferase) activities, as shown in Figure 1.

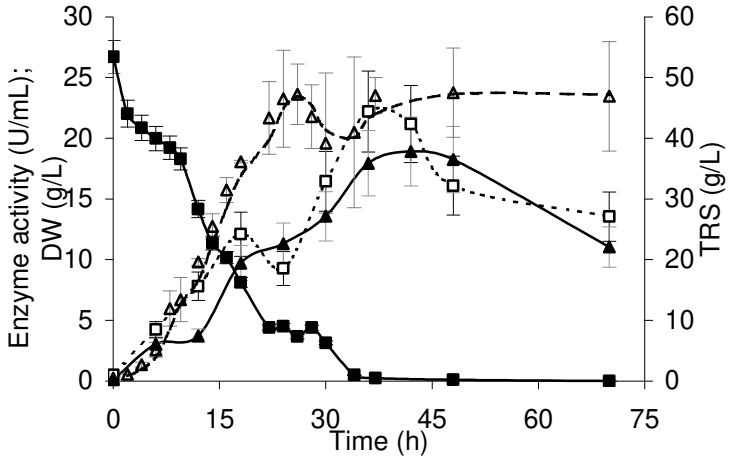

Figure 1: Enzyme production by *Rhodotorula* sp.
Transfructosilating activity - FTA(▲); Hydrolytic activity - FA(□);
Total reducing sugar - TRS(■); Dry weight - DW(△)

3.2 Optimum enzyme conditions

The experimental conditions and the results for FA and FTA activity, in the factorial design, are shown in Table 2. Both types of enzyme activity were analysed in order to obtain high FOS production, since for this purpose, the enzyme requires high fructosyl transferase activity and low hydrolytic activity.

An estimate of the main effect is obtained by evaluating the difference in process performance caused by a change from the low (-1) to the high (+1) level of the corresponding factor (Haaland, 1989). The statistical parameters t-test and p-value were used to confirm the significance of studied factors, in this case, $p<0.05$ suggested significance at the 0.05 level, or a 95% confidence level.

All factors had a statistically significant effect on fructofuranosidase (FA) activity. The analysis of variance (ANOVA), shown in Table 3, did not validate the mathematical model, since the residual value was high and hence the F-test value was small. Nevertheless, it

was still of interest to analyse the effect of each factor on the fructofuranosidase activity (Figure 2), where it can be seen that all the factors, except for temperature (linear factor), were negative. These results mean that an increase in pH from 3.3 to 4.7 decreased the enzyme activity.

Table 2: Experimental design and results of the 2^2 central composite design

Trial	Coded levels (real values)		Enzyme activity (U. ml^{-1})	
	pH	T (℃)	FA	FTA
1	-1 (3.5)	-1 (60)	40.31	52.68
2	+1 (4.5)	-1 (60)	23.02	30.02
3	-1 (3.5)	+1 (70)	53.92	149.86
4	+1 (4.5)	+1 (70)	15.49	52.20
5	-α (3.3)	0 (65)	95.85	101.64
6	+α (4.7)	0 (65)	85.24	212.05
7	0 (4.0)	-α (58)	19.94	30.47
8	0 (4.0)	+α (72)	51.18	67.46
9	0 (4.0)	0 (65)	83.70	339.08
10	0 (4.0)	0 (65)	81.90	349.37
11	0 (4.0)	0 (65)	81.90	345.19

[a] $\alpha = \sqrt{2} = 1.41$

Table 3: Analysis of variance for fructofuranosidase activity (ANOVA)

	SS	df	MS	F[b]
Regression (R)	6679.99	5	1336.00	3.11
Residual[a] (r)	2150.12	5	430.02	
Lack of fit	2147.96	3		
Pure Error	2.16	2		
Total	8830.11	10		

[a] Residual = Lack of fit + Pure error; [b] $F_{0.05;5;5}$= 5.05; R^2 = 0.76

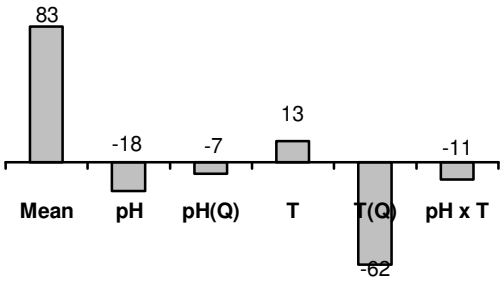

Figure 2: Effect of each factor on FA

For transfructosylating activity (FTA), the factor pH x temperature was not statistically significant at 90% of confidence. The analysis of variance (Table 4), showed good performance in the F-test and a high correlation coefficient, and made it possible to use the data to construct a coded mathematical model (Equation 2), describing how the temperature and pH affected transfructosylating activity.

Table 4: Analysis of variance (ANOVA) for transfructosylating activity

	SS	df	MS	F[b]
Regression (R)	161591.37	4	40397.84	19.96
Residual [a](r)	12146.54	6	2024.42	
Lack of fit	12092.98	4	3023.25	
Pure Error	53.56	2	26.78	
Total	173737.91	10	17373.79	

[a] Residual = Lack of fit + Pure error; [b] $F_{0.10;4;6}= 4.53$; $R^2 = 0.93$

$$FTA \cdot ml^{-1} = 344.67\text{-}102.09 \cdot pH^2 + 21.50 \cdot T - 156.35 \cdot T^2 - 18.75 \cdot pH \cdot T \qquad \text{(eq. 2)}$$

The response surface and contour plot for this experimental design are shown in Figure 3. It can be seen that the optimal condition for transfructosylating activity is around the central point. The temperature required for maximum reaction rate is between 63 and 67 ºC with a pH value between 3.8 and 4.3.

The synthesis of FOS by this enzyme under these optimised conditions, resulted in 48% conversion of sucrose to oligosaccharides. In 72 hours, the initial solution containing 500 g L^{-1} sucrose was converted into a solution containing (per litre): 103.4 g fructose, 156.7 g glucose, 240.0 g FOS (94% kestose, 1.5% nystose and 4.5% fructosylnystose).

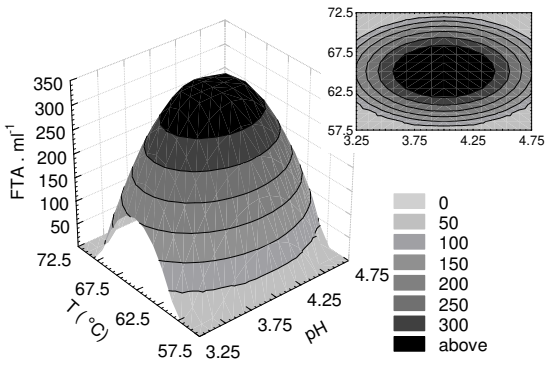

Figure3: Response surface and contour plot from the experimental design for transfructosylating activity (FTA.ml^{-1}) as a function of pH and temperature

3.3 Prebiotic effect

The prebiotic effect is another important piece of information about FOS, and one way of evaluating this is by studying the growth of probiotic bacteria in a particular synthesized FOS solution. Table 5 shows the microorganisms used in this test, how they grew in the MRS-FOS medium as compared to their growth in the MRS-basal and MRS-G (glucose) media, and the amount of FOS consumed. The composition of the culture medium, after sterilization at 121℃ (15 min) and a ddition of the FOS solution, was (per litre): 4.9 g glucose, 4.3 g fructose and 16 g FOS.

Some bacteria grew well but with poor FOS consumption, due to the presence of other carbon sources in the MRS basal and in the FOS solution. However, the opposite behaviour, that is, poor growth but with good FOS consumption, as in the case of *Bifidobacterium bifidum,* could only be explained by carrying out a pertinent metabolic study for these strains.

The FOS produced failed to show a beneficial effect on the growth of the B-548, B-4240, B-1917 and B 14171 strains. The best effect was observed with the strains B-4495, B-4564, B-1976 and BL-04, well-known probiotic bacteria belonging to the *Lactobacilli* and *Bifidobacteria* genera.

Table 5: Effect of synthesized FOS on the development of probiotic bacteria

Microorganisms	Code	Growth [a] (595 nm)	Consumption FOS (%)	Final pH
Lactobacillus delbrueckii subsp. *bulgaricus*	NRRL: B-548	- (0.06)	67	5.9
Lactobacillus acidophilus	NRRL: B-4495	++ (0.39)	85	5.3
Lactobacillus gasseri	NRRL: B-4240	- (0.06)	38	5.4
Lactobacillus casei subsp. *Casei*	NRRL: B-1922	++ (0.39)	50	5.3
Lactobacillus sakei	NRRL: B-1917	- (0.06)	0	6.0
Lactobacillus casei subsp. *lactosus*	NRRL: B-4564	++ (0.66)	100	5.2
Lactobacillus brevis	NRRL: B-4527	++ (0.65)	52	6.2
Lactobacillus acidophilus	NRRL: B-14168	+ (0.10)	21	5.1
Lactobacillus reuteri	NRRL: B-14171	- (0.06)	10	6.0
Lactococcus lactis subsp. *Lactis*	NRRL: B-1821	+(0.10)	20	5.4
Bifidobacterium longum	Rhodia: BL-04	++ (0.20)	91	5.3
Bifidobacterium bifidum	NRRL: B-1976	+ (0.10)	99	5.7

[a] the values given are means of two assays

[a] Growth: – = same as MRS-basal; + = same as MRS-G, ++ = higher then MRS-G

Concerning the digestibility of the prebiotic product synthesized, it would also be interesting to know how the oligosaccharide would pass through the digestive solutions. This was verified using an artificial gastric mixture containing commercial enzymes. This study was made with the synthesized FOS solution, and compared with the degradation of dextran (4~6 KDa – Sigma), inulin ("Raftline" – Orafti - Belgium) and commercial FOS (Wako Pure Chemical Industries – Japan). The results are shown in Table 6, where it can be seen that the oligosaccharides produced by the *Rhodotorula* sp. extra-cellular enzyme were only 10% degraded, whereas all the other products were considerably more degraded and transformed into mono and disaccharides.

Table 6. Degradation of oligosaccharides by an artificial gastric solution

Carbohydrates	Digestibility (%)
Dextran	51.0 ± 5.6
Inulin	43.7 ± 1.5
FOS (commercial)	31.7 ± 6.1
FOS (*Rhodotorula* sp. LEB-V10)	10.0 ± 4.4

4 Discussion

The production of oligosaccharides using mainly fructofuranosidases and fructosyltransferase enzymes has been studied by a number of workers in the last decades. These studies investigated all kinds of organisms, from lactic acid and ethanol producing bacteria to moulds, yeasts and vegetables.

Chen and Liu (1996) studied the β-fructofuranosidase from *Aspergillus japonicus*, an enzyme showing both hydrolytic and transfructosilating activities, optimum activity being achieved at pH 5.0-6.0 and temperatures between 55 and 65 °C for fructose transfer, and at pH 4.0 and 65°C for the hydrolytic activity.

The optimum pH and temperature for the fructosyltransferase from *Aspergillus niger* were found to be 5.8 and 50°C, whereas the optimum values for hydrolytic activity were pH 4.4 and 55°C. In addition, in a 50% (w/w) sucrose solution, the enzymes from the crude extract produced a yield of 54% in FOS, and the purified fructosyltranferase a yield of 62%. (L'Hocine et al, 2000).

Whole cells of *Bifidobacterium bifidum* are also used for the synthesis of galacto-oligosaccharides from lactose, reaching a conversion rate of 35%. The optimum activity was achieved at 40°C and pH 6.8-7.0 (Tzortis et al., 2005).

Rhodotorula yeasts were found to produce extracellular invertase, β-glucosidases and levanases, their activity on cellobiose and lactose resulting in gluco- and galacto-oligosaccharides with yields from 36 to 38%. The optimum pH and temperature for the enzymes of *Rhodotorula minuta* were pH 4.0 – 6.0 and a temperature of 70°C (Rubio et al, 2002; Onishi and Tanaka, 1996; Onishi and Yokoseki, 1996).

The extra-cellular enzyme used in this work showed two types of activity: hydrolytic and transfructosylating. Using a central composite design (CCD), the effects of pH and

temperature on the enzyme activity were evaluated, and the optimal conditions were found to be a temperature of 65ºC (± 2ºC) and pH value between 3.8 and 4.3. Under these conditions, the transfructosylating activity was about 10 times higher than the hydrolytic activity.

By means of a digestibility study, it was also shown that the oligosaccharides synthesized were able to resist gastric conditions fairly well, and by means of another study on the growth of lactic acid bacteria, it was shown that the FOS synthesized were able to stimulate the growth of probiotic bacteria. The best effects (good growth and high FOS consumption) were observed with *Lactobacillus acidophilus* (NRRL B-4495), *Lactobacillus casei* subsp. *alactosus* (NRRL B-4564), *Bifidobacterium bifidum* (NRRL B-1976) and *Bifidobacterium longum* (Rhodia: BL-04), all well known probiotic bacteria.

The efforts made to screen for extra-cellular transfuctosylating enzyme producing yeasts gave very promising results. In this work, the *Rhodotorula sp*. isolated from tropical Brazilian biomass was shown to be capable of producing an extra-cellular fructosyltransferase enzyme with good prospects for industrial application, since it can convert sucrose into FOS, giving high yields.

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ARTIGO 3 :

PROPERTIES OF FRUCTOOLIGOSACCHARIDE-PRODUCING FRUCTOFURANOSIDASE FROM RHODOTORULA SP.

Propriedades da fructofuranosidase de *Rhodotorula* sp. produtora de frutooligossacarídeos

(Journal of Biotechnology)

PROPERTIES OF FRUCTOOLIGOSACCHARIDE-PRODUCING FRUCTOFURANOSIDASE FROM RHODOTORULA SP.

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Abstract

The present work was devoted to investigations concerning the fructooligosaccharide (FOS) producing activity of *Rhodotorula* sp. LEB-V10 (Laboratory of Bioprocess Engineering – Unicamp- Brazil) and its extra-cellular fructosyl transferase. After cell separation, the enzyme was purified by ethanol precipitation and anion exchange chromatography. The molecular weight was estimated to be 170 kDa by preparative gel filtration and 77 kDa by SDS-PAGE. The molecular mass determination indicated that the native enzyme exists as a dimer. The enzyme showed both fructofuranosidase / hydrolytic (FA) and fructosyl transferase (FTA) activities. With sucrose as substrate, the data failed to fit the Michaellis-Mentem model, rather showing a sigmoid shape, similar to that of the alosteric enzymes (cooperative behaviour), requiring high sucrose concentrations to obtain high reaction rates. The optimum pH and temperature for FA activity were found to be around 4.0 and 72-75°C, respectively, while FTA showed optimum activity at pH 4.5 and 65-70°C. Both activities were very stable at temperatures below 66°C, while for FA the enzyme was more stable at pH 4.0 and for FTA at pH 5.0. This enzyme showed high transfructoslating activity, catalysing high conversion of sucrose into FOS, very similar to the current industrial process with immobilized cells from fungi like *Aspergillus* and *Aureobasidium*.

Keywords: Rhodotorula sp.; fructooligosaccharides, enzyme characterization

1 Introduction

The human being is currently becoming more and more concerned with health improvement and a good diet, including many natural products and mainly functional foods, is one of the most important factors related to this concern. The ingestion of functional fructooligosaccharides (FOS) is one way to promote health, creating a good balance in the intestinal flora and inducing the proliferation of intestinal bifidobacteria (probiotics). These oligosaccharides are made of 1 to 3 fructose units bound to a sucrose unit, and are classified as 1-kestose (GF2), nystose (GF3) and fructofuranosyl-nystose (GF4) (Gibson & Roberfroid, 1995).

The concept of functional food is quite new to the consumers, but this increasing awareness in combination with scientific knowledge provides unique opportunities in the development of functional foods. Despite considerable knowledge about the production of invertase, little can be found about the fructooligosaccharide producing enzymes from yeasts, most studies involving bacteria (*Bacillus macerans*, *Zymomonas mobilis*, *Lactobacillus reuteri*) or fungi/molds (*Aspergillus* sp, *Penicillium citrinum* and *Aureobasidium* sp.) fructosyl transferases (Sageetha *et al*., 2005). Risso (2004) and Santos (2003) made an extensive study of FOS production by yeasts, but the trials carried out with *Kluyveromyces* species resulted in low FOS production, the maximum yield being about 12% in aqueous media and 18% in organic solvents. Food-grade FOS is produced commercially from sucrose or inulin using intracellular enzymes from fungi like *Aspergillus* and *Aureobasidium* (Yun, 1996). Due to the increasing demand for FOS as a functional food, it is of interest to find alternative processes for the production of oligosaccharides.

Having the advantage of working in Brazil, one of the most extensive sources of all kinds of living organisms and also a great sucrose producing country (sugar-cane), the present paper describes the production of a fructofuranosidase with high transfructosilating activity by *Rhodotorula* sp. (LEB-V10: Laboratory of Bioprocess Engineering – UNICAMP - Brazil) isolated from the Atlantic Forest or “Mata Atlântica”, a biome with an extremely diverse and unique mixture of vegetation and forest types, that stretches along the Brazilian coast.

2 Material and Methods

2.1 Microrganisms and their cultivation conditions

Rhodotula sp. (LEB-V10: Laboratory of Bioprocess Engineering – UNICAMP - Brazil) was isolated from flowers found in the Brazilian Atlantic Forest (São Paulo). The culture was maintained on GYMP agar medium at 4℃.

The same medium was used for both the inoculum and for enzyme production, the standard medium consisting of (per litre): 50 g sucrose, 20 g yeast extract, 10 g $NaNO_3$, 0.5 g $MgSO_4.7H_2O$ and 5 g K_2HPO_4 (pH 5.0). The inoculum was incubated at 30℃ and 150 rpm for 24 hours. For enzyme production, the same conditions were used except for the fermentation time, which was 48 hours.

2.2 Enzyme recovery

A clear supernatant was obtained by centrifuging the culture broth at 5℃, (4000 G, 10 min) and the enzyme recovered by adding ethanol at to a final concentration of 70% (0 – 4℃). The precipitate was removed by centrifugati on (2℃, 6000G, 10 min.), dissolved in 50 mM sodium acetate buffer (pH 4.5) and stored at -18℃.

A two-step ethanol fractionation was also carried out. In this case 95% ethanol was slowly added to the cell-free supernatant to 50% saturation. The precipitate was removed by centrifugation and more ethanol added to the supernatant to a final concentration of 70%, and the enzyme-rich precipitate again collected by centrifugation.

2.3 Enzyme purification

The crude enzyme solution was applied to an anionic column (HiLoad™ 16/10 Q Sepharose® Fast Flow - Pharmacia Biotech) and equilibrated with 50 mM sodium acetate buffer (pH 5 - 6). The non-adsorbed proteins were eluted with the starting buffer and the adsorbed proteins and enzymes eluted with a linear gradient of NaCl (0 to1 M) in the same buffer at a flow rate of 1 $ml.min^{-1}$.

2.4 Enzyme assay

The reaction media used to determine enzyme activity consisted of 50% (w/v) sucrose (in 50 mM sodium acetate buffer, pH 4.5) and 10% (v/v) of adequately diluted enzyme suspension at 50 ºC. Samples were collected at constant time intervals for 30 minutes of reaction time, and used to quantify glucose with commercial glucose-oxidase kits and reducing sugars by the Somogi-Nelson method.

Sucrose conversion by fructofuranosidase yields glucose and fructose, but when the transfructosilating activity is present, part of the fructose is built into a fructan polymer. By measuring the amounts of glucose and reducing sugars released into the reaction medium, the hydrolytic and transfructosylating activities can thus be assessed (Chen and Liu, 1996). The equation below (eq.1) allows for the determination of the activities by estimating glucose (G) and reducing sugars (R) in the reaction media (F = fructose, F' = transferred fructose):

$$R = G + F \Leftrightarrow F = R - G \qquad F' = G - F \Leftrightarrow F' = 2\,G - R \qquad \text{(eq. 1)}$$

One unit of fructofuranosidase activity (FA) is defined as the amount of enzyme required to hydrolyse 1 µmol of sucrose per minute. One unit of transfructosylating activity (FTA) is defined as the amount of enzyme required to transfer one µmol of fructose (F') per minute.

2.5 Estimation of protein concentration

Protein concentration was measured according to the Lowry method, using bovine serum albumin as the standard (Lowry, 1951).

2.6 Molecular mass estimation

Sodium dodecil sulphate-poliacrylamide gel electrophoresis (SDS-PAGE) was used to determine protein purity and the molecular mass of the enzymes was determined under denaturing conditions using a 7.5% acrylamide gel, as described by Laemnli. The proteins were silver stained according to the manufacturer's instructions (Silver staining kit for proteins - Pharmacia Biotech). A mixture of high molecular weight proteins (HMW electrophoresis standard – Sigma) was used as molecular mass markers. Electrophoresis was performed at 150 V for 30 – 40 minutes.

The molecular mass of the native enzyme was estimated by gel filtration using a Superdex 200 preparative column (10 x 60cm; Amersham Bioscience Co.) equilibrated with 50mM sodium phosphate buffer (pH 6.8) containing 100 mM NaCl. The enzyme was eluted using the same buffer at a flow rate of 1 $ml.min^{-1}$. The column was calibrated using the high molecular weight standard for GPC (PW – 1000 GPC - Sigma).

2.7 Characterization of fructofuranosidase

The activity and stability of the enzyme were measured at various pH values from 3.0 to 7.0 using sodium acetate buffer for pH values from 3.0 to 5.5 and sodium phosphate

buffer for pH 6.0 to 7.0. The enzyme solution was maintained at 50 ℃ in each buffer and the residual activity measured using standard assay conditions.

The effect of temperature on the maximum reaction rate was studied from 30 to 80 ℃, the reaction media consisting of 50% (w/v) sucr ose in 50mM sodium acetate buffer (pH 4.5). Heat stability was determined by incubating the enzyme (crude preparation) in a 50 mM sodium acetate buffer (pH 4.5) at temperatures from 60 to 80 ℃. The residual enzyme activity (using standard assay conditions) was checked until a significant decrease was noted.

The effect of various metal ions on the transfructosylating activity was studied by incubating the enzyme in 50% (w/v) sucrose solutions in 50 mM sodium acetate buffer (pH 4.5) with 10 mM of the salt. All experiments were made with a diluted enzyme solution containing approximately 15 FTA.ml^{-1}.

2.8 Determination of the kinetic parameters

The initial reaction rates were determined for the two activities under their optimum conditions (pH 4.5, 60℃) for various sucrose conce ntrations (0.5 – 50%).

2.9 Fructooligosaccharide production

The crude enzyme preparation (1 FTA.ml^{-1}) was incubated in a 50% (w/v) sucrose solution in 50 mM sodium acetate buffer (pH 4.5), at 50 ℃, for 72 hours to produce the fructooligosaccharides. The synthesis media was analysed for its carbohydrate composition by high performance liquid chromatography with pulsed amperometric detection (HPLC-PAD), using a Dionex (USA) equipment and a Carbopac PA-100 column equilibrated with 50 mM NaOH, and eluted with a linear gradient (0 – 30%) of 500 mM sodium acetate in 50 mM NaOH (1 ml.min^{-1}).

3 Results

3.1 Enzyme production, recovery and purification

The maximum enzyme production, about 20 ± 3 FTA.ml^{-1} and 21 ± 2 FTA.ml^{-1}, was reached after 40-48 hours of cultivation, for both the hydrolytic and transfructosylating activities. About 75% of the transfructosilating activity and 40% of the hydrolytic activity could be recovered by extracting with 70% ethanol, but almost all the proteins precipitated at the same time, resulting in poor purification, although a considerable concentration of the enzyme was achieved. Using two-step ethanol fractionation as an initial purification

step, only about 25% of the transfructosylating activity was recovered, with a purification factor of 2.

The results of the purification procedure are reported in Table 1. After the tests with different buffer pH values, the best value for enzyme purification was shown to be between 5.5 and 6.0. In sodium acetate buffer (pH 5.5) the recovery was slightly more efficient, but at pH 6.0 (sodium phosphate buffer) the purification factor was higher.

Table 1: Purification of *Rhodotorula* sp. fructofuranosidase

Purification step	Total activity (U)	Protein content (mg)	Specific activity ($U.mg^{-1}$)	Purification (fold)	Recovery (%)
Crude extract					
FA	830 ± 10	1561 ± 5	0.5	1.0	100
FTA	910 ± 6		0.6	1.0	100
Ethanol precipitation (70%)					
FA	337 ± 5	1445 ± 3	0.2	0.4	40
FTA	683 ± 6		0.5	0.8	75
Q-sepharose (pH6.0)					
FA	110 ± 5	3 ± 2	35.9	71.9	13
FTA	460 ± 7		150.9	251.5	51
Q-sepharose (pH5.5)					
FA	85 ± 4	8 ± 2	10.5	21.0	10
FTA	603 ±6		75.4	150.8	66

The elution profile on Q-sepharose at pH 6.0 (Figure 1) revealed that the majority of the protein content was not the target enzyme. A noteworthy occurrence was that in all the chromatographic purifications, the enzyme was eluted as broad, asymmetric peaks. This behaviour is quite typical of polydispersed glycoproteins, as reported by other authors (L'Hocine *et al.*, 2000).

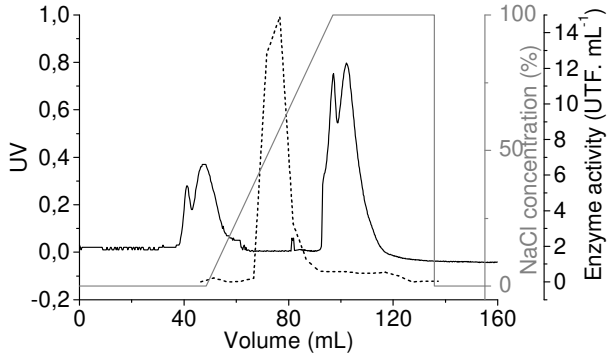

Figure 1: Enzyme purification on a Q-sepharose column

(—) Protein (Abs 180nm); (—) NaCl gradient; (…) transfructosylating activity.

3.2 Molecular weight determination (GPC and SDS-PAGE)

Denaturing electrophoresis (SDS-PAGE) with a 7.5% polyacrylamide gel, indicated that, after purification, two bands of proteins showed different mobility, the first one was equivalent to a molecular weight of about 77 kDa, ranging between 70 and 85 kDa, and the second one was about 124 kDa, ranging between 119 and 129 kDa (Figure 2).

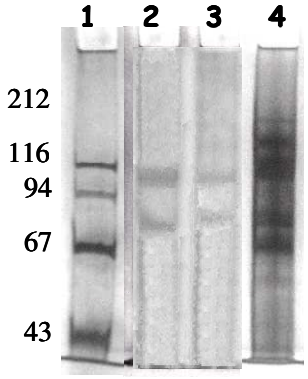

Figure 2: SDS-PAGE (7.5%) of *Rhodotorula* sp. fructofuranosidase.

Lane 1, protein standards; lane 2, Q-sepharose purification pH 6.0 (pool of 2 fractions); lane 3, Q-sepharose purification pH 5.5 (fraction number 7); lane 4, crude extract

The FPLC gel permeation elution profile showed a good distribution of the peaks, but only one showed enzyme activity (Figure 3). When compared with the protein markers, the supernatant fructofuranosidase eluted as a peak with a molecular weight of approximately 170 kDa. Considering the SDS-Page results, it appears that the enzyme corresponds to a dimer of 170 kDa, composed of two monomers each of about 80 kDa. The difference

between the values is probably due to the early elution of glycoproteins, and hence an overestimation of the real weight of the complex.

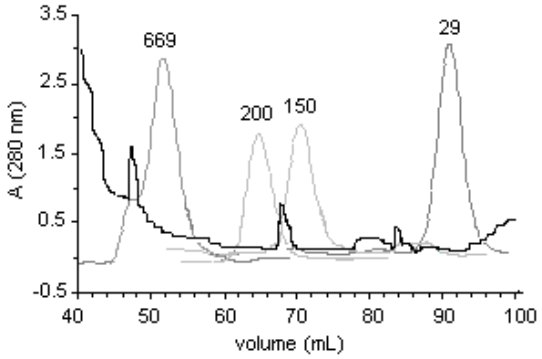

Figure 3: FPLC gel filtration of the crude enzyme extract

(— crude extract, — protein markers)

3.3 Enzyme characterization

Effect of pH on enzyme activity and stability

Both enzyme activities (FA and FTA) were measured in the pH range from 3 to 7 (using 50 mM sodium acetate and sodium phosphate buffers). As shown in Figure 4*a*, the optimum pH was found to be around 4.0 – 4.5 for both kinds of activity.

In order to determine the effect of pH on enzyme stability, the ethanol precipitated enzyme was incubated in the same buffers at 50ºC for 200 hours. The residual enzyme activities were then assayed by the standard method (Figure 4*b*). In the case of the transfructosylating activity, the highest stability was around pH 5.0, a decrease in activity only being detected after 90 hours. For the hydrolytic activity, the best stability was found at around pH 4.0.

Effect of temperature on enzyme activity and stability

As shown in Figure 4*c*, the enzyme had the highest FTA activity at around 65ºC - 70ºC, in contrast to the maximum hydrolytic activity that was at around 72 - 75ºC. Concerning heat stability, it was found that the FTA activity was slightly less stable at high temperatures (Figure 4*d*), although the enzyme was quite stable at temperatures below 66 ºC. The half-life of the enzyme decreased very quickly at temperatures above 66ºC. The activation and denaturation energies for the transfructosylating activity were 13.48 Kcal.mol^{-1} and 150.8 Kcal.mol^{-1}, respectively, and for the hydrolytic activity, 17.21 and 167.27 Kcal.mol^{-1}, respectively.

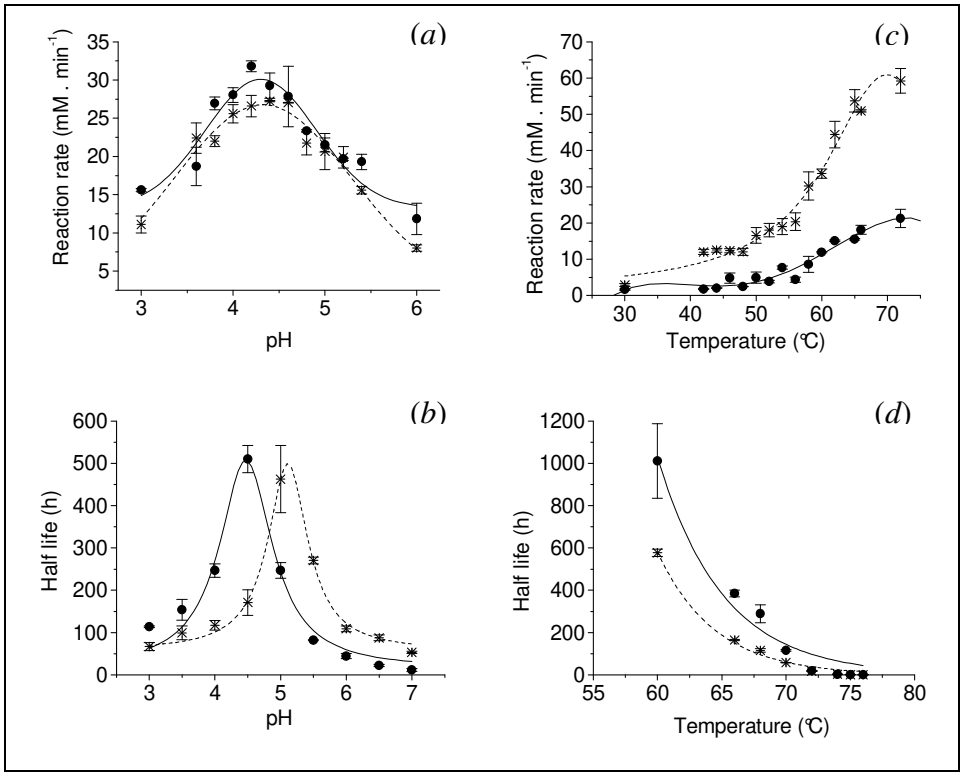

Figure 4: Effect of pH and temperature on enzyme activity and stability

(a) pH-enzyme activity; (b) pH – enzyme stability; (c) Temperature – enzyme activity; (d) Temperature – enzyme stability. (● FA; ✷FTA)

Effect of metals

The FTA and FA activities were studied in terms of the influence of different ions. The values for activity were assayed by the standard method. As shown in Table 3, Na^{+} and Mn^{2+} ions inhibited the hydrolytic activity at 10 mM. Both ions reduced the activity to approximately 30% of its initial value. On the other hand, the transfructosylating activity was not affected by Mn^{2+} ions, while in the presence of Na^{+} it retained 74% of its initial activity and lost about 30% in the presence of citric acid, Ca^{+2} and Zn^{+2}. Potassium ions reduced its activity to 40%.

Of those tested, Cu^{2+} was the only ion that stimulated FTA activity. For FA activity, most of the ions tested produced a positive effect, the exceptions being Na^{+} and Mn^{+2}. It was not verified whether these effects were relating to ion binding to the enzyme causing conformational changes, or the requirement by the enzyme for a metal ion at the active site. Further work is necessary to ascertain the mode of action.

Table 3: Effect of ions on FTA and FA activity

Ion	% residual activity*	
	FA	FTA
None	100	100
Citric acid	167	65
$CuSO_4$	125	121
NaCl	29	74
KCl	304	40
$MnCl_2$	33	99
$CaCl_2$	133	69
$ZnSO_4$	438	68

*mean of duplicates

3.4 Determination of kinetic parameters

The kinetic parameters for each kind of activity were determined using non-linear estimation procedures (Figure 5). The Michaellis-Mentem model did not fit the experimental data, and the use of that model led to meaningless parameters. The enzyme showed a rather sigmoid cooperative type behaviour, similar to that expressed by the Hill's model (Eq.2), where v is the reaction rate, v_{max} the limiting reaction rate, h the Hill's coefficient and $k_{0,5}$ the substrate concentration to reach the reaction rate for half v_{max}. The $k_{0,5}$,h and v_{max} values were determined to be 263.5 g.l^{-1}, 1.5 and 129.7 µmol.ml^{-1}.min^{-1} for FA activity and 299.0 g.l^{-1}, 2.2 and 236.1 µmol.ml^{-1}.min^{-1} for FTA activity respectively.

$$v = v'_{\max} \cdot \frac{S^h}{k_{0,5}^h + S^h} \qquad \text{(eq. 2)}$$

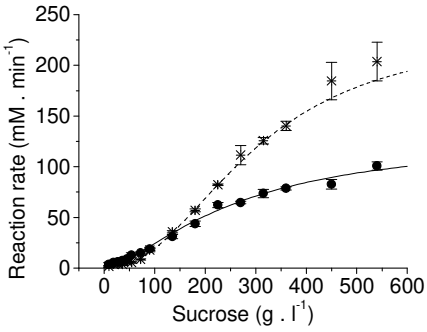

Figure 5: Kinetic behaviour of *Rhodotorula sp*. LEB-V10 enzyme

∗FTA (R^2 = 99.1%); ● FA (R^2 = 99.4%)

3.5 Fructooligosaccharide production

A typical time course for the sugar concentration data is presented in Figure 6, the synthesis was done in triplicate, resulting in a medium standard error of 11%. A FOS production of 240 $g.l^{-1}$ was achieved at the end of the reaction with sucrose practically disappearing and glucose and fructose concentrations of about 160 and 100 $g.l^{-1}$ respectively.

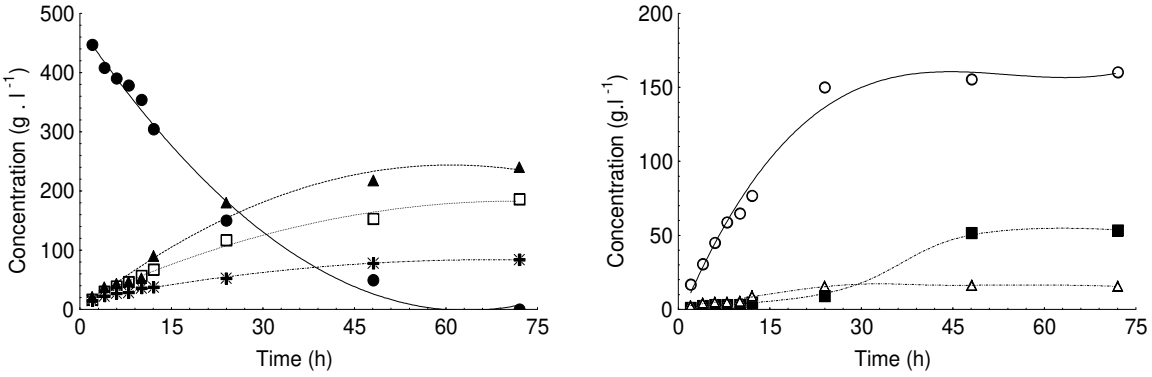

Figure 6: The time course for the sugar concentrations during the enzymatic synthesis of fructooligosaccharides

(▲ FOS; • Sucrose, ∗ fructose, □ glicose, ○ GF2, ■ GF3, △ GF4)

4 Discussion

The first important result of this study was that the fructofuranosidade enzyme with high transfructosylating activity from *Rhodotorula* sp. LEB-V10 (Laboratory of Bioprocess Engineering – Unicamp- Brazil) showed some interesting features, revealing a potential for new enzyme applications in fructooligosaccharide production. The enzyme was purified

with 50-66% recovery and a 150-250 purification fold factor, using HiLoad™ 16/10 Q Sepharose® Fast Flow (Pharmacia Biotech).

The gel permeation and electrophoretic mobility data suggested that the enzyme was a dimer with an approximate mean subunit size of 77 kDa. From the catalytic properties of the *Rhodotorula* sp. fructofuranosidase, it was shown that parameters such as temperature, pH and metal ion activation/inhibition had different effects on the respective activities. Besides indicating residues involved in the active sites, the study of the behaviour of the enzyme in solutions containing metal ions is important when working with two kinds of activity. Considering that the ratio between the transfructosilating and hydrolytic activities is a major characteristic in determining the production of fructooligosaccharides (Hidaka *et al*, 1988), the use of manganese or sodium chloride should be beneficial to the process in this case, since both of these caused considerable inactivation of the fructofuranosidase activity with little loss of the transfructosilating activity.

When comparing this enzyme with yeasts enzymes, the *Rhodotorula* sp. (LEB-V10) enzyme had optimum pH values (4.2 - 4.4 for FTA; 3.8 - 4.2 for FA) and temperatures (72-75℃ for FA; 65-70℃ for FTA), similar to those of the invertase from *Candida utilis* (Belcarz *et al*., 2002) and the invertase (Rubio *et al*, 2002) or β-glucosidase (Onishi and Tanaka, 1996) from *Rhodotorula minuta*.

Both the invertase and fructosyltranferases from fungi and yeasts reported as being capable of producing oligosaccharides, showed kinetics that could be explained by the Michaellis-Menten model (with or without substrate inhibition), like the glicosyltransferase, frutosyltransferase and glicosydase from *Aspergillus foetidus* (Wang and Rakshit, 2000) and frutosyltransferase and invertase from *Aspergillus niger* (L'Hocine *et al*., 2000).

β-fructofuranosidases usually possess both fructosyltransferase and hydrolysing activities, and the hydrolysis is a particular case of transfer to water in dilute reaction systems (Paul and Monsan, 1995). However, it was observed that the enzyme from *Rhodotorula* sp. (LEB-V10) only showed both activities in high sucrose concentrations, and both activities showed a sigmoid shape for the initial reaction rate as a function of sucrose concentration, behaviour very similar to that of cooperative enzymes, when the enzyme has two active sites.

In terms of FOS synthesis, although the enzyme from *Rhodotorula* sp. LEB-V10 had both activities, it achieved a 48% conversion of sucrose to FOS, similar to that of the enzymes from *Aspergillu*s and *Aureobasidium*, where the reaction reached 50 to 60% of FOS (Chen and Liu, 1996 and Sangeetha *et al*., 2004). The synthesis of FOS from sucrose by the *Kluyveromyces* genus yielded a maximum of 18% in organic solvents, being even lower in aqueous solutions (Risso, 2004). Therefore the *Rhodotorula* sp. (LEB-V10) may show potential for its industrial application in fructooligosaccharide production, and this study offers an understanding of its properties and the requirements for process optimisation.

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Acknowledgments

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ARTIGO 4 :

PROPERTIES OF FRUCTOOLIGOSACCHARIDE-PRODUCING FRUCTOFURANOSIDASE FROM Cryptococcus SP.

Propriedades da fructofuranosidase de *Cryptococcus* sp. (produtora de frutooligossacarídeos)

(Enzyme and Microbial Technology)

FRUCTOOLIGOSACCHARIDES PRODUCTION BY *CRYPTOCOCCUS* SP.

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Abstract

The present work was devoted to investigations concerning the fructooligosaccharide (FOS) producing activity of *Cryptococcus* sp. LEB-V2 (Laboratory of Bioprocess Engineering – Unicamp- Brazil) and its extra-cellular fructofuranosidase. After cell separation, the enzyme was purified by ethanol precipitation and anion exchange chromatography. The enzyme showed both hydrolytic, or fructofuranosidase (FA) activity, and fructosyl transferase (FTA) activity. With sucrose as substrate, the data failed to fit the Michaellis-Mentem behaviour, showing a substrate inhibitory model. The K_m, K_i and v_{max} values were shown to be 21.9 $g.l^{-1}$, 1082.3 $g.l^{-1}$ and 159.6 $\mu mol.ml^{-1}.min^{-1}$ for FA activity and 44.9 $g.l^{-1}$, 546.2 $g.l^{-1}$ and 377.8 $\mu mol.ml^{-1}.min^{-1}$ for FTA activity respectively. The optimum pH and temperature were found to be around 4.0 and 65°C, while the best stability was achieved at pH 4.5 and temperatures below 60°C, for both the FA and FTA activities. Despite the strong fructofuranosidase activity, causing hydrolysis of the fructooligosaccharides, the high transfructosylating activity allowed for good FOS production from sucrose (35% yield).

Keywords: *Cryptococcus* sp.; fructooligosaccharides, enzyme characterization

1. Introduction

It is well known that there is a strong relationship between what we eat and our health. The fructooligosaccharides (FOS) are functional ingredients, being able to promote a good balance in the intestinal flora, inducing the proliferation of intestinal bifidobacteria (probiotics). These oligosaccharides are made of 1 to 3 fructose units bonded to one molecule of sucrose, and they are classified as 1-kestose (GF2), nystose (GF3) and fructofuranosyl-nystose (GF4) (Gibson & Roberfroid, 1995).

Despite considerable knowledge about invertase production, little can be found about the fructooligosaccharide producing enzymes from yeasts. Most studies deal with bacterial (*Bacillus macerans*, *Zymomonas mobilis*, *Lactobacillus reuteri*) or fungal (*Aspergillus* sp, *Penicillium citrinum* and *Aureobasidium* sp.) fructosyl transferases (Sageetha *et al*., 2005). Food-grade FOS are produced commercially from sucrose or inulin using intracellular enzymes from fungi like *Aspergillus* and *Aureobasidium* (Yun, 1996).

Tropical environments (forests) are one of the greatest sources of all kinds of living organisms, including yeasts, unicellular fungi with a ubiquitous distribution in almost all ecosystems. Brazil has four biomes of great biodiversity, the Amazon Forest, Atlantic Forest (stretches along the Brazilian coast with an extremely diverse and unique mix of vegetation and forest types), Cerrado (tropical savanna eco-region) and Pantanal (the world's largest wetland). It is also a big sucrose producing country (sugar-cane) and due to the increasing demand for FOS as a functional food, it would be of interest to find alternative processes for oligosaccharide production.

The *Cryptococcus* genus has been studied for the production of α amylase (Wanderley *et al*, 2004), xylanase (Iefuji, 1996), β galactosidase (Ohtsuka *et al*, 1990) and lipase (Iefuji *et al*., 2002). In a previous study (Hernalsteens and Maugeri, 2006), the isolation of yeast strains aimed at FOS production by extra cellular yeasts enzymes resulted in the selection of four strains, one of which was a *Cryptococcus* strain (*Cryptococcus* sp. LEB-I3).

In the present study we investigated the fructosyl-transferase activity of a *Cryptococcus* sp. (LEB-V2: Laboratory of Bioprocess Engineering – UNICAMP - Brazil)

isolated from Brazilian indigenous flora (Atlantic Forest), and some of the biochemical properties of the extracellular enzyme.

2. Materials and Methods

2.1. Microrganisms and cultivation conditions

Cryptococcus sp. (LEB-V2: Laboratory of Bioprocess Engineering – UNICAMP - Brazil) was isolated from flowers from the Brazilian Atlantic Forest (São Paulo). The culture was maintained on GYMP agar at 4℃.

The same medium was used for both the inoculum and enzyme production, and consisted of (per litre): 50 g sucrose, 20 g yeast extract, 10 g $NaNO_3$, 0.5 g $MgSO_4.7H_2O$ and 5 g K_2HPO_4 (pH 5.0). The flasks were incubated at 30℃ and 150 rpm for both growth and enzyme production, the fermentation time being 24 hours for the inoculum and 48 hours for the enzyme producing cultures.

2.2. Enzyme recovery

A clear supernatant was obtained by centrifuging the culture broth at 5℃, (4000 G, 10 min) and the enzyme recovered by the addition of ethanol to a final concentration of 70% (0 – 4℃). The precipitate was recovered by centrifugation (2℃, 6000G, 10 min.), dissolved in 50 mM sodium acetate buffer (pH 4.5) and stored at -18℃.

A two-step ethanol fractionation was also carried out. In this case 95% ethanol was added slowly to the cell-free supernatant to 50% saturation. The precipitate was recovered by centrifugation, and more ethanol added to the supernatant to a final concentration of 70%, and the enzyme-rich precipitate recovered by centrifugation.

2.3. Enzyme purification

The crude enzyme solution was applied to an anionic column (HiLoad™ 16/10 Q Sepharose® Fast Flow - Pharmacia Biotech) equilibrated with 50 mM sodium acetate buffer (pH5 - 6). The non-adsorbed proteins were eluted with the starting buffer and the adsorbed proteins and enzymes eluted with a linear gradient of NaCl (0 to1 M) in the same buffer at a flow rate of 1 $mL.min^{-1}$.

2.4. Enzyme assay

The reaction medium used to determine the enzyme activity consisted of 50% (w/v) sucrose (in 50 mM sodium acetate buffer, pH 4.5) and 10% (v/v) of a diluted enzyme suspension at 50 ºC. Samples were collected at regular intervals for 30 minutes during the reaction, and the amount of glucose quantified using commercial glucose-oxidase kits and amount of reducing sugars by the Somogi-Nelson method.

Sucrose conversion by fructofuranosidase yields glucose and fructose. However, when fructosyl transferase is present, part of the fructose produced is built into a fructan polymer. The hydrolytic and the transfructosylating activities can thus be assessed from the amounts of glucose and reducing sugars released into the reaction medium (Chen and Liu, 1996). The equations below (eq 1) allow for the determination of the activities by estimating glucose (G) and reducing sugar (R) in the reaction media (F = fructose, F' = transferred fructose):

$$R = G + F \Leftrightarrow F = R - G$$

$$F' = G - F \Leftrightarrow F' = 2\,G - R \qquad \text{(eq. 1)}$$

One unit of fructofuranosidase activity (FA) is defined as the amount of enzyme required for the hydrolysis of 1 µmol of sucrose per minute. One unit of transfructosylating activity (FTA) is defined as the amount of enzyme required to transfer one µmol of fructose (F') per minute.

2.5. Estimation of protein concentration

Protein concentration was measured according to the Lowry method, using bovine serum albumin as standard (Lowry, 1951).

2.6. Molecular mass estimation

Sodium dodecil sulphate-poliacrylamide gel electrophoresis (SDS-PAGE) was used to determine protein purity and the molecular mass of the enzyme under denaturing conditions, using 7.5% acrylamide gel as described by Laemnli (1970). Proteins were silver stained according to the manufacturer's instructions (Silver staining kit for proteins-Pharmacia Biotech). A mixture of high molecular weight proteins (HMW electrophoresis

standard – Sigma) was used as molecular mass markers. Electrophoresis was performed at 150 V for 30 – 40 minutes.

2.7. Characterization of fructofuranosidase

The activity and stability of the enzyme were measured at various pH values from 3.0 to 7.0 using sodium acetate buffer for pH values from 3.0 to 5.5 and sodium phosphate buffer for pH 6.0 to 7.0. The enzyme solution was maintained at 50 °C in each buffer and the residual activity was measured for up to 90 hours using standard assay conditions.

The effect of temperature on the maximum reaction rate was studied from 30 to 80 °C, the reaction medium consisting of 50% (w/v) sucrose in 50mM sodium acetate buffer (pH 4.5). Thermo-stability was determined by incubating the enzyme (crude preparation) in a 50 mM sodium acetate buffer (pH 4.5) at temperatures from 60 to 80 °C. The residual enzyme activity was checked until a significant decrease was noted (using standard assay conditions).

A central composite experimental design (CCRD) was used to confirm the optimal pH and temperature for enzyme activity. Factorial designs and response surface analyses are important tools to determine optimal process conditions. Factorial designs are advantageous as compared to the conventional method, which handles a single parameter per trial. The conventional method does not consider the effect of possible interactions between factors, which are very important in biological and biochemical process and when substrates such as agro-industrial by-products and surpluses are used (Kalil *et al.*, 2000).

The effect of various metal ions on the transfructosylating activity was studied, by incubating the enzyme in 50% (w/v) sucrose in 50 mM sodium acetate buffer (pH 4.5) plus 10 mM of the salt. All experiments were carried out using a dilute enzyme solution containing approximately 15 FTA.ml^{-1}.

2.8. Determination of kinetics parameters

The initial reaction rates were determined for the two kinds of activity using different sucrose concentrations (0.5 – 50%) under standard conditions (pH 4.5, 60°C).

2.9. Fructooligosaccharides production

The crude enzyme preparation (1 FTA.ml^{-1}) was incubated in a 50% (w/v) sucrose solution (50 mM sodium acetate buffer, pH 4.5) at 50 ºC for 72 hours. The synthesis medium was analysed for its carbohydrate composition by high performance liquid chromatography with pulsed amperometric detection (HPLC-PAD), using a Dionex (USA) chromatograph and a Carbopac PA-100 column equilibrated with 50 mM NaOH and eluted with a linear gradient (0 – 30%) of 500 mM sodium acetate in 50 mM NaOH (1 ml.min^{-1}).

3. Results

3.1. Enzyme production, recovery and purification

The maximum enzyme production was 16.2 (± 1.7) FTA.ml^{-1} and 13.0 (± 2) FA.ml^{-1}, obtained after 36-42 hours of cultivation for both the hydrolytic and transfructosylating activities. About 80% of FTA and 30% of FA could be recovered using 70% ethanol extraction, but almost all the protein present also precipitated, resulting in poor purification and in difficulties with the chromatographic procedure. The use of the two step ethanol extraction (50 – 70%) resulted in the recovery of almost 60% of the transfructosilating activity and 20% of the hydrolytic activity, and this process eliminated almost 90% of the proteins, resulting in an efficient initial purification step.

The results of the purification procedure are reported in Table 1. Performing assays with different buffers pH values, the best value for enzyme purification was shown to be between 5.5 and 6.0. At pH 5.5 (sodium acetate buffer) the highest purification factor and recovery were obtained, about 178 fold and 55%, respectively.

The elution profile on Q-sepharose (Figure 1) revealed that the majority of the protein content was not the target enzyme and that the enzyme was eluted as a well-defined and sharp peak.

Table 1: Purification of *Cryptococcus* sp. fructofuranosidase

Step of purification	Total activity (U)	Protein content (mg)	Specific activity ($U.mg^{-1}$)	Purification Fold	Recovery (%)
Crude extract					
FA	1061 ± 10	1354 ± 18	0.8	1.0	100
FTA	900 ± 15		0.7	1.0	100
Ethanol precipitation (50 – 70%)					
FA	184 ± 5	28 ± 3	6.7	8.4	17
FTA	567 ± 6		20.5	29.3	63
Q-sepharose (pH6.0)					
FA	254 ± 5	7 ± 2	37.6	47.0	24
FTA	427 ± 7		63.1	90.1	47
Q-sepharose (pH5.5)					
FA	136 ± 4	4 ± 2	34.0	42.5	13
FTA	499 ± 6		124.8	178.2	55

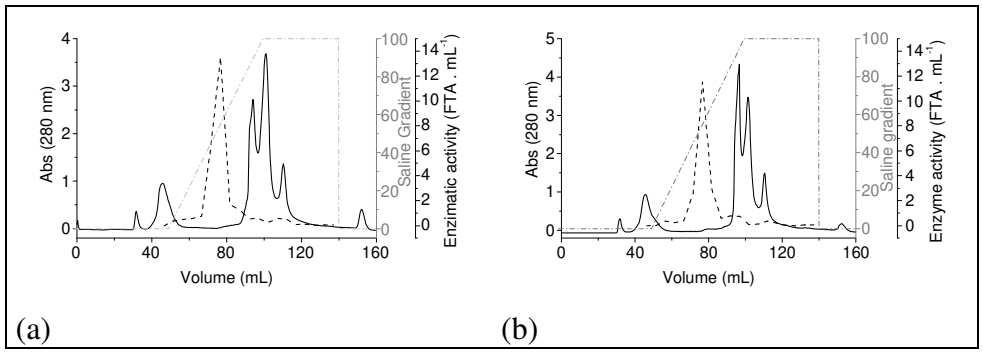

Figure 1: Chromatogram of the crude preparation on the Q-sepharose column

(a) pH 5.5 and (b) pH 6.0; (—) Protein (A180); (—) NaCl gradient; (⋯) transfructosylating activity.

3.2. Molecular weight determination

Denaturing electrophoresis (SDS-PAGE) on 7.5% polyacrylamide gel indicated that after purification, two or three protein bands showed different mobility values. However, all the samples that presented FTA activity showed one polydispersed band equivalent to a molecular weight of about 90 kDa, and another band of 130 kDa (Figure 2).

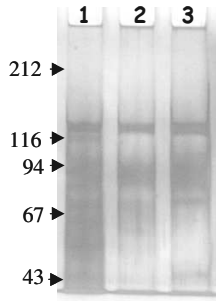

Figure 2: SDS-PAGE (7.5%) of *Cryptococcuss* sp. fructofuranosidase.

Lane 1: crude extract; lane 2, Q-sepharose purification (pH 5.5) ; lane 3, Q-sepharose purification (pH 6.0)

3.3. Enzyme characterization

Effects of pH on enzyme activity and stability

Both enzyme activities were measured in the pH range from 3 to 7 (using 50 mM sodium acetate and sodium phosphate buffers). As shown in Figure 3*a*, the optimum pH was found to be around 4.0 for both kinds of activity.

In order to determine the effect of pH on the enzyme stability, the ethanol precipitated enzyme was incubated in the same buffers at 50℃ for 200 hours (Figure 3 *b*). The residual enzyme activities were then assayed by the standard method. In both cases (FTA and FA), the greatest stability was found to be around pH 4.5.

Effect of temperature on enzyme activity and stability

As shown in Figure 3*c*, the enzyme showed the highest FTA and FA activities at 65℃. Concerning heat stability of the enzyme, it w as found that the FTA and FA activities were very similar (Figure 3*d*). Although the enzyme was quite stable at temperatures below 60ºC, the half-lives of the enzyme activities decreased very quickly at temperatures above 60℃, as shown in Figure 3 *d*. The activation and denaturation energies for FTA were 8.74 Kcal.mol^{-1} and 171.20 Kcal.mol^{-1}, respectively, and for FA, 5.28 Kcal.mol^{-1} and 156.72 Kcal.mol^{-1}, respectively.

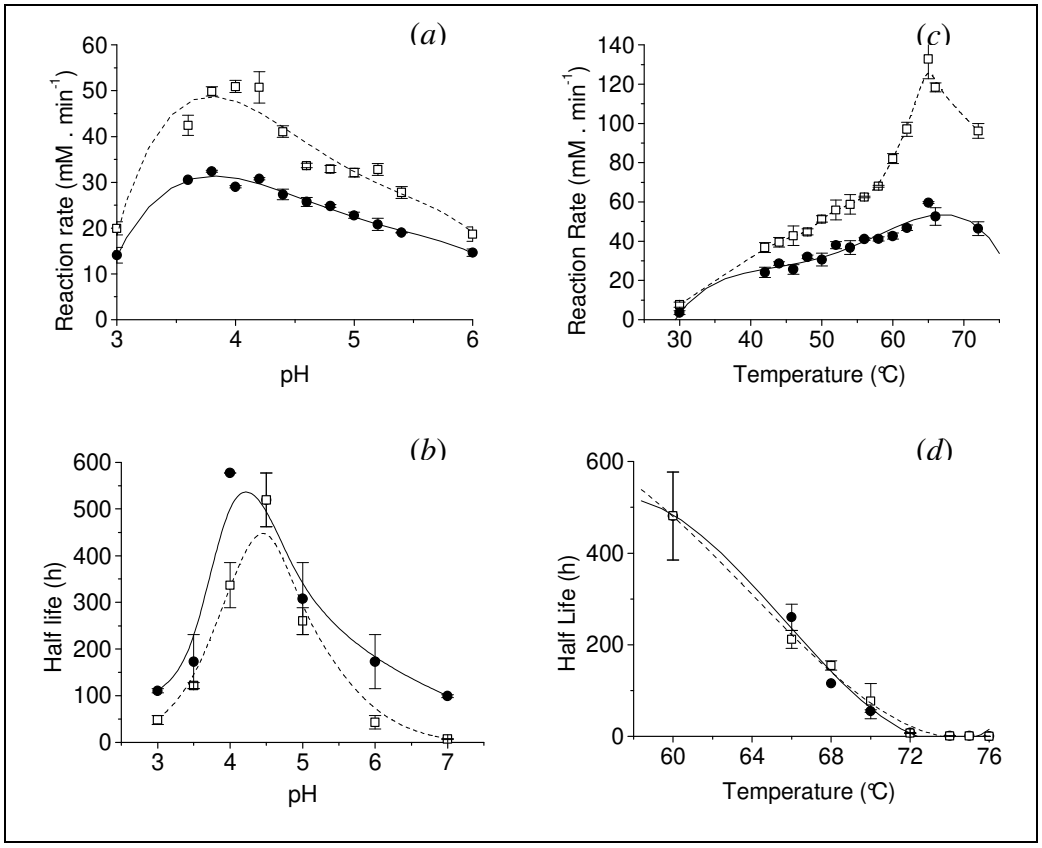

Figure 3: Effect of pH and temperature on enzyme activity and stability

(a) pH-enzyme activity; (b) pH – enzyme stability; (c) Temperature – enzyme activity; (d)Temperature – enzyme stability. (• FA; □ FTA)

Optimum enzyme condition

Considering the optimum values for pH and temperature, a factorial design was used to confirm the above results. Table 2 shows the experimental conditions and the results for the FA and FTA activities according to the factorial design. Both types of enzyme activity were analysed in order to obtain high FOS production, since for this purpose, the enzyme requires high fructosyl transferase activity and low hydrolytic activity.

An estimate of the main effect is obtained by evaluating the difference in process performance caused by a change from the low (-1) to the high (+1) level of the corresponding factor (Haaland, 1989). The statistical parameters t-test and p-value were used, to confirm the significance of the factors studied. In this case, $p<0.05$ suggested significance at the 0.05 level, or a 95% confidence level.

Table 2: Experimental design and results of the 2^2 central composite design

Trial	Coded levels (real values)		Enzyme activity (U. ml^{-1})	
	pH	T (℃)	FA	FTA
1	-1 (3.5)	-1 (60)	89.09	98.90
2	+1 (4.5)	-1 (60)	39.54	60.16
3	-1 (3.5)	+1 (70)	54.69	221.66
4	+1 (4.5)	+1 (70)	28.67	175.22
5	-α (3.3)	0 (65)	109.89	100.86
6	+α (4.7)	0 (65)	134.53	196.25
7	0 (4.0)	-α (58)	48.87	64.12
8	0 (4.0)	+α (72)	72.74	132.42
9	0 (4.0)	0 (65)	117.08	437.72
10	0 (4.0)	0 (65)	115.79	434.11
11	0 (4.0)	0 (65)	115.28	420.67

[a] $\alpha = \pm 1.41$

All factors showed a statistically significant effect on FA, as shown in Table 3. The analysis of variance (ANOVA) did not validate the mathematical model, since the residual value was high and hence the F-test value was small.

Nevertheless, it was still of interest to analyse the effect of each factor on the fructofuranosidase activity. All the factors, except for the interaction between pH and temperature, were negative. These results mean that an increase in pH from 3.3 to 4.7 or in temperature from 58 to 72℃ decreased the enzyme activity.

Table 3: Main effects analysis for FA activity from the factorial design

Factor	Effect	Std. Error	t-value	p-value
pH (L)	-10.236	0.657	-15.580	0.0041[a]
pH (Q)	-13.253	0.784	-16.905	0.0035[a]
T (L)	-2.912	0.657	-4.432	0.0473[a]
T (Q)	-75.024	0.784	-95.699	0.0001[a]
pH x T	11.765	0.928	12.681	0.0062[a]

[a] Significant factors ($p<0,05$)

A fitted model was accomplished for the FTA response: the independent and dependent variables were fitted to the second-order model equation and examined in terms of goodness of fit. ANOVA (Table 4) was used to evaluate the adequacy of the fitted model. The R-squared value provided a measure of how much the model could explain the variability in the observed response.

Table 4: Analysis of variance for FTA activity (ANOVA)

	SS	df	MS	F[b]
Regression (R)	212292.43	3	70764.14	51.50
Residual (r)	9617.82	7	1373.97	
Lack of fit	9456.37	5		
Pure Error	161.46	2		
Total	221910.25	10		

[a] Residual = Lack of fit + Pure error; [b] $F_{0.05;3;7}$= 4.34; R-sqr = 0.96

On the basis of the analysis of variance (ANOVA), a second order model was established, describing the enzyme activity as a function of pH and temperature (eq. 2). Based on the F-test, the model was predictive, since the calculated F value was higher than the critical F value and the R-squared value was 0.96. The coded model was used to generate response surfaces to analyse the effects of the variables on FTA activity (Figure 4).

Therefore, to maximize the FTA activity, both variables should be maintained at the central levels, as tested. Thus the optimised conditions for the fructosyl transferase activity were around the central points, as can be seen in Figure 4, with a temperature between 63 and 67 ºC and a pH value between 3.8 and 4.3.

$$FTA \cdot ml^{-1} = 430.76 - 137.57 * pH^2 + 41.89 * T - 162.86 * T^2 \qquad \text{(eq.2)}$$

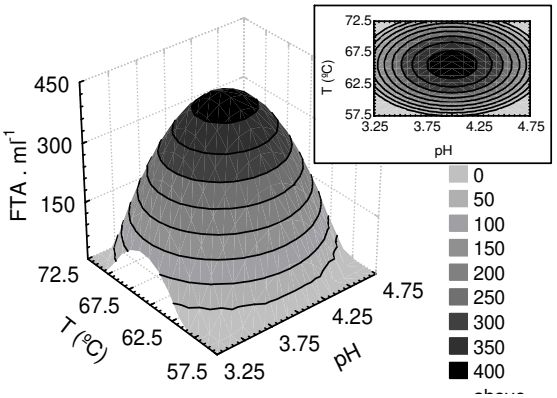

Figure 4: Response surface and contour diagrams for the fructosyl transferase activity (FTA) as a function pH and temperature

Effect of metals

The FTA and FA activities were studied in terms of the influence of different ions, the activity values being assayed by the standard methods. As shown in Table 5, almost all the ions stimulated FA and inhibited FTA, with the exception of copper, which inhibited both activities.

It was not verified whether these effects were relating to ion binding to the enzyme causing conformational changes, or requirements by the enzyme for a metal ion at the active site. Further work is necessary to ascertain the mode of action.

Table 5: Effect of ions on FTA and FA activity

Ion	% residual activity*	
	FA	FTA
None	100	100
Citric acid	113	73
$CuSO_4$	39	36
NaCl	99	85
KCl	122	77
$MnCl_2$	102	93
$CaCl_2$	181	57
$ZnSO_4$	147	60

* means of duplicates

3.4. Determination of kinetics parameters

The kinetic parameters for each kind of activity were determined using non-linear estimation procedures. The substrate inhibition model (eq. 3) fitted the experimental data as shown in Figure 5. Considering *v* as the reaction rate, v_{max} the limiting reaction rate, *Km* the substrate concentration to reach a reaction rate of half v_{max} and *Ki* a factor related to the inhibitory effect of the substrate:

$$v = \frac{v_{max}\, S}{S + K_m + \frac{S^2}{K_i}} \qquad \text{(eq. 3)}$$

The K_m, K_i and v_{max} values were determined to be 21.87 g.l^{-1}, 1082.3 g.l^{-1} and 159.6 µmol.ml^{-1}.min^{-1} for FA activity and 44.9 g.l^{-1}, 546.2 g.l^{-1}and 377.8 µmol.ml^{-1}.min^{-1} for FTA activity respectively.

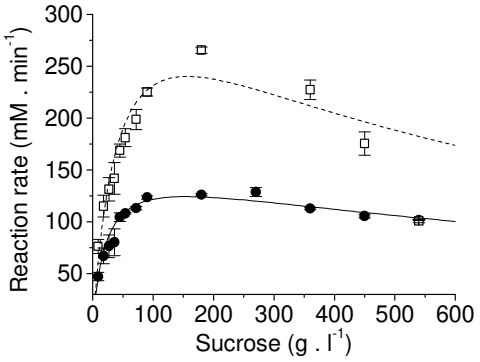

Figure 5: Kinetic behavior of *Cryptococcus sp*. (LEB-V2) enzymes
○ FTA (R^2 = 89.1%); ▲ FA (R^2 = 91.4%)

3.5. Fructooligosaccharide production

The crude enzyme preparation (1 FTA.ml^{-1}) was incubated in a 50% (w/v) sucrose solution (50 mM sodium acetate buffer, pH 4.5) at 50 ℃. A typical time course for the sugar concentration data is presented in Figure 6. The synthesis was done in triplicate, resulting in a mean standard error of 12%. A FOS production of 170 g.l^{-1} was achieved after 48 hours of reaction, when the residual sucrose content was approximately 10% (50 g/l) and the glucose and fructose concentrations 190 and 90 g.l^{-1} respectively.

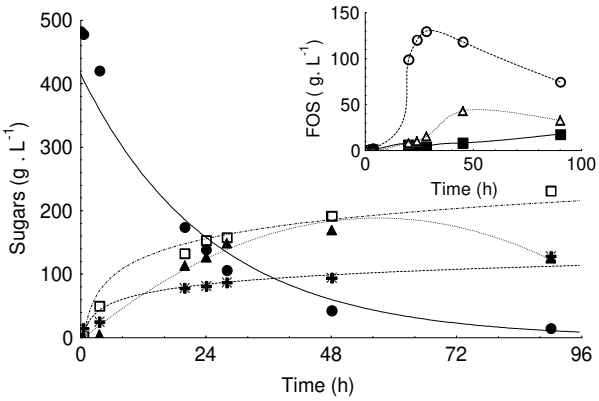

Figure 6: The time course for sugar concentration during enzymatic synthesis of fructooligosaccharides
(▲ FOS; ● Sucrose, ✶ fructose, □ glicose, ○ GF2, ■ GF3, △ GF4)

4. Discussion

The first important result of this study was that the fructofuranosidase enzyme with high transfructosylating activity obtained from *Cryptococcus* sp. LEB-V2 (Laboratory of Bioprocess Engineering – Unicamp- Brazil) showed interesting features, revealing its potential for new enzyme applications in fructooligosaccharide production. The enzyme was purified obtaining 50-55% recovery and a 90-180 fold purification factor, using HiLoad™ 16/10 Q Sepharose® Fast Flow (Pharmacia Biotech). The electrophoretic mobility under denaturing conditions (SDS-PAGE), showed two protein bands, one poly-dispersed band equivalent to a molecular weight of about 90 kDa, and the other of about 130 kDa, further studies on the purification process being required to determine the enzyme molecular weight, and hence the presence of one or two enzymes with different characteristics.

Besides the indication of the involvement of amino acid residues at the active site, the study of enzyme behaviour in solutions containing metal ions is important when working with two kinds of activity, considering the fact that the ratio between the FTA and FA activities is of major importance in determining the production of fructooligosaccharides (Hidaka *et al*, 1988).

When comparing this enzyme to the yeast enzymes, the *Cryptococcus* sp. (LEB-V2) enzyme had similar optimum pH (around 4.5 for FTA and FA) and temperature (65 ℃ for FA and FTA) values to the invertase from *Candida utilis* (Belcarz *et al*., 2002) and to the

invertase (Rubio *et al*, 2002) and β-glycosidase (Onishi and Tanaka, 1996) from *Rhodotorula minuta*.

β-fructofuranosidase usually possesses both fructosyl transferase (FTA) and hydrolysing (FA) activities, the hydrolytic reaction being a particular case involving the transfer to water in dilute reaction systems (Paul and Monsan, 1995). However, it was observed that the enzyme from *Cryptococcus* sp. (LEB-V2) had both activities in both high and low sucrose concentrations. The kinetics of both activities was explained by the Michaellis-Mentem with substrate inhibition model.

The kinetics of both the invertase and fructosyl transferases obtained from moulds and yeasts, related to oligosaccharide production, were explained by the Michaellis-Mentem model (with or without substrate inhibition), such as the glucosyltransferase, fructosyl transferase and glycosidase from *Aspergillus foetidus* (Wang and Rakshit, 2000) and the fructosyl transferase and invertase from *Aspergillus niger* (L'Hocine *et al*., 2000).

In terms of FOS synthesis, a sucrose conversion to FOS of 35% was achieved after 48 hours of reaction, a lower yield then that with the enzymes from *Aspergillu*s and *Aureobasidium*, in which yields of from 50 to 60% of FOS were achieved (Chen and Liu, 1996). The yield obtained was smaller because of the intense FA activity observed in the synthesis time course (after 72 hours of reaction, the fructooligosaccharides were hydrolysed).

Although the enzyme from *Cryptococcus* sp. (LEB-V2) showed both fructofuranosidase and fructosyl transferase activities, the 35% FOS yield is promising, and further studies on the synthesis conditions may show its potential for industrial applications. This study is the first step in a process development, offering an understanding of the properties of the extra cellular enzymes and the requirements for process optimisation.

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ARTIGO 5 :

FRUCTOOLIGOSACCHARIDES PRODUCTION BY RHODOTORULA SP. LEB-U5

Produção de frutooligossacarídeos por *Rhodotorula* sp. LEB-U5

(Biochemical Engineering Journal)

FRUCTOOLIGOSACCHARIDES PRODUCTION BY RHODOTORULA SP.

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Abstract

In the present work, the production of fructooligosaccharides (FOS) by an extra-cellular fructosyl transferase enzyme from a strain of *Rhodotorula* (LEB-U5, Laboratory of Bioprocess Engineering – Unicamp- Brazil) was investigated. After cell separation, the enzyme was purified by ethanol precipitation and anion exchange chromatography. The enzyme showed both fructofuranosidase (FA) and fructosyl transferase (FTA) activities. With sucrose as substrate, the data failed to fit the Michaellis-Mentem behaviour, showing a substrate inhibitory model. The K_m, K_i and v_{max} values were shown to be 4.7 $g.l^{-1}$, 463.1 $g.l^{-1}$ and 150.3 $\mu mol.ml^{-1}.min^{-1}$ for FA and 13.6 $g.l^{-1}$, 246.4 $g.l^{-1}$and 281.8 $\mu mol.ml^{-1}.min^{-1}$ for FTA, respectively. The optimum pH and temperature for FA activity were found to be around 4.0 and 55-60°C, respectively, while FTA showed optimum activity at pH 4.0 and 65-70°C. Both activities were very stable at temperatures below 60°C, while FA was most stable at pH 5.0 and FTA at pH 4.5-5.0. Despite the relatively intense fructofuranosidase activity, causing hydrolysis of the fructooligosaccharides, the high transfructosylating activity allowed for high FOS production from sucrose (40%).

Keywords: *Rhodotorula* sp.; fructooligosaccharides, enzyme characterization

1. Introduction

Considering that the state of well being and reduction in the risk of diseases are associated with target gastrointestinal functions, which are related to a balanced microflora together with an optimal gut associated lymphoid tissue, the development of food ingredients with the capacity to enhance health has attracted much interest.

Prebiotics are defined as non-digestible food ingredients that may beneficially affect the host by selectively stimulating the growth and/or activity of a limited number of bacteria in the colon (Gibson & Roberfroid, 1995). Prebiotics are principally oligosaccharides, including fructooligosaccharides, galacto-oligosaccharides, some glucooligosaccharides and others.

Fructooligosaccharides (FOS) are well known functional ingredients, promoting a good balance in the intestinal flora by inducing the proliferation of intestinal bifidobacteria (probiotics). These oligosaccharides are made of 1 to 3 fructose units bound to a sucrose unit: 1-kestose (GF2), nystose (GF3) and fructofuranosyl-nystose (GF4) (Gibson & Roberfroid, 1995).

FOS are produced from sucrose on a commercial scale using fungal fructosyl transferase enzymes. The majority of the studies have been carried out using the enzyme from moulds such as *Aspergillus* and *Aureobasidium* (Yun, 1996). Although it is not very common, some bacterial strains are also capable of producing FOS, such as *Bacillus macerans*, *Zymomonas mobilis* and *Lactobacillus reuteri* (Sageetha *et al*., 2005). Despite considerable knowledge about the yeast enzymes invertase and inulinase (associated with growth on sucrose), little can be found about the fructooligosaccharide producing enzymes from yeasts. Risso (2004) and Santos (2003) extensively studied the use of inulinase from *Kluyveromyces* sp. to produce FOS, but due to the high hydrolytic activity, the process resulted in low FOS production (12% of yield). The *Rhodotorula* genus has been studied for the production of fats, single-cell protein (Yech, 1996), carotenoids (Buzzini and Martini, 1999), epoxide hydrolases (Kronenburg *et al*, 1999) and invertase (Rubio *et al*., 2002). There are also some studies on a cell-wall β-glycosidase with strong transglycosilation activity from *Rhodotorula minuta*, producing galacto and gluco oligosaccharides (Onishi and Tanaka, 1996).

In a previous study (Hernalsteens and Maugeri, 2006), the isolation of yeast strains aiming at the production of FOS by extra cellular yeast enzymes resulted in the selection of four strains, one of which was a *Rhodotorula* strain (*Rhodotorula* sp. LEB-U5). This *Rhodotorula* strain produces a high activity transfructosylating enzyme, producing almost 200 $g.l^{-1}$ of FOS from a 50% sucrose solution within 24 hours of reaction.

In the present work, the fructosyl-transferase from *Rhodotorula* sp. (LEB-U5) was studied and some of the biochemical properties of this extra cellular enzyme determined, aiming at a better understanding of its characteristics and of the conditions necessary for its future application in process development.

2. Material and Methods

2.1. Microorganisms and cultivation conditions

Rhodotorula sp. (LEB-U5: Laboratory of Bioprocess Engineering – UNICAMP - Brazil) was isolated from flowers from the Brazilian Atlantic Forest (São Paulo). The culture was maintained on GYMP agar medium at 4℃.

The same medium was used for both the inoculum and for enzyme production, the standard medium consisting of (per litre): 50 g sucrose, 20 g yeast extract, 10 g $NaNO_3$, 0.5 g $MgSO_4.7H_2O$ and 5 g K_2HPO_4 (pH 5.0). The inoculum was incubated at 30℃ for 2 4 hours at 150 rpm. For the enzyme producing culture, the same conditions were used, except for the fermentation time, which was 48 hours.

2.2. Enzyme recovery

A clear supernatant was obtained by centrifuging the culture broth at 5℃, (4000 G, 10 min) and the enzyme was recovered by adding ethanol to a final concentration of 70% (0 – 4℃). The precipitate was removed by centrifug ation (2℃, 6000G, 10 min.), dissolved in 50 mM sodium acetate buffer (pH 4.5) and stored at -18℃.

A two-step ethanol fractionation was also carried out. In this case, 95% ethanol was slowly added to the cell-free supernatant to 50% saturation. The precipitate was removed by centrifugation and more ethanol was added to the supernatant to a final concentration of 70%, and the enzyme-rich precipitate again collected by centrifugation.

2.3. Enzyme purification

The crude enzyme solution was applied to an anionic column (HiLoad™ 16/10 Q Sepharose® Fast Flow - Pharmacia Biotech) equilibrated with 50 mM sodium acetate buffer (pH5 - 6). The non-adsorbed proteins were eluted with the starting buffer and the adsorbed proteins and enzymes eluted with a linear gradient of NaCl (0 to1 M) in the same buffer, at a flow rate of 1 $mL.min^{-1}$.

2.4. Enzyme assay

The reaction media used to determine the enzyme activity consisted of: 50% (w/v) sucrose (in 50 mM sodium acetate buffer, pH 4.5) and 10% (v/v) of the diluted enzyme solution, at 50 ºC. Samples were collected at regular intervals for 30 minutes and used for the quantitative determination of glucose using commercial glucose-oxidase kits and of reducing sugars by the Somogi-Nelson method.

Sucrose conversion by fructofuranosidase yields glucose and fructose, but when fructosyl transferase activity is present, part of the fructose is built into a fructan polymer. By measuring the amounts of glucose and reducing sugars released into the reaction medium, the hydrolytic and transfructosylating activities can be assessed (Chen and Liu, 1996). The equations below (eq 1) allow for the determination of the activities by estimating glucose (G) and reducing sugars (R) in the reaction medium (F = fructose, F' = transferred fructose):

$$R = G + F \Leftrightarrow F = R - G$$

$$F' = G - F \Leftrightarrow F' = 2\,G - R \qquad \text{(eq. 1)}$$

One unit of fructofuranosidase activity (FA) is defined as the amount of enzyme required for the hydrolysis of 1 µmol of sucrose per minute. One unit of transfructosylating activity (FTA) is defined as the amount of enzyme required to transfer one µmol of fructose (F') per minute.

2.5. Estimation of protein concentration

Protein concentration was measured according to the Lowry method, using bovine serum albumin as the standard (Lowry *et al*, 1951).

2.6. Molecular mass estimation

Sodium dodecil sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to determine protein purity and the molecular mass of the enzymes under denaturing conditions using a 7.5% acrylamide gel, as described by Laemnli (1970). The proteins were silver stained according to the manufacturer's instructions (Silver staining kit proteins- Pharmacia Biotech). A mixture of high molecular weight proteins (HMW

electrophoresis standard – Sigma) was used as molecular mass markers. Electrophoresis was performed at 150 V for 30 – 40 minutes.

The molecular mass of the native enzyme was estimated by gel filtration using a Superdex 200 preparative column (10 x 60cm; Amersham Bioscience Co.), equilibrated with a 50mM sodium phosphate buffer (pH 6.8) containing 100 mM of NaCl. The enzyme was eluted in the same buffer at a flow rate of 1 $mL.min^{-1}$. The column was calibrated using the high molecular weight standard for GPC (PW – 1000 GPC - Sigma).

2.7. Characterization of fructofuranosidase

The activity and stability of the enzyme were measured at various pH values from 3.0 to 7.0 using sodium acetate and sodium phosphate buffers. The enzyme solution was maintained at 50 ℃ in each buffer and the residual activity measured using standard assay conditions.

The effect of temperature on the maximum reaction rate was studied from 30 to 80 ℃, the reaction medium consisting of 50% (w/v) suc rose in 50mM sodium acetate buffer (pH 4.5). Heat stability was determined by incubating the enzyme (crude preparation) in a 50 mM sodium acetate buffer (pH 4.5) at temperatures from 60 to 80 ℃. The residual enzyme activity was checked until a significant decrease was noted.

A central composite rotatable design (CCRD) of experiments was used to confirm the optimal pH and temperature for enzyme activity. Factorial designs and response surface analyses are important tools to determine optimal process conditions. The factorial design is an improvement on the conventional method, which handles a single parameter per trial. Also the conventional method does not consider the effect of possible interactions between factors, which is very important in biological and biochemical processes and when agro-industrial by-products and surpluses are used as substrates (Kalil *et al.*, 2000).

The effect of various metal ions on FA and FTA was studied by measuring the enzyme activity in a solution of 50% (w/v) sucrose in 50 mM sodium acetate buffer (pH 4.5) containing 10 mM of the salt. All experiments were carried out with a diluted enzyme solution containing approximately 15 $FTA.ml^{-1}$.

2.8. Determination of the kinetic parameters

The initial reaction rates were determined for the two kinds of activity with various sucrose concentrations (0.5 – 50%), under standard conditions (pH 4.5, 60℃).

2.9. Fructooligosaccharide production

Fructooligosaccharides were produced by incubating the crude enzyme preparation (1 FTA.ml^{-1}) in a 50% (w/v) sucrose solution in 50 mM sodium acetate buffer, pH 4.5, at 50 ℃ for 72 hours. The synthesis medium was analysed for its carbohydrate composition by high performance liquid chromatography with pulsed amperometric detection (HPLC-PAD) using a Dionex (USA) chromatograph and a Carbopac PA-100 column, equilibrated with 50 mM NaOH and eluted with a linear gradient (0 – 30%) of 500 mM sodium acetate in 50 mM NaOH (1 ml.min^{-1}).

3. Results

3.1. Enzyme production, recovery and purification

The maximum enzyme production, which was about 15.4 (± 1.3) FTA.ml^{-1} and 6.9 (± 1.0) FA.ml^{-1}, was achieved after 16-20 hours of cultivation for FA and 30-36 hours for FTA. Approximately 80% of the FTA and 65% of the FA could be recovered by extraction with 70% ethanol, but in this process almost 90% of the proteins were precipitated, resulting in a poor initial purification step. The use of two-step ethanol extraction (50 – 70%) was also inefficient, despite showing a good purification factor (6), resulting in FTA recovery of only 35%.

The results of the purification procedure are reported in Table 1. The tests were performed with different pH value buffers, and the best pH range for enzyme purification was between 5.5 and 6.0. At pH 5.5 (sodium acetate buffer), the purification factor was higher (104 fold), but at pH 6.0 (sodium phosphate buffer), the recovery was higher (74%).

The elution profile from Q-sepharose (Figure 1) revealed that the target enzyme was only a small part of the protein content. A noteworthy occurrence was that although the enzyme was eluted as a broad and asymmetric peak at pH 5.5, typical of a poly-dispersed glycoprotein, as reported by other authors (L'Hocine *et al.*, 2000), at pH 6.0 a well-defined and sharp peak could be observed.

Table 1: Purification of *Rhodotorula* sp. fructofuranosidase

Step of purification	Total activity (U)	Protein content (mg)	Specific activity ($U.mg^{-1}$)	Purification Fold	Recovery (%)
Crude extract					
FA	916 ± 10	654 ± 5	1.4	1.0	100
FTA	1020 ± 6		1.6	1.0	100
Ethanol precipitation (70%)					
FA	598 ± 5	614 ± 3	0.9	0.6	65
FTA	790 ± 6		1.3	0.8	77
Q-sepharose (pH6.0)					
FA	394 ± 5	13 ± 2	30.3	21.6	43
FTA	756 ± 7		58.8	36.8	74
Q-sepharose (pH5.5)					
FA	415 ± 4	4 ± 2	104.2	74.4	45
FTA	667 ±6		167.3	104.2	65

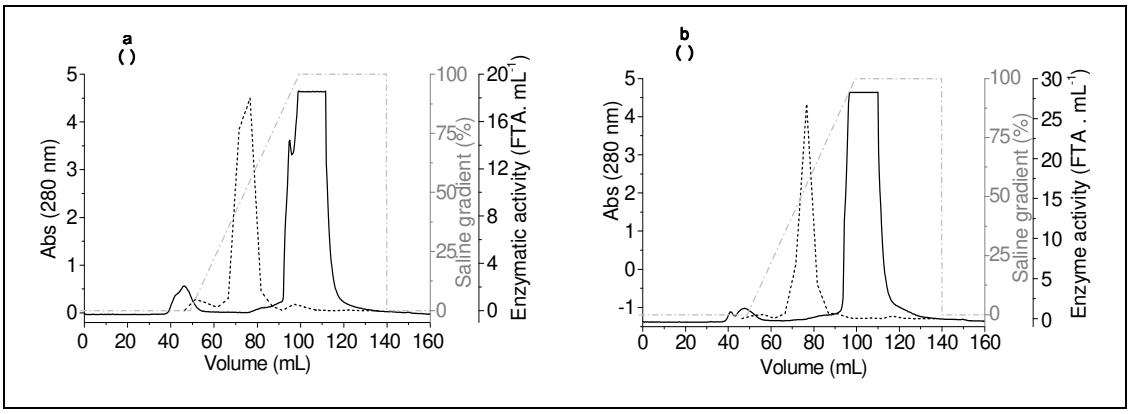

Figure 1: Chromatogram of the crude preparation from the Q-sepharose column
(a) pH 5.5 and (b) pH 6.0; (—) Protein (A_{180}); (—) NaCl gradient; (···) transfructosylating activity.

3.2. Molecular weight determination (GPC and SDS-PAGE)

Electrophoresis under denaturing conditions (SDS-PAGE) on 7.5% polyacrylamide gel indicated that, after purification, two protein bands showing different mobility were

obtained, the first being a poly-dispersed band equivalent to a molecular weight of about 88 kDa, and the second corresponding to about 125 kDa (Figure 2).

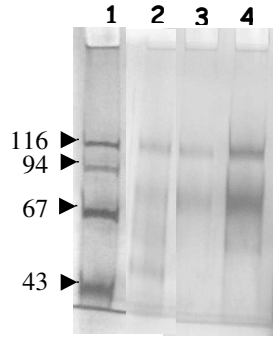

Figure 2: SDS-PAGE (7.5%) of *Rhodotorula* sp. fructofuranosidase.

Lane 1, protein standards; lane 2, crude extract (pool of 2 fractions); lane 3, Q-sepharose purification (pH 5.5) (fraction number 7); lane 4, Q-sepharose purification (pH 6.0)

The FPLC elution profile showed a good peak distribution (Figure 3) when compared to the protein markers, the purified enzyme being eluted as two peaks with molecular weights of approximately 115 and 175 kDa. Although the purification process was not as efficient as expected, considering the SDS-Page results the enzyme would correspond either to a monomer of approximately 120 kDa or to a dimmer of 88 kDa. There is also the possibility of it being, in fact, two different enzymes.

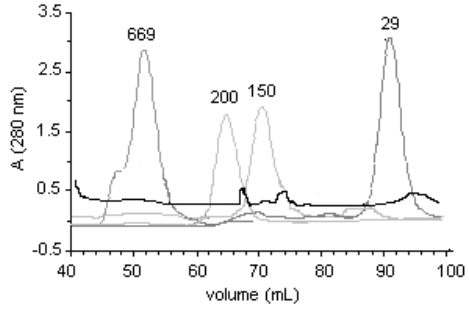

Figure 3: FPLC gel filtration of the crude enzyme extract

(— crude extract, — protein markers)

3.3. Enzyme characterization

Effect of pH on enzyme activity and stability

Both enzyme activities were measured in a pH range from 3 to 7 (using 50 mM sodium acetate and sodium phosphate buffers). As shown in Figure 4a, the optimum pH was found to be around 3.5 for FTA and 4.0 for FA.

In order to determine the effect of pH on enzyme stability, the ethanol precipitated enzyme was incubated in the same buffers at 50℃ fo r 200 hours (Figure 4b). The residual enzyme activities were then assayed by the standard method. In the case of the fructosyl transferase activity, the highest stability was around pH 4.5 and for fructofuranosidase activity, the best stability was found at pH 5.0.

Effect of temperature on enzyme activity and stability

As shown in Figure 4c, the enzyme showed the highest FTA activity at temperatures above 62℃ and maximum FA activity at 55℃. With re spect to heat stability, the FTA activity was more stable then FA at temperatures between 60 and 66℃ (Figure 4d), although the enzyme was quickly deactivated at temperatures above 70 ºC.

The half-life of the enzyme decreased very quickly at temperatures above 60℃ for both FTA and FA activities, as shown in Figure 4d. The activation and denaturation energies for FTA were 12.00 Kcal.mol^{-1} and 170.52 Kcal.mol^{-1}, respectively, and for FA, 7.00 and 150.50 Kcal.mol^{-1}, respectively.

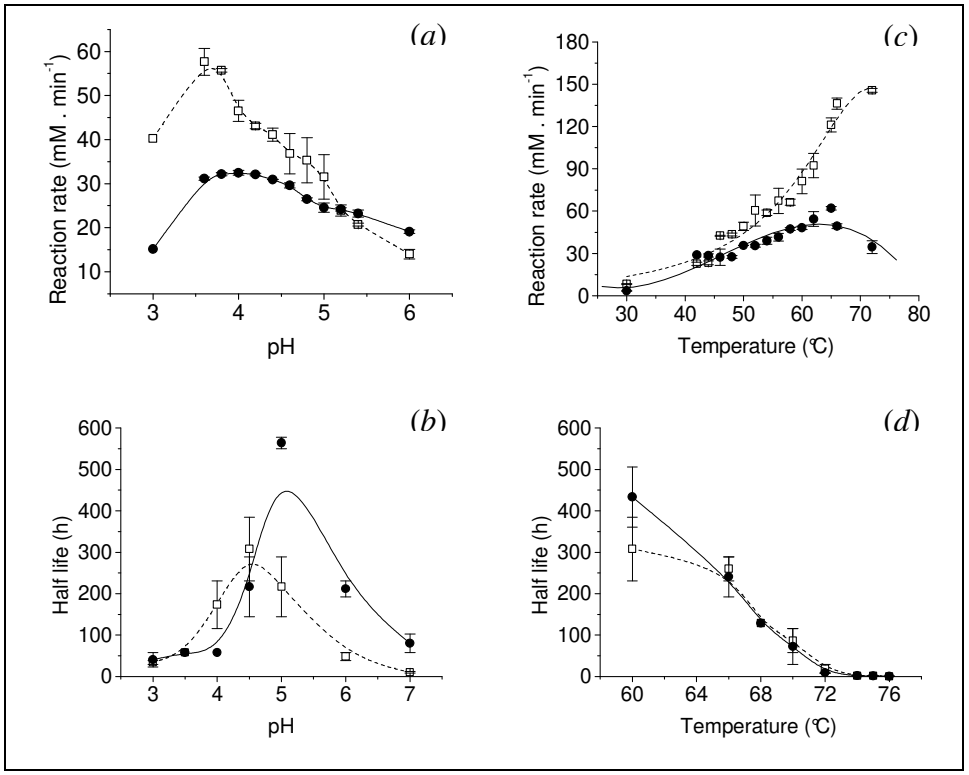

Figure 4: Effect of pH and temperature on enzyme activity and stability
(a) pH-enzyme activity; (b) pH – enzyme stability; (c) Temperature – enzyme activity; (d) Temperature – enzyme stability. (● FA; □ FTA)

Optimum enzyme conditions

Considering the above results for the effects of pH and temperature on enzyme activity, a factorial design was used to confirm these results. The experimental conditions and the results for the FA and FTA activities according to the factorial design, are shown in Table 2. Both activities were analysed in order to obtain high FOS production, since for this purpose the enzyme requires high fructosyl transferase activity and low hydrolytic activity.

An estimate of the main effect is obtained by evaluating the difference in process performance caused by a change from the low (-1) to the high (+1) levels of the corresponding factor (Haaland, 1989). The statistical parameters t-test and p-value were used to confirm the significance of the factors studied, $p<0.05$ suggesting significance at the 0.05 level, or a 95% confidence level.

Table 2: Experimental design and results of the 2^2 central composite design

Trial	Coded levels (real values)		Enzyme activity (U. ml^{-1})	
	pH	T (℃)	FA	FTA
1	-1 (3.5)	-1 (60)	90.20	144.78
2	+1 (4.5)	-1 (60)	51.01	117.18
3	-1 (3.5)	+1 (70)	76.60	284.89
4	+1 (4.5)	+1 (70)	34.75	20.30
5	-α (3.3)	0 (65)	168.68	478.23
6	+α (4.7)	0 (65)	143.78	218.41
7	0 (4.0)	-α (58)	47.16	78.70
8	0 (4.0)	+α (72)	89.52	128.21
9	0 (4.0)	0 (65)	109.12	477.08
10	0 (4.0)	0 (65)	119.64	472.84
11	0 (4.0)	0 (65)	110.14	461.40

[a] $\alpha = \pm 1.41$

A fitted model was accomplished for the FA and FTA responses: the independent and dependent variables were fitted to a second-order model equation and examined in terms of goodness of fit. ANOVA (Tables 4 and 5) was used to evaluate the adequacy of

the fitted model. The R-squared value provided a measure of how much the model could explain the variability of the response observed.

Table 3: Analysis of variance for FA activity (ANOVA)

	SS	df	MS	F[b]
Regression (R)	10837.25	3	3612.42	4.05
Residual (r)	6250.42	7	892.92	
Lack of fit	6183.10	5		
Pure Error	67.32	2		
Total	17087.67	10		

[a] Residual = Lack of fit + Pure error; [b] $F_{0.10;3;7}$= 3.07; R-sqr = 0.63

Table 4: Analysis of variance for FTA activity (ANOVA)

	SS	df	MS	F[b]
Regression (R)	307409.54	5	61481.91	21.24
Residual (r)	14473.87	5	2894.77	
Lack of fit	14396.06	3		
Pure Error	77.81	2		
Total	321883.40	10		

[a] Residual = Lack of fit + Pure error; [b] $F_{0.05;5;5}$= 5.05; R-sqr = 0.96

Based on the analysis of variance (ANOVA), a second order model was established, describing the enzyme activity as a function of pH and temperature (eqs. 2 and 3), since the calculated F value was higher than the critical F value. The coded model was used to generate response surfaces to analyse the effects of the variables on FA (Figure 5) and FTA (Figure 6).

$$FA \cdot ml^{-1} = 113.16 - 14.56 * pH + 9.30 * pH^2 - 34.90 * T^2 \quad \text{(eq. 2)}$$

$$FTA \cdot ml^{-1} = 468.88 - 82.56 * pH - 81.43 * pH^2 + 14.17 * T - 204.59 * T^2 - 59.25 * pH*T \quad \text{(eq.3)}$$

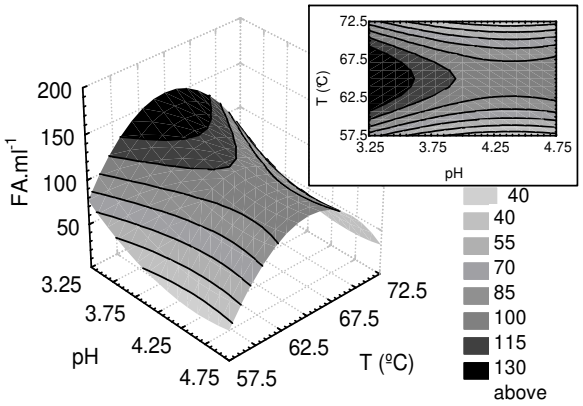

Figure 5: Response surface and contour diagrams for the FA as a function pH and temperature

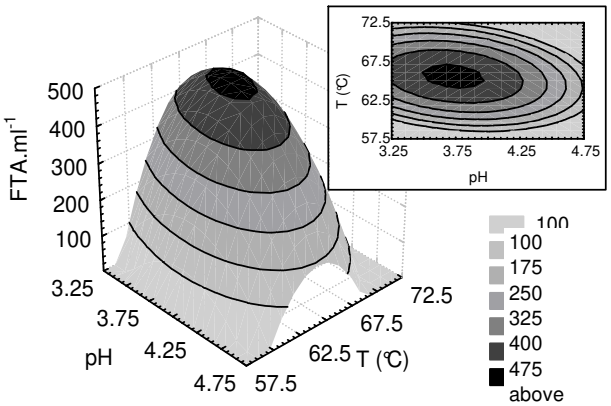

Figure 6: Response surface and contour diagrams for the FTA as a function pH and temperature

The temperature required for maximum FA activity (Figure 5) was between 62 and 68 ºC, with a pH value between 3.25 and 3.5. Therefore to maximize the FTA activity (Figure 6), both these variables must be kept near the central levels tested. The optimised condition for fructosyl transferase activity was thus: 65 ℃ ± 2℃ and pH values around 3.75 (± 0.25).

Effect of metals

The influence of various ions on the FTA and FA activities was studied. The enzyme activities were assayed using the standard methods. As shown in Table 2, Zn^{+} and Mn^{2+} were the only two ions that did not inhibit the hydrolytic activity at 10 mM. On the other hand, Na^{+}, K^{+} and Ca^{2+} ions stimulated the transfructosylating activity, whilst in the

presence of Mn^{+2} only 63% of the initial activity was retained, and the ion Zn^{+} caused the loss of 60% of the initial activity.

It was not verified whether these effects were relating to ion binding to the enzyme causing conformational changes, or the requirement by the enzyme for a metal ion at the active site. Further work is necessary to ascertain the mode of action.

Table 2: Effect of ions on FTA and FA activity

Ion	% residual activity*	
	FA	FTA
None	100	100
Citric acid	87	81
$CuSO_4$	32	92
NaCl	66	139
KCl	79	120
$MnCl_2$	93	63
$CaCl_2$	82	122
$ZnSO_4$	100	40

* Means of duplicates

3.4. Determination of kinetics parameters

The kinetic parameters for each kind of activity were determined using non-linear estimation procedures. The Michaellis-Mentem model did not fit the experimental data. As can be seen in Figure 7, substrate inhibition behaviour was observed, where v was the reaction rate, v_{max} the limiting reaction rate, K_m the substrate concentration to reach a reaction rate of half v_{max} and K_i a factor related to the inhibitory effect of the substrate (eq. 3).

$$v = \frac{v_{max}\, S}{S + K_m + \frac{S^2}{K_i}} \qquad \textbf{(eq. 3)}$$

The values for K_m, K_i and v_{max} were found to be 4.7 $g.l^{-1}$, 463.1 $g.l^{-1}$ and 150.3 $\mu mol.ml^{-1}.min^{-1}$ for FA activity and 13.6 $g.l^{-1}$, 246.4 $g.l^{-1}$and 281.8 $\mu mol.ml^{-1}.min^{-1}$ for FTA activity, respectively.

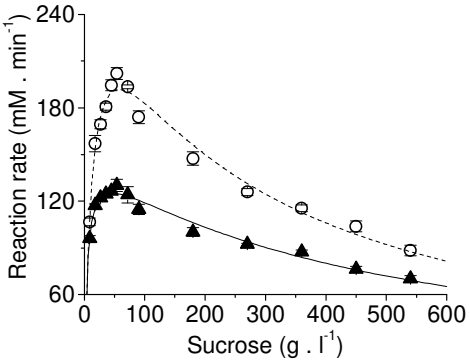

Figure 7: Kinetic behavior of *Rhodotorula sp*. (LEB-U5) enzymes

○ FTA (R^2 = 93.1%); ▲ FA (R^2 = 95.2%)

3.5. Fructooligosaccharides production

The typical time course for the sugar concentration data is presented in Figure 8. The synthesis was carried out in triplicate, resulting in a mean standard error of 7%. The reaction was carried out by incubation of the crude enzyme preparation (1 FTA.ml^{-1}) in a 50% (w/v) sucrose solution in 50 mM sodium acetate buffer, pH 4.5, at 50 ℃. A FOS yield of 195 g.l^{-1} was achieved, but only at the end of the reaction when the sucrose was almost totally consumed, the glucose and fructose concentrations being 170 and 120 g.l^{-1}, respectively.

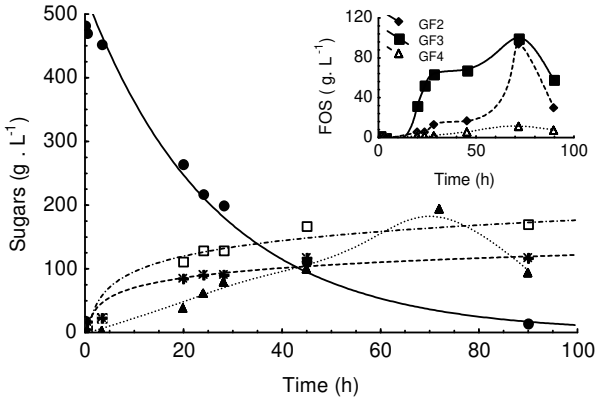

Figure 8: The time course for sugar concentration during enzymatic synthesis of fructooligosaccharides

(▲ FOS; • Sucrose, ✶ fructose, □ glicose)

4. Discussion

An enzyme with high transfructosylating activity obtained from *Rhodotorula* sp. (LEB-U5) was studied, and shown to have a potential for application in fructooligosaccharide

production. The enzyme was purified with 65-75% of recovery and a 40-100 purification fold factor, using HiLoad™ 16/10 Q Sepharose® Fast Flow (Pharmacia Biotech). The gel permeation and electrophoretic mobility data were not conclusive, suggesting that the enzyme was either a 120kDa monomer or a dimer with an approximate average subunit size of 88 kDa.

The effects of temperature, pH and metal ion activation/inhibition on the catalytic properties of the *Rhodotorula* sp. fructosyl transferase were studied. The use of copper ($CuSO_4$), potassium (KCl), calcium ($CaCl_2$) and sodium (NaCl) might be beneficial to the process, because all of these caused inhibition of the FA activity with little loss, or even slight activation, of the FTA activity. In addition to indicating the amino acid residues involved in the active site, the study of the enzyme behaviour in solutions containing different metal ions was important, since we were working with two kinds of activity and the production of fructooligosaccharides. Concerning FOS production, one of the most important characteristics is the ratio between the two types of activity (Hidaka *et al*, 1988).

When comparing this enzyme with other yeast enzymes, the *Rhodotorula* sp. (LEB-U5) enzyme had a similar pH optimum (around 4.0 for FTA and FA) and temperature optimum (55 - 60℃ for FA; 65 - 70℃ for FTA), to t hose of the invertase from *Candida utilis* (Belcarz *et al*., 2002) and the invertase (Rubio *et al*, 2002) and β-glucosidases (Onishi and Tanaka, 1996) from *Rhodotorula minuta*.

β-fructofuranosidase usually possesses both fructosyl transferase and hydrolytic activity, the hydrolytic activity being a particular case involving transfer to water in dilute reaction systems (Paul and Monsan, 1995). However, it was observed that the enzyme from *Rhodotorula* sp. (LEB-U5) showed both activities at high and low sucrose concentration, behaviour observed in transferase type enzymes. A Michaellis-Mentem model with substrate inhibition could explain the kinetics of both activities.

The Michaellis-Mentem model (with or without substrate inhibition) can explain the kinetics of both the invertase and fructotransferases from moulds and yeasts related to oligosaccharide production, such as the glucosyltransferase, fructosyl transferase and glycosidase from *Aspergillus foetidus* (Wang and Rakshit, 2000), and the fructosyl transferase and invertase from *Aspergillus niger* (L'Hocine *et al*., 2000).

In terms of FOS synthesis, a 40% sucrose conversion to FOS was achieved after 72 hours of reaction, a yield lower than that obtained with the enzymes from *Aspergillus* and *Aureobasidium*, which reached from 50 to 60% conversion to FOS (Chen and Liu, 1996 and Sageetha *et al*., 2005). In the present study, the yield was affected by the strong hydrolase activity occurring at the low sucrose concentrations found after 72 hours of reaction, as shown in Figure 8, when the fructooligosaccharides started being hydrolysed at a high rate.

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ARTIGO 6 :

FRUCTOOLIGOSACCHARIDES PRODUCTION BY CANDIDA SP.

Produção de frutooligossacarídeos por *Candida* sp.

(Bioresource technology)

FRUCTOOLIGOSACCHARIDES PRODUCTION BY *CANDIDA* SP.

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Abstract

The present work was carried out with the aim of investigating fructooligosaccharide (FOS) production by an extra-cellular fructofuranosidase from *Candida* sp. LEB-I3 (Laboratory of Bioprocess Engineering – Unicamp- Brazil). The enzyme was produced by fermentation. After cell separation from the fermented medium, the enzyme was concentrated by ethanol precipitation and then purified by anion exchange chromatography. The enzyme exhibited both fructofuranosidase (FA) and fructosyl transferase (FTA) activities in both low and high sucrose concentrations. With sucrose as substrate, the data fitted the Michaellis-Mentem model for FA, but showing a rather substrate inhibitory form for the fructosyl transferase activity. The K_m and v_{max} values were shown to be respectively 13.4 $g.l^{-1}$ and 21.0 $\mu mol.ml^{-1}.min^{-1}$ for FA, and 25.5 $g.l^{-1}$ and 52.5 $\mu mol.ml^{-1}.min^{-1}$ for FTA. FTA presented an inhibitory factor K_i of 729.8 $g.l^{-1}$. The optimum conditions for FA activity were found to be pH 3.25 – 3.5 and temperatures of around 69°C, while for FTA the optimum conditions were 65°C (±2°C) and pH 4.00 (±0.25). Both activities were very stable at temperatures below 60°C, while for FA the greatest stability occurred at pH 5.0 and for FTA at pH 4.5-5.0. Despite the strong fructofuranosidase activity, causing hydrolysis of the fructooligosaccharides, the high transfructosilating activity allowed for high FOS production from sucrose (44%).

Keywords: *Candida* sp.; fructooligosaccharides, enzyme characterization

1. Introduction

The development of foods with health benefits is a trend that is becoming popular, consumer demand being the propelling force for the functional food market. The good intestinal flora balance associated with the consumption of fructooligosaccharides (FOS), inducing the proliferation of intestinal bifidobacteria (probiotics), plus other properties of FOS, have led to their popularity as food ingredients, including as alternative sweeteners. FOS are oligosaccharides made up of 1 to 3 fructose units bound to a sucrose unit: 1-kestose (GF2), nystose (GF3) and fructofuranosyl-nystose (GF4) (Gibson & Roberfroid, 1995).

Several microorganisms are capable of producing FOS, most studies involving bacterial or fungal fructosyl transferases (Sageetha *et al*., 2005). The bacterial strains are capable of producing certain types of FOS, the final yield being around 30%. *Bacillus macerans* EG-6 produced GF5 and GF6 selectively (Park *et al*, 2001), while *Zymomonas mobilis* produced GF2, GF3, 6-kestose and neokestose (Beker *et al*., 2002).

Of the fungal strains the most important are *Aspergillus* sp., reported to produce between 50 and 60% of FOS using 40 to 60% of sucrose as substrate (L'Hocine *et al*., 2000, Chien *et al*., 2001, Sangeetha *et al*., 2005). Food-grade FOS is produced commercially from sucrose or inulin using intracellular enzymes from fungi like *Aspergillus* and *Aureobasidium* (Yun, 1996).

The production of FOS by yeasts is not very common apart from the well-known growth on sucrose associated with the extra cellular enzymes invertase and inulinase. Risso (2004) and Santos (2003) carried out extensive studies on FOS production by *Kluyveromyces* strains, however, because of the high hydrolytic activity, these trials resulted in low FOS production (12% of yield).

Fungi are generally accepted as the largest group of organisms on the Earth after the insects, but only recently are they being noticed with respect to biodiversity stakes. Tropical environments (forests) are one of the greatest sources of all kinds of living organisms, including the yeasts, unicellular fungi with a ubiquitous distribution throughout almost all ecosystems. Brazil has four biomes of great biodiversity, including the Amazon Forest, Atlantic Forest (stretches along the Brazilian coast with an extremely diverse and unique mix of vegetative types), Cerrado (tropical savanna ecoregion) and Pantanal (the world's largest wetland area).

Since the tropical fungi are essential to the survival of other organisms, they are crucial in global ecological processes, being a source of novel bioactive compounds and able to contribute to sustainable development (Hawksworth, 2002). Since Brazil is also a big sucrose producing country (sugar-cane), and also considering the increasing demand for FOS in functional foods, it would be of interest to find alternative processes for the production of oligosaccharides from sucrose.

Candida strains have been studied since the 70s for the production of biomass and biodegradation of industrial and agricultural by-products and surpluses, because of their great ability to use a large variety of carbon sources (Rajoka *et al*., 2005). The productions of lipase, β-fructofuranosidase and xylitol by *Candida* sp. are also well known (Belcarz *et al*., 2002; Carvalho *et al*., 2005). In previous work (Hernalsteens and Maugeri, 2006), the isolation of yeast strains aiming at FOS production by extra cellular yeast enzymes resulted in the selection of four strains, one of which was a *Candida* strain (*Candida* sp. LEB-I3 - Laboratory of Bioprocess Engineering – UNICAMP - Brazil).

The present work describes the fructosyl-transferase activity of an extra cellular enzyme from the strain of *Candida* (LEB-I3) isolated from Brazilian indigenous flora (Pantanal), and some of its biochemical properties.

2. Materials and Methods

2.1. Microorganism and cultivation conditions

The *Candida* sp. (LEB-I3: Laboratory of Bioprocess Engineering – UNICAMP - Brazil) was isolated from flowers found in the Pantanal. The culture was maintained on GYMP agar medium at 4℃.

The same medium was used for both the inoculum and for enzyme production, the standard medium consisting of (per litre): 50 g sucrose, 20 g yeast extract, 10 g $NaNO_3$, 0.5 g $MgSO_4.7H_2O$ and 5 g K_2HPO_4 (pH 5.0). The inoculum was incubated at 30℃ for 2 4 hours at 150 rpm. For the enzyme producing culture, the same conditions were used, except for the fermentation time, which was 48 hours.

2.2. Enzyme recovery

A clear supernatant was obtained by centrifuging the culture broth at 5℃, (4000 G, 10 min) and the enzyme recovered by adding ethanol to a final concentration of 70% (0 – 4℃). The precipitate was removed by centrifugation (2℃, 6000G, 10 min.), dissolved in 50 mM sodium acetate buffer (pH 4.5) and stored at -18℃.

A two-step ethanol fractionation was also carried out. In this case 95% ethanol was slowly added to the cell-free supernatant to 50% saturation. After the removal of the precipitate, by centrifugation, more ethanol was added to the supernatant to a final concentration of 70%, and the enzyme-rich precipitate again collected by centrifugation.

2.3. Enzyme purification

The crude enzyme solution was applied to an anionic column (HiLoad™ 16/10 Q Sepharose® Fast Flow - Pharmacia Biotech) equilibrated with 50 mM sodium acetate buffer (pH 5 - 6). The non-adsorbed proteins were eluted with the starting buffer and the adsorbed proteins and enzymes were eluted with a linear gradient of NaCl (0 to1 M) in the same buffer, at a flow rate of 1 $mL.min^{-1}$.

2.4. Enzyme assay

The reaction media used to determine the enzyme activity consisted of 50% (w/v) sucrose (in 50 mM sodium acetate buffer, pH 4.5) and 10% (v/v) of an adequately diluted enzyme suspension at 50 ºC. Samples were collected at constant time intervals for 30 minutes of reaction time, and used to quantify glucose using commercial glucose-oxidase kits, and reducing sugars by the Somogi-Nelson method.

Sucrose conversion by fructofuranosidase yields glucose and fructose, but when the transfructosilating activity is also present, part of the fructose is built into a fructan polymer. By measuring the amounts of glucose and reducing sugars released into the reaction medium, the hydrolytic and transfructosylating activities can thus be assessed (Chen and Liu, 1996). The equation below allows for the determination of the activities by estimating glucose (G) and reducing sugars (R) in the reaction medium (F = fructose, F' = transferred fructose):

$$R = G + F \Leftrightarrow F = R - G$$

$$F' = G - F \Leftrightarrow F' = 2\,G - R \qquad \text{(eq. 1)}$$

One unit of fructofuranosidase activity (FA) was defined as the amount of enzyme required for the hydrolysis of 1 µmol of sucrose per minute. One unit of fructosyl transferase activity (FTA) was defined as the amount of enzyme required to transfer one µmol of fructose (F') per minute.

2.5. Estimation of protein concentration

Protein concentration was measured according to the Lowry method, using bovine serum albumin as the standard (Lowry *et al*, 1951).

2.6. Molecular mass estimation

Sodium dodecil sulphate-poliacrylamide gel electrophoresis (SDS-PAGE) was used to determine protein purity and the molecular mass of the enzymes under denaturing conditions, using a 7.5% acrylamide gel, as described by Laemnli (1970). The proteins were silver stained according to the manufacturer's instructions (Silver staining kit proteins- Pharmacia Biotech). A mixture of high molecular weight proteins (HMW electrophoresis standard – Sigma) was used as molecular mass markers. Electrophoresis was performed at 150 V for 30 – 40 minutes.

2.7. Characterization of fructofuranosidase

The activity and stability of the enzyme were measured at various pH values from 3.0 to 7.0 using sodium acetate and sodium phosphate buffers. The enzyme solution was maintained at 50 ℃ in each buffer and the residual activity measured using standard assay conditions.

The effect of temperature on the maximum reaction rate was studied from 30 to 80 ℃, the reaction media consisting of 50% (w/v) sucr ose in 50mM sodium acetate buffer (pH 4.5). Thermo-stability was determined by incubating the enzyme (crude preparation) in 50 mM sodium acetate buffer (pH 4.5) at temperatures from 60 to 80 ℃. The residual enzyme activity was checked until a significant decrease was noted.

A central composite rotatable design (CCRD) of experiments was used to confirm the optimal pH and temperature for enzyme activity. Factorial design and response surface analysis are important tools to determine optimal process conditions. Factorial design is an advantage as compared to the conventional method, which only handles a single parameter per trial. The conventional method does not consider the effect of possible interactions between factors, very important in biological and biochemical processes and when substrates such as agro-industrial by-products and surpluses are used (Kalil et al., 2000).

The effect of various metal ions on the transfructosylating activity was studied by determining the enzyme activity in a solution consisting of 50% (w/v) sucrose in 50 mM

sodium acetate buffer (pH 4.5) plus 10 mM of the salt. All experiments were carried out with a diluted enzyme solution containing approximately 15 FTA.ml^{-1}.

2.8. Determination of the kinetic parameters

The initial reaction rates were determined for the two kinds of activity under their optimum conditions (pH 4.5, 60℃) for various sucro se concentrations (0.5 – 50%). All experiments were carried out using a diluted enzyme solution containing approximately 10 FTA.ml^{-1}.

2.9. Fructooligosaccharide production

The crude enzyme preparation (1 FTA.ml^{-1}) was incubated in 50% (w/v) sucrose solution, using 50 mM sodium acetate buffer (pH 4.5) at 50 ℃ for 72 hours. The synthesis medium was analysed for its carbohydrate composition by high performance liquid chromatography equipped with pulsed amperometric detection (HPLC-PAD), using a Dionex (USA) chromatograph and a Carbopac PA-100 column equilibrated with 50 mM NaOH. Elution was carried out using a linear gradient (0 – 30%) of 500 mM sodium acetate in 50 mM NaOH (1 mL.min^{-1}).

3. Results

3.1. Enzyme production, recovery and purification

Maximum enzyme production was achieved after 30-40 hours of cultivation, for both hydrolytic and transfructosylating activities, being 8.0 ± 1.0 FTA.ml^{-1} in the case of the latter. About 90% of the transfructosilating activity and 50% of the hydrolytic activity could be recovered using two-step ethanol extraction. This process eliminated almost 90% of the proteins, resulting in an efficient initial purification step. The addition of 70% ethanol as a single step procedure resulted in the precipitation of all the high molecular compounds from the fermented broth, which made the use of chromatographic purification difficult.

The results of the purification procedure are reported in Table 1. The tests in buffers with different pH values showed that the best enzyme purification was achieved at a pH value between 5.5 and 6.0. At pH 5.5 (sodium acetate buffer) the purification factor was higher (20 fold), but at pH 6.0 (sodium phosphate buffer) the recovery was slightly more efficient.

The elution profile on Q-sepharose (Figure 1) revealed that almost all the protein content was not the target enzyme, but it was noteworthy that the enzyme eluted as a

broad, asymmetric peak, a behaviour quite typical of polydispersed glycoproteins, as reported by other authors (L'Hocine *et al.*, 2000).

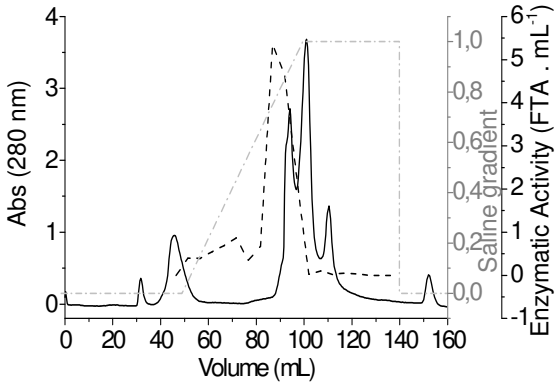

Figure 1: Crude preparation chromatogram on a Q-sepharose column (pH 5.5)
(—) Protein (A_{180}); (–•–) NaCl gradient; (···) transfructosylating activity.

Table 1: Purification of *Candida* sp. fructofuranosidase

Step of purification	Total activity (U)	Protein content (mg)	Specific activity ($U.mg^{-1}$)	Purification Fold	Recovery (%)
Crude extract					
FA	2416 ± 05	1178 ± 15	2.0	1.0	100
FTA	3291 ± 12		2.8	1.0	100
Ethanol precipitation (50 – 70%)					
FA	1147 ± 50	109 ± 5	10.6	5.2	48
FTA	3037 ± 60		28.0	10.0	92
Q-sepharose (pH6.0)					
FA	596 ± 20	55 ± 4	10.9	5.3	25
FTA	1857 ± 25		34.0	12.2	56
Q-sepharose (pH5.5)					
FA	387 ± 8	30 ± 2	12.7	6.2	16
FTA	1680 ±10		55.2	19.7	51

3.2. Molecular weight determination (GPC and SDS-PAGE)

Denaturing electrophoresis (SDS-PAGE) on 7.5% polyacrylamide gel indicated that, after purification, 3 to 6 different protein bands were present (Figure 2): 35, 47, 53, 68, 78, and 120 kDa. These results indicated that although the purification procedures were quite

efficient, the purification factors were still low and the quantity and variety of proteins in the samples still high.

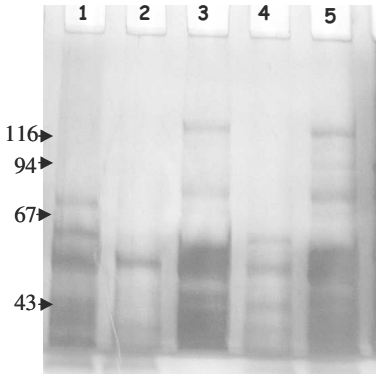

Figure 2: SDS-PAGE (7.5%) of *Candida* sp. fructofuranosidase.
Lane 1: Q-sepharose purification pH 5.5- pool of three fractions; lane 2: Q-sepharose purification pH 5.5 – fraction number 15; lane 3: Q-sepharose purification (pH 6.0) - pool of three fractions; lane 4, Q-sepharose purification (pH 6.0) - fraction number 16; lane 5: crude extract

3.3. Enzyme characterization

Effects of pH on enzyme activity and stability

Both enzyme activities were measured in the pH range from 3 to 7 (using 50 mM sodium acetate and sodium phosphate buffers). As shown in Figure 3*a*, the optimum pH was found to be between 3.5 and 4.5 for both kinds of activity.

In order to determine the effect of pH on enzyme stability, the ethanol precipitated enzyme was incubated in the same buffers at 50°C for 200 hours (Figure 3*b*). The residual enzyme activities were then assayed by the standard method. In the case of FTA, the greatest stability was around pH 4.5 – 5.0. For FA the greatest stability was found to be at pH 5.0.

Effects of temperature on enzyme activity and stability

As shown in Figure 3*c*, the enzyme showed the highest FTA and FA activities at the highest temperature (72°C). With respect to heat stability (Figure 3*d*), it was found that both kinds of activity were stable at 60°C, and at lower temperatures, both FTA and FA showed no decrease in activity after 200 hours of incubation.

The half-life of the enzyme decreased very quickly at temperatures above 60°C for FTA or above 65°C for FA, as shown in Figure 4 *d*. The activation and denaturation energies for FTA were 12.83 Kcal.mol^{-1} and 146.79 Kcal.mol^{-1}, respectively, and for FA, they were 7.09 and 140.30 Kcal.mol^{-1}, respectively.

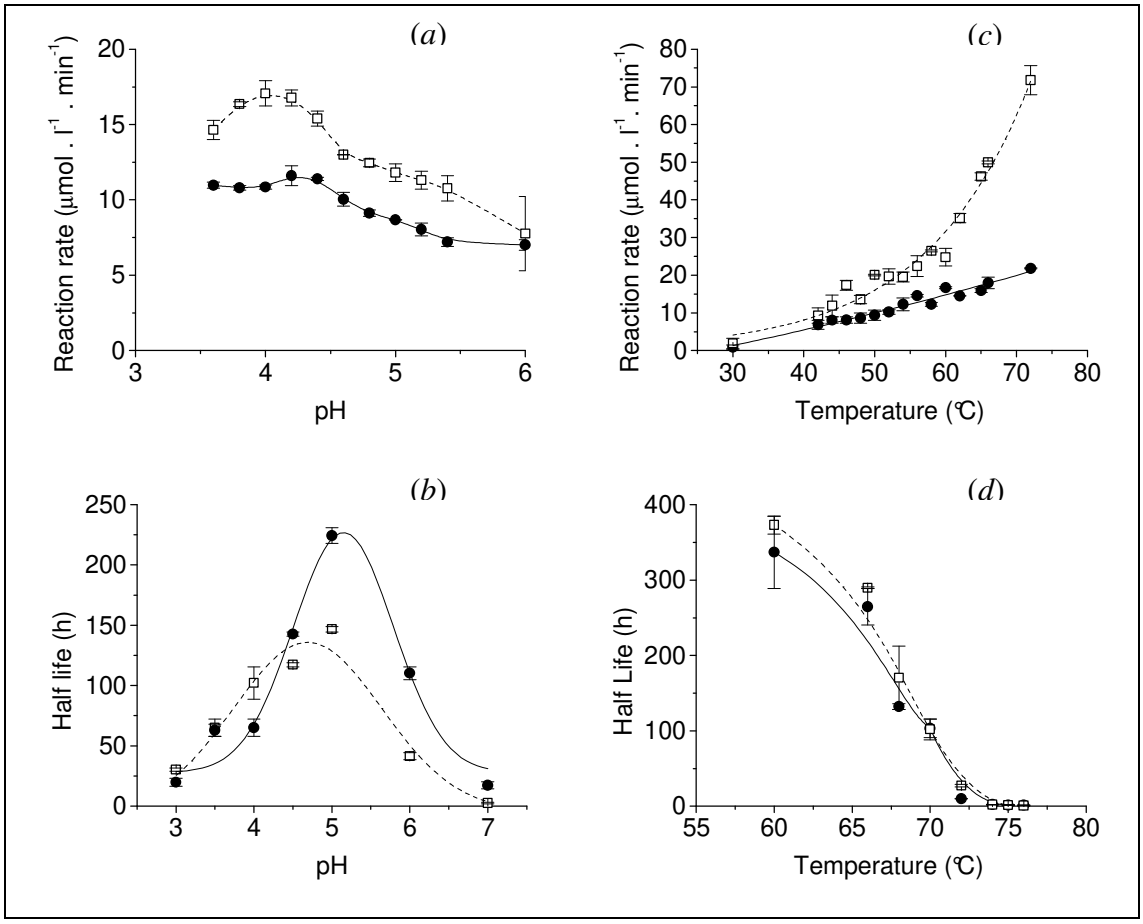

Figure 3: Effect of pH and temperature on enzyme activity and stability
(a) pH-enzyme activity; (b) pH – enzyme stability; (c) Temperature – enzyme activity; (d) Temperature – enzyme stability. (• FA; □ FTA)

Optimum enzyme conditions

Considering the results obtained for the optimum pH and temperature for the enzyme activities, a factorial design was done to confirm these results. The experimental conditions and the results for the FA and FTA activities in the factorial design are shown in Table 2. Both types of enzyme activity were analysed in order to obtain high FOS production, since for this purpose, the enzyme requires high fructosyl transferase activity and low hydrolytic activity.

An estimate of the main effect is obtained by evaluating the difference in process performance caused by a change from the low (-1) to the high (+1) level of the corresponding factor (Haaland, 1989). The statistical parameters t-test and p-value were

used to confirm the significance of the factors studied and in this case, $p<0.05$ suggested significance at the 0.05 level, or a 95% confidence level.

Table 2: Experimental design and results of the 2^2 central composite design

Trial	Coded levels (real values)		Enzyme activity (U. ml^{-1})	
	pH	T (℃)	FA	FTA
1	-1 (3.5)	-1 (60)	43.05	102.31
2	+1 (4.5)	-1 (60)	19.77	29.58
3	-1 (3.5)	+1 (70)	105.44	94.15
4	+1 (4.5)	+1 (70)	23.79	31.34
5	-α (3.3)	0 (65)	103.04	72.82
6	+α (4.7)	0 (65)	44.16	167.29
7	0 (4.0)	-α (58)	17.63	31.35
8	0 (4.0)	+α (72)	46.39	72.25
9	0 (4.0)	0 (65)	82.16	267.47
10	0 (4.0)	0 (65)	83.19	268.08
11	0 (4.0)	0 (65)	86.01	269.90

[a] $\alpha = \pm 1.41$

A fitted model was accomplished for the FA and FTA responses: the independent and dependent variables fitting the second-order model equation, and were examined in terms of goodness of fit. The ANOVA (Tables 3 and 4) was used to evaluate the adequacy of the fitted model. The R-squared value provided a measure of how much the model could explain the variability in the response observed.

Based on the analysis of variance (ANOVA), a second order model was established, describing the enzyme activity as a function of pH and temperature (eqs. 2 and 3), since the calculated F value was higher than the critical F value. The coded model was used to generate response surfaces to analyse the effects of the variables on FA (Figure 4) and FTA (Figure 5).

Table 3: Analysis of variance for fructofuranosidase activity (ANOVA)

	SS	df	MS	F[b]
Regression (R)	10878.75	5	2175.75	57.54
Residual (r)	189.08	5	37.82	
Lack of fit	181.12	3		
Pure Error	7.95	2		
Total	11067.83	10		

[a] Residual = Lack of fit + Pure error; [b] $F_{0.05;5;5}$= 5.05; R-sqr = 0.98

Table 4: Analysis of variance for FTA activity (ANOVA)

	SS	df	MS	F[b]
Regression (R)	86646.64	3	28882.21	19.38
Residual (r)	10434.98	7	1490.71	
Lack of fit	10431.79	5		
Pure Error	3.19	2		
Total	97081.62	10		

[a] Residual = Lack of fit + Pure error; [b] $F_{0.05;37}$= 4.34; R-sqr = 0.89

$$FA.ml^{-1} = 83,8 - 23,56*pH - 6,29*pH^2 + 13,41*T - 27,21*T^2 - 14,59*pH*T \quad \text{(eq. 2)}$$

$$FTA{\cdot}ml^{-1} = 268,56 - 79,85 * pH^2 + 6,43 * T - 114,18 * T^2 \quad \text{(eq.3)}$$

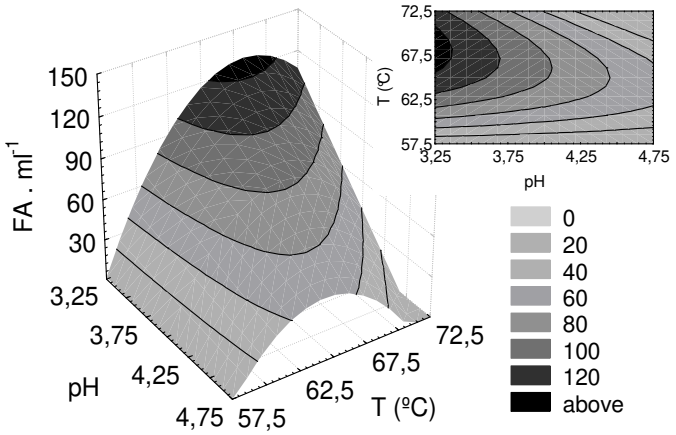

Figure 4: Response surface and contour diagrams for the FA as a function pH and temperature

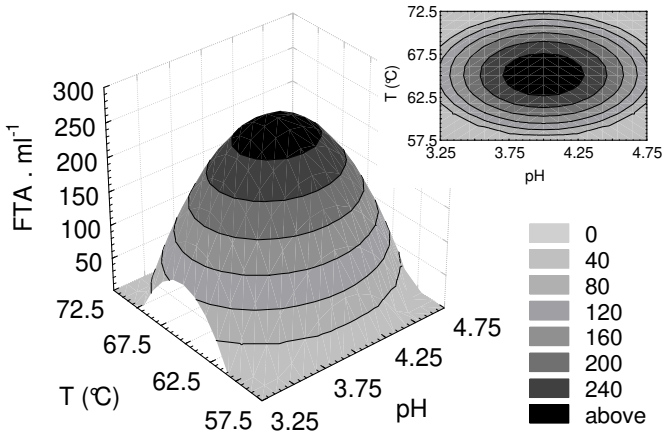

Figure 5: Response surface and contour diagrams for the FTA as a function pH and temperature

The temperature required for maximum FA was between 68 and 70 ºC with a pH value between 3.25 and 3.5. Therefore to maximize the FTA activity, both variables should be kept near the central levels tested, so the optimised condition for fructosyl transferase activity was: 65 ℃ ± 2℃ and pH values around 4.00 (± 0.25).

Effect of metals

The FTA and FA activities were studied in terms of the influence of different ions. The activities were determined by the standard method. As shown in Table 5, the only ions that did not inhibit the fructofuranosidase activity at 10 mM were K^{+}, Ca^{+2} and Mn^{2+}. On the other hand, the fructosyl transferase activity was only slightly affected by the ions, apart from the ion Cu^{+2}, which caused the loss of 38% of the initial activity.

It was not verified whether these effects were related to the ion binding to the enzyme causing conformational changes, or the requirement by the enzyme for a metal ion at the active site. Further work is necessary to ascertain their mode of action.

Table 5: Effect of ions on FTA and FA activity

Ion	% Residual activity*	
	FA	FTA
None	100	100
Citric acid	90	92
$CuSO_4$	23	62
NaCl	72	84
KCl	120	90
$MnCl_2$	100	81
$CaCl_2$	96	103
$ZnSO_4$	87	78

*** Means of duplicates**

3.4. Determination of kinetic parameters

The kinetic parameters for each kind of activity were determined using non-linear estimation procedures (Figure 6). Although it was possible to use the Michaellis-Mentem model (eq.4) for FA activity, this model did not fit the experimental data of FTA, for which substrate inhibition was observed (eq.5). The mathematical models are described below, where *v* is the reaction rate, v_{max} the limiting reaction rate, K_m the substrate concentration to attain a reaction rate of half v_{max} and K_i related to the inhibitory effect of the substrate.

$$v = v_{max} \frac{[S]}{K_m + [S]} \qquad \textbf{(eq.4)}$$

$$v = \frac{v_{max} S}{1 + K_m + \frac{S^2}{K_i}} \qquad \textbf{(eq.5)}$$

The K_m and v_{max} values were determined to be 13.4 $g.l^{-1}$ and 21.0 $\mu mol.ml^{-1}.min^{-1}$ for FA activity and 25.5 $g.l^{-1}$, and 52.5 $\mu mol.ml^{-1}.min^{-1}$ for FTA activity, respectively, and the K_i value for FTA was 729.8 $g.l^{-1}$.

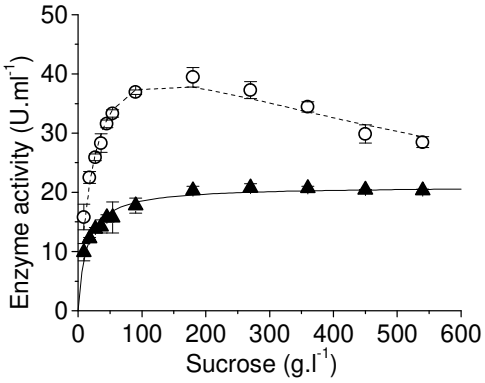

Figure 6: Kinetic behavior of *Candida* sp. (LEB-I3) enzymes
○ FTA (R^2 = 85.6%); ▲ FA (R^2 = 92.8%)

3.5. Fructooligosaccharide production

The crude enzyme preparation (1 FTA.ml^{-1}) was incubated in a 50% (w/v) sucrose solution (50 mM sodium acetate buffer, pH 4.5), at 50 ℃. A typical time course for the sugar concentration data is presented in Figure 7, the synthesis being done in triplicate and resulting in a medium standard error of 8.4%. FOS production of 220 g.l^{-1} was achieved after 42 hours of reaction, at which point the residual sucrose concentration was 10% of the initial content and the glucose and fructose concentrations were 150 g.l^{-1} and 70 g.l^{-1} respectively.

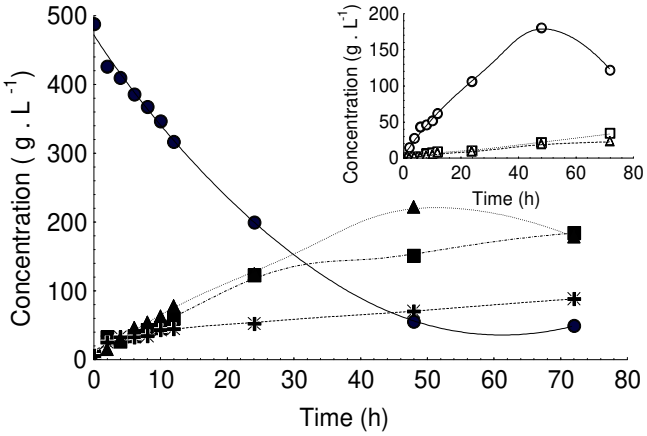

Figure 7: The time course for sugar concentration during enzymatic synthesis of fructooligosaccharides
(▲ FOS; ● Sucrose, ✶ fructose, ■ glicose, ○ GF2, □ GF3, △ GF4)

4. Discussion

The fructofuranosidade enzyme with high transfructosylating activity from *Candida* sp. LEB-I3 (Laboratory of Bioprocess Engineering – Unicamp- Brazil) showed interesting features, revealing potentials for new enzyme applications in fructooligosaccharide production. The enzyme was purified with 50-55% recovery and a 12-20 fold purification factor, using HiLoad™ 16/10 Q Sepharose® Fast Flow (Pharmacia Biotech), but the electrophoretic mobility suggested that the purification process was far from ideal, showing a diversity of proteins in the samples, from 35 to 120 kDa. Although the purification process used showed satisfactory recovery, further studies should be done for a more accurate molecular weight determination.

Kinetic parameters such as temperature, pH, and metal ion activation/inhibition had different effects on the fructofuranosidase and fructosyltransferase activities of the *Candida* sp. LEB-I3 extra cellular enzyme.

Besides indicating the amino acid residues involved in the active site, the study of the behaviour of the enzymes in solutions containing metal ions is important when working with two kinds of activity. Considering that the ratio between the fructosyl transferase and fructofuranosidase activities is a major characteristic in determining the production of fructooligosaccharides (Hidaka *et al*, 1988), the use of copper sulphate might be beneficial to this process, because it causes great inactivation of the fructofuranosidase activity, with little loss of transfructosilating activity.

When comparing this enzyme with the commonly reported *Candida* invertase, the *Candida* sp. LEB-I3 enzyme showed a similar optimum pH (around 4.0 for FTA and FA) and temperature (72ºC for FA and FTA), to the inver tase from *Candida utilis* (Belcarz *et al*., 2002). These values were slightly different from the well-known *Aspergillus* sp. enzymes used in the production of FO, which showed an optimum pH at 4.5 – 6.0 and optimum temperature between 50 and 60ºC (L'Hocine *et al*., 2000; Wang and Rakshit, 2000).

It was observed that the enzyme from *Candida* sp. (LEB-I3) showed both activities in both low and high sucrose concentrations, a behaviour typical of fructosyl-transferase enzymes. β-fructofuranosidase usually possesses both fructosyl transferase and hydrolysing activities, but its hydrolysis is a particular case of transference to water in

dilute reaction systems, the fructosyl transferase activity requiring high sucrose concentrations (Paul and Monsan, 1995).

The Michaellis-Mentem model could explain the kinetics of both activities of the *Candida* sp. LEB-I3 extra cellular enzyme (with substrate inhibition for FTA). The kinetics of the invertase and fructosyl transferase from moulds and yeasts reported in the literature for use in oligosaccharide production could also be explained by the Michaellis-Mentem model (with or without substrate inhibition), such as the glucosyl transferase, fructosyl transferase and glycosidase from *Aspergillus foetidus* (Wang and Rakshit, 2000) and the fructosyl transferase and invertase from *Aspergillus niger* (L'Hocine *et al.*, 2000).

In terms of FOS synthesis, a sucrose conversion to FOS of 44% was achieved after 48 hours of reaction, a lower yield then the process with enzymes from *Aspergillu*s and *Aureobasidium*, in which values of from 50 to 60% of FOS were reported (Chen and Liu, 1996). The yield was not bigger because of the strong invertase activity that could be observed in the synthesis time course (after 72 hours of reaction one could observe the hydrolysis of fructooligosaccharides).

Although the enzyme from *Candida* sp. (LEB-I3) has both activities, fructooligosaccharide production from sucrose was efficient, with a yield of 44%, and after studying the optimal conditions for synthesis and enzyme production, a higher yield could be obtained. This work offers an understanding of its properties and demonstrates the need for process optimisation.

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ARTIGO 7 :

OPTIMIZATION OF EXTRACELLULAR FRUCTOSYL TRANSFERASE PRODUCTION BY RHODOTORULA SP. LEB-V10

Otimização da produção de frutosiltranferase extracelular por *Rhodotorula* sp.

(Process Biochemistry)

OPTIMIZATION OF EXTRACELLULAR FRUCTOSYL TRANSFERASE PRODUCTION BY RHODOTORULA SP.

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Abstract

Response surface methodology was employed to study the effect of potassium phosphate, sugar cane molasses, corn steep liquor and yeast hydrolysate (Prodex-Lac®) on fructosyl transferase production by *Rhodotorula* sp. LEB-V10 (Laboratory of Bioprocess Engineering – UNICAMP - Brazil). The experiment included a 2^{7-3} factorial design and two central composite rotatable designs (2^3 and 2^2). Response surface methodology was applied to the optimisation of the nutrient concentrations in the culture medium, which led to an enzyme activity of 96 FTA.ml^{-1}. On the basis of the experimental results, the optimum conditions to obtain high fructosyl transferase activity from *Rhodotorula* sp. LEB-V10 were: 250 rpm, 30-35°C, 9% (± 1%) corn steep liquor and 7.5% (± 0.7%) of total reducing sugar from sugar cane molasses.

Keywords: *Rhodotorula* sp.; fructosyl transferase production; factorial design, surface response analysis; optimization

1.Introduction

The human being is currently more and more concerned with his health improvement and one way of promoting health is by ingesting functional fructooligosaccharides (FOS), creating a good balance in the intestinal flora. These oligosaccharides consist of 1 to 3

fructose units bound to a sucrose unit: 1-kestose (GF2), nystose (GF3) and fructofuranosyl-nystose (GF4) (Gibson & Roberfroid, 1995).

Fructooligosaccharides with a low degree of polymerisation (D.P 2 ~ 4) can be produced by the action of enzymes with transfructosylating activity (fructofuranosidase or fructosyl transferases) on sucrose solutions, or by the enzymatic hydrolysis of inulin using the inulinase from *Kluyveromyces* sp. or *Aspergillus* sp. (Roberfroid, 2000). Although the process using high transfructosylating activity fungal enzymes (*Aspergillus* sp. and *Aureobasidium* sp.*)* has been well studied and already scaled-up, these microorganisms show mainly high intracellular activity, and the process is carried out using immobilized cells, which is a complex industrial process (Shin *et al*., 2004, Yun, 1996).

From an economic point of view, yeasts are the most important microorganisms exploited by men, with many technical advantages over filamentous fungi, but their ability to produce fructosyl transferase (FTA) enzymes has scarcely been studied. Studies on *Kluyveromyces* sp. and its inulinase enzyme (Hensing at al., 1993) and on *Rhodotorula* sp. have referred to gluco- and galactooligosaccharide production (Onishi & Yokoseki, 1996). Vranešić at al. (2002), Risso (2004) and Treichel (2004) carried out a considerable number of studies on inulinase and FOS production by *Kluyveromyces* sp, but despite all their efforts, and having obtained high enzyme production (1300 FTA.ml^{-1}), the maximum yield of FOS was only about 12% in aqueous media and 18% in organic solvents.

The genus *Rhodotorula* produces large amounts of fatty acids (Davoli *et al*., 2004) and has been used in the production of single-cell protein (biomass) from different substrates, such as from ethanol and glutamate fermentation wastewater (Yech. 1996; Zheng *et al*., 2005). It has also been reported in the production of lipase (Cardenas *et al*., 2001), epoxide hydrolases (Kotik *et al*., 2005), carotenoids (Buzzini and Martini, 1999; Aksu and Tuğba Eren, 2005) and expo-polysaccharides (Pavlova and Grigorova, 1999). More recently, interest in alternative diesel fuels led to some studies on the production of biodiesel, such as the lipid compounds synthesized by *Rhodotorula glutinis* in glutamate wastewater media, which can be used as raw materials for biodiesel production (Xue *et al*, 2006).

Rubio *et al*. (2002), studying the invertase from a strain of *Rhodotorula minuta*, reported that this enzyme had high hydrolytic activity in high sugar concentrations (1M) but

no oligosaccharide synthetic properties. On the other hand, the *Rhodotorula minuta* IFO879 cell wall enzymes showed strong transglycosilation activity, producing galacto and gluco-oligosaccharides from lactose and cellobiose, respectively (Onishi and Tanaka, 1996).

In a previous study on the screening of yeasts (Hernalsteens and Maugeri, 2005) from fruits and flowers (from Brazilian tropical forests) for extra-cellular fructosyl transferase activity, a *Rhodotorula* sp. LEB-V10 (Laboratory of Bioprocess Engineering – UNICAMP – Brazil) strain was isolated. The enzyme excreted from this *Rhodotorula* sp. was able to produce more then 250 $g.l^{-1}$ of FOS from a 500 $g.l^{-1}$ sucrose solution, a yield not previously detected in yeasts.

One of the possible options to reduce the costs of bioprocess products is to use low-cost non-conventional agro-industrial residues as culture medium ingredients. Two major by-products from food industries widely used in the fermentation industry, are molasses and corn steep liquor. Their use not only reduces potential environmental pollution, but also serves as a low cost and powerful source of nitrogen, carbon and vitamins. Molasses is a by-product of the sugar industry and is defined as a solution of sugar and organic and inorganic matters, containing from 45 to 70% (w/w) of sugars, and is widely used as a substrate in fermentation, mainly in the production of ethanol and baker's yeast. Corn steep liquor is a by-product from the wet-milling corn industry. On average, corn steep liquor contains 20% (w/w) carbon and 5% (w/w) nitrogen, besides minerals and vitamins.

Rhodotorula strains produce carotenoids such as β-carotene and torularhodin, and there are a number of studies aiming at optimising the carotenoid production. The use of industrial by-products as nutrient sources for carotenoid production by *Rhodotorula mucilaginosa,* was reported by Aksu and Eren (2005). In this case optimum culture growth was achieved at 30℃, with an initial pH value of 7 .0 and aeration rate of around 2.5 vvm. In general, an increase in sugar concentration increased yeast growth, the strain's ability to grow on a variety of carbon sources, such as glucose, sucrose and lactose, being remarkable.

Factorial design and response surface analysis are important tools in the determination of optimal process conditions. Factorial design shows advantages over the conventional method, which only handles a single parameter per trial. In addition, the

conventional method does not consider the effect of possible interactions between factors, very important in biological and biochemical process and when agro-industrial by-products and surpluses are used as substrates (Kalil *et al*., 2000; Burkert at al, 2004).

In a previous study, enzyme production by the *Rhodotorula* sp. LEB-V10 strain on a synthetic medium (consisting of sucrose, peptone, yeast extract and salts) was low, about 10 FTA.ml^{-1}. Therefore, in this work the conditions for FTA production were studied using a factorial design and response surface analysis.

2. Material and Methods

2.1. Microorganism, maintenance and inoculum preparation

Rhodotula sp. LEB-V10 (Laboratory of Bioprocess Engineering – UNICAMP – Brazil) was isolated from flowers from the Brazilian biota (Hernalsteens and Maugeri, 2006). The working culture was grown on GYMP medium (2% glucose, 0.5% yeast extract, 1% malt extract, 0.2% KH_2PO_4 - pH 5.5) and maintained in 10% glycerol at -20℃.

The inoculum was produced by two step activation using a medium containing 2% glucose, 1% yeast extract, 2% peptone, 0.5% KH_2PO_4 (pH 4.5). The cultures were first incubated in 500 ml flasks containing 100 ml of the inoculum medium at 30 ℃ in a rotary shaker (150 rpm). A 24-hour old inoculum culture (10% v/v) was transferred to another inoculum flask, which was then incubated for 16 hours before use.

2.2. Enzyme assay

The culture broth was harvested by centrifugation (6000 G, 10 min, 5℃) and the clear supernatant used to determine enzyme activity.

The reaction media used to determine enzyme activity consisted of 50% (w/v) sucrose (in 50 mM sodium acetate buffer, pH 4.5) and 10% (v/v) of an adequately diluted enzyme suspension at 50 ºC. Samples were collected at constant time intervals for 30 minutes of reaction time, and used to quantify glucose using commercial glucose-oxidase kits, and reducing sugars by the Somogi-Nelson method.

Sucrose conversion by fructofuranosidase yields glucose and fructose, but when transfructosilating activity is present, part of the fructose is built into a fructan polymer. By measuring the amounts of glucose and reducing sugars released into the reaction

medium, the hydrolytic and transfructosylating activities can thus be assessed (Chen and Liu, 1996). The equation below allows one to determine these activities by estimating glucose (G) and reducing sugars (R) in the reaction medium (F = fructose, F' = transferred fructose):

$$R = G + F \Leftrightarrow F = R - G$$

$$F' = G - F \Leftrightarrow F' = 2\,G - R \qquad \text{(eq. 1)}$$

One unit of fructofuranosidase activity (FA) was defined as the amount of enzyme required for the hydrolysis of 1 µmol of sucrose per minute. One unit of fructosyl transferase activity (FTA) was defined as the amount of enzyme required to transfer one µmol of fructose (F') per minute.

2.3. Factorial design

Three factorial designs were carried out. In the first one, a 2^{7-3} fractional factorial design was performed in order to study the effects of corn steep liquor (CSL) concentration (Corn Products International Inc., São Paulo – Brazil), sugar cane molasses concentration (SM), Prodex-lac® yeast hydrolysate concentration (YEP), potassium phosphate concentration, K_2HPO_4 (KP), agitation (RPM), initial pH and temperature (T). Enzyme production (FTA activity) was used as the response. Additionally, a 2^3 central composite rotatable design (CCRD) was performed, in which the concentrations of corn steep liquor, sugar cane molasses and potassium phosphate were studied. To obtain maximum enzymatic activity, another CCRD was performed to determine the optimum concentrations of the corn steep liquor and sugar cane molasses.

In the first experimental design, 19 experimental runs were carried out, including 16 factorial points and 3 central points. The second design was used to study 3 variables at 3 levels, requiring 11 runs and including 3 central points. Two variables at five levels were studied in the final CCRD, which required 12 assays, including 8 factorial points and 4 central points. The independent variables and their levels are shown together with the results.

Central points provide additional degrees of freedom for estimating errors, which increases the power when testing the significance of the effects. All data were treated with the aid of STATISTICA 5.0 (Statsoft Inc., USA). Two other experiments were carried out to

confirm the optimised conditions, four runs under the optimised conditions and three assays at a lower temperature (30℃).

2.4. Pre-treatment process for the sugar cane molasses (SM) and corn steep liquor (CSL)

Molasses and other by-products from the “agro-food” industry should be pre-treated before use as substrates In biological and biochemical processes, so that contaminant and inhibitor concentrations can be minimized. The use of corn steep liquor and sugar cane molasses in fermentation by *Kluyveromyces* (aiming at the production of inulinase) caused flocculation of the media, an undesirable effect since it makes the downstream processes more difficult (Treichel, 2004). Therefore, both the corn steep liquor and sugar cane molasses were clarified using activated charcoal as established by Treichel (2004), in a reciprocal shaker (150 rpm) with 8% (w/w) activated charcoal at 70 ℃ for one hour. The activated charcoal was removed from the media by filtration and centrifugation (6000 G, 10 min).

3. Results and discussion

Table 1 shows the experimental conditions and results for FTA activity in the first factorial design. The statistical analyses were performed with data obtained from 72 hour-fermentation samples, since the enzyme activity reached its maximum point in this fermentation time. This first factorial design, a 2^{7-3} fractional design, was a preliminary and screening step, intended to test the effect of each medium component. The level of FTA activity in the 19 runs varied from 1.1 to 66.1 FTA.ml^{-1}. The best results were achieved in runs 2, 3, 10, 11, 12 and 14.

Table 1: Real values (coded levels) and FTA activity for the first factorial design

Run	Independent variable							Enzyme activity[d]
	CSL[a]	SM[b]	YEP[a]	KP[a]	RPM	pH	T[c]	
1	40 (-1)	80 (-1)	0 (-1)	0 (-1)	100 (-1)	3.0 (-1)	35 (-1)	16.45
2	60 (1)	80 (-1)	0 (-1)	0 (-1)	300 (1)	3.0 (-1)	45 (1)	48.17
3	40 (-1)	120 (1)	0 (-1)	0 (-1)	300 (1)	5.0 (1)	35 (-1)	57.40
4	60 (1)	120 (1)	0 (-1)	0 (-1)	100 (-1)	5.0 (1)	45 (1)	10.37
5	40 (-1)	80 (-1)	4 (1)	0 (-1)	300 (1)	5.0 (1)	45 (1)	13.48
6	60 (1)	80 (-1)	4 (1)	0 (-1)	100 (-1)	5.0 (1)	35 (-1)	24.36
7	40 (-1)	120 (1)	4 (1)	0 (-1)	100 (-1)	3.0 (-1)	45 (1)	6.64
8	60 (1)	120 (1)	4 (1)	0 (-1)	300 (1)	3.0 (-1)	35 (-1)	1.08
9	40 (-1)	80 (-1)	0 (-1)	2 (1)	100 (-1)	5.0 (1)	45 (1)	11.04
10	60 (1)	80 (-1)	0 (-1)	2 (1)	300 (1)	5.0 (1)	35 (-1)	66.13
11	40 (-1)	120 (1)	0 (-1)	2 (1)	300 (1)	3.0 (-1)	45 (1)	52.82
12	60 (1)	120 (1)	0 (-1)	2 (1)	100 (-1)	3.0 (-1)	35 (-1)	41.63
13	40 (-1)	80 (-1)	4 (1)	2 (1)	300 (1)	3.0 (-1)	35 (-1)	6.26
14	60 (1)	80 (-1)	4 (1)	2 (1)	100 (-1)	3.0 (-1)	45 (1)	54.04
15	40 (-1)	120 (1)	4 (1)	2 (1)	100 (-1)	5.0 (1)	35 (-1)	11.58
16	60 (1)	120 (1)	4 (1)	2 (1)	300 (1)	5.0 (1)	45 (1)	32.96
17	50 (0)	100 (0)	2 (0)	1 (0)	200 (0)	4.0 (0)	40 (0)	17.21
18	50 (0)	100 (0)	2 (0)	1 (0)	200 (0)	4.0 (0)	40 (0)	17.44
19	50 (0)	100 (0)	2 (0)	1 (0)	200 (0)	4.0 (0)	40 (0)	16.45

[a] [g . l^{-1}]; [b] [g total reducing sugar . l^{-1}]; [c] [°C]; [d] [FTA . ml^{-1}].

An estimate of the main effect is obtained by evaluating the difference in process performance caused by a change from the low (-1) to the high (+1) level of the corresponding factor (Haaland, 1989). The estimate of the effects for each variable were determined and reported in Table 2. The t-test and p-value for the statistical parameters were used to confirm the significance of the factors studied, and in this case, $p<0.05$ suggested significance at the 0.05 level, or a 95% confidence level.

It can be seen that the most relevant variables with respect to FTA activity were corn steep liquor, yeast hydrolysate, potassium phosphate and agitation (Table 2). Increases in corn steep liquor, potassium phosphate concentration or agitation speed had positive effects, increasing FTA activity. On the other hand, the addition of yeast hydrolysate and increase in sugar cane molasses concentration had negative effects. Furthermore, variations in the initial pH and temperature were not statistically significant at the 95% confidence level.

Table 2: Main effects analysis for FTA activity from the first factorial design at 72 hours of fermentation

Factor	Effect	Std. Error	t-value	p-value
Mean	26.61	0.119	223.794	0.0000 [a]
CSL	12.88	0.259	49.717	0.0004 [a]
SM	-3.18	0.259	-12.281	0.0066 [a]
YEP	-19.20	0.259	-74.108	0.0002 [a]
KP	12.32	0.259	47.531	0.0004 [a]
RPM	12.77	0.259	49.294	0.0004 [a]
pH	0.03	0.259	0.107	0.9242
T	0.58	0.259	2.227	0.1559

[a] Significant factors ($p<0,05$)

Based on the previous results, in the second experimental design the concentration ranges of the corn steep liquor and of the potassium phosphate were increased and of the sugar cane molasses, decreased. The yeast hydrolysate was excluded from the culture medium (negative effect) and the initial pH and temperature fixed at 4.5 - 5.0 and 35℃, respectively. The agitation speed was fixed at 250 rpm.

Table 3 shows the experimental conditions and results for FTA production obtained in the second factorial design. The estimates of the effects for each variable and the interactions between them were determined and reported for the maximum activity achieved in each run (Table 4) and for productivity (Table 5). The FTA activity level in the 11 runs varied between 14 and 100 FTA.ml^{-1}. The maximum value for FTA activity was significantly higher than that of the maximum activity obtained in the medium before optimisation (10 – 15 FTA.ml^{-1}). It is noteworthy that the decrease in enzyme activity

observed after 48 hours of cultivation was concurrent with an increase in the pH value from 4.5 – 5.0 to 8.0 or above.

Table 3: Real values (coded levels) and FTA activity during the fermentation for the second factorial design

Run	Independent variable			Enzyme activity[c] (h)			
	CSL [a]	SM[b]	KP[a]	12	24	36	48
1	50 (-1)	40 (-1)	0.5 (-1)	29.21	28.05	61.54	14.93
2	90 (1)	40 (-1)	0.5 (-1)	36.58	58.57	63.62	45.79
3	50 (-1)	70 (1)	0.5 (-1)	36.34	72.29	71.56	53.38
4	90 (1)	70 (1)	0.5 (-1)	39.98	99.69	82.70	58.25
5	50 (-1)	40 (-1)	3.5 (1)	13.68	50.68	55.57	44.93
6	90 (1)	40 (-1)	3.5 (1)	20.62	39.96	63.85	42.71
7	50 (-1)	70 (1)	3.5 (1)	50.16	54.27	55.13	60.61
8	90 (1)	70 (1)	3.5 (1)	53.37	64.88	63.40	65.38
9	70 (0)	55 (0)	2 (0)	24.89	58.86	58.54	60.94
10	70 (0)	55 (0)	2 (0)	21.13	46.96	56.40	64.65
11	70 (0)	55 (0)	2 (0)	32.49	53.85	51.51	62.21

[a] [g. l^{-1}]; [b] [g total reducing sugar . L^{-1}]; [c] [FTA.ml^{-1}]

Productivity (FTA.l^{-1}.h^{-1}) was analysed because each run achieved its maximum enzyme production at a different fermentation time. In both of these analyses, the corn steep liquor and sugar cane molasses concentrations were shown to be significant and an increase in concentration of these variables led to an increase in FTA activity. On the other hand, an increase in potassium phosphate concentration had no beneficial effect.

Table 4: Main effects analysis for maximum FTA activity from the second factorial design

Factor	Effect	Std. Error	t-value	p-value
Mean	66.40	0.568	116.791	0.0001 [a]
CSL	10.63	1.333	7.975	0.0154 [a]
SM	13.34	1.333	10.011	0.0098 [a]
KP	-12.93	1.333	-9.700	0.0105 [a]
CSL x SM	5.45	1.333	4.090	0.0549
CSL x KP	-4.11	1.333	-3.081	0.0912
SM x KP	-10.06	1.333	-7.547	0.0171 [a]

[a] Significant factors (p<0,05)

Table 5: Main effects analysis for productivity from the second factorial design

Factor	Effect	Std. Error	t-value	p-value
Mean	1863.36	11.844	157.329	0.0000 [a]
CSL	382.20	27.776	13.760	0.0052 [a]
SM	749.18	27.776	26.972	0.0014 [a]
KP	-1175.12	27.776	-42.307	0.0006 [a]
CSL x SM	238.32	27.776	8.580	0.0133 [a]
CSL x KP	-217.52	27.776	-7.831	0.0159 [a]
SM x KP	-1095.40	27.776	-39.437	0.0006 [a]

[a] Significant factors (p<0,05)

The interactions between these three factors (sugar cane molasses, corn steep liquor and potassium phosphate) were not all statistically significant, but the negative influence of the interaction between potassium phosphate and all the other variables was noteworthy. This salt is used not only to fulfil the cell requirements of the yeast but also as a buffering agent, controlling the pH of the medium during fermentation. In the first design, when the concentration varied from 0 to 2 $g.l^{-1}$, the statistical analysis showed a positive effect of this salt on enzyme production, but in the second design, the increase in K_2HPO_4 from 0.5 to 3.5 $g.l^{-1}$ had a negative effect, showing that if this salt is necessary, its concentration should be kept low at around 0.5 $g.l^{-1}$. Anther important consideration is that in this second design, the concentration of the corn steep liquor was increased, and this

substrate is very rich in buffering constituents and in potassium salts, making the addition of potassium phosphate unnecessary and inhibitory.

In the third experimental design, the concentrations of the constituents were adjusted according to the results of the second experimental design. The ranges for corn steep liquor and sugar cane molasses were increased and potassium phosphate was not added to the medium. This design tested the two components at five levels, which required 12 assays, aiming at optimisation of the medium by constructing a mathematical model and response surfaces. Table 6 shows the experimental conditions and the results for FTA production obtained in the third factorial design.

Table 6: Real values (coded levels) and FTA activity during the fermentation for the third factorial design

Run	Independent variable		Enzyme activity[c] (h)			
	CSL[a]	SM[b]	12	24	36	48
1	70 (-1)	60 (-1)	34.2	58.8	64.8	71.5
2	110 (1)	60 (-1)	19.8	37.8	50.5	58.8
3	70 (-1)	90 (1)	33.9	49.6	84.8	79.5
4	110 (1)	90 (1)	35.3	67.5	66.9	79.0
5	61.8 (-1.41)	75 (0)	34.1	42.6	56.8	64.2
6	118.2 (1.41)	75 (0)	20.5	52.7	50.8	58.2
7	90 (0)	53.8 (-1.41)	17.1	52.9	50.9	57.1
8	90 (0)	96.2 (1.41)	29.6	57.7	82.8	66.6
9	90 (0)	75 (0)	32.6	67.4	84.9	98.7
10	90 (0)	75 (0)	46.0	68.4	85.9	92.0
11	90 (0)	75 (0)	37.3	62.9	76.2	92.3
12	90 (0)	75 (0)	24.5	57.6	78.4	96.9

[a] [g. l^{-1}]; [b] [g total reducing sugar . l^{-1}]; [c] [FTA.ml^{-1}]

The level of maximum FTA activity in the runs varied between 57 and 99 FTA.ml^{-1}. The maximum value for FTA activity was no higher than that obtained in the second experimental design (100 FTA.ml^{-1}), indicating that this value may be the limit of FTA activity production by this microorganism in a shaker (500 ml flasks), using sugar cane molasses and corn step liquor as substrates.

A fitted model was accomplished in the third experimental design: the independent and dependent variables were fitted to a second-order model equation and checked for goodness of fit. ANOVA (Tables 7 and 8) was used to evaluate the adequacy of the fitted model. The R-squared value provided a measure of how much the model could explain the variability in the observed response.

Table 7: ANOVA for the third design (maximum FTA activity)

Souce of variation	Sum of square	Degree of Freedom	Mean Square	F-ratio
Regression	2496.36	4	624.09	22.96
Residual	190.29	7	27.18	
Lack of fit	156.70	4	39.17	
Pure error	33.59	3	11.20	
Total	2686.64	11		

*Residual = Lack of Fit + Pure error; $F_{0,10;4;7} = 2,96$; R-sqr = 0,93

Table 8: ANOVA for the third design (productivity)

Souce of variation	Sum of square	Degree of Freedom	Mean Square	F-ratio
Regression	$1.85 \cdot 10^6$	5	$3.70 \cdot 10^5$	13.62
Residual	$1.63 \cdot 10^5$	6	$2.71 \cdot 10^4$	
Lack of fit	$1.48 \cdot 10^5$	3	$4.94 \cdot 10^4$	
Pure error	$1.46 \cdot 10^4$	3	$4.86 \cdot 10^3$	
Total	$2.01 \cdot 10^6$	11		

*Residual = Lack of Fit + Pure error; $F_{0,10;5;6} = 3,10$; R-sqr = 0,91

According to the analysis of variance (ANOVA), a second order model was established, describing the enzyme activity (or the productivity) as a function of corn steep liquor and total reducing sugar (sugar cane molasses) concentrations (Eqs. 1 and 2). Based on the F test, the model was predictive, since the calculated F value was higher than the critical F value and the R-squared test was about 0.9. The coded model was used to generate response surfaces (Figures 1 and 2) for the analyses of the effects of the variables on enzyme activity, FTA (FTA . ml^{-1}) and productivity, P (FTA . l^{-1} . h^{-1}).

$$FTA = - 499.13 + 6.55*[CSL] - 0.04*[CSL]^2 + 7,53*[SM] - 0.05*[SM]^2 \quad (Eq.1)$$

$$P = - 5496.15+123.69*[CSL]+89.41*[SM]-0.37*[CSL]*[SM]- 0.33*[SM]^2-0.78*[CSL]^2 \quad (Eq.2)$$

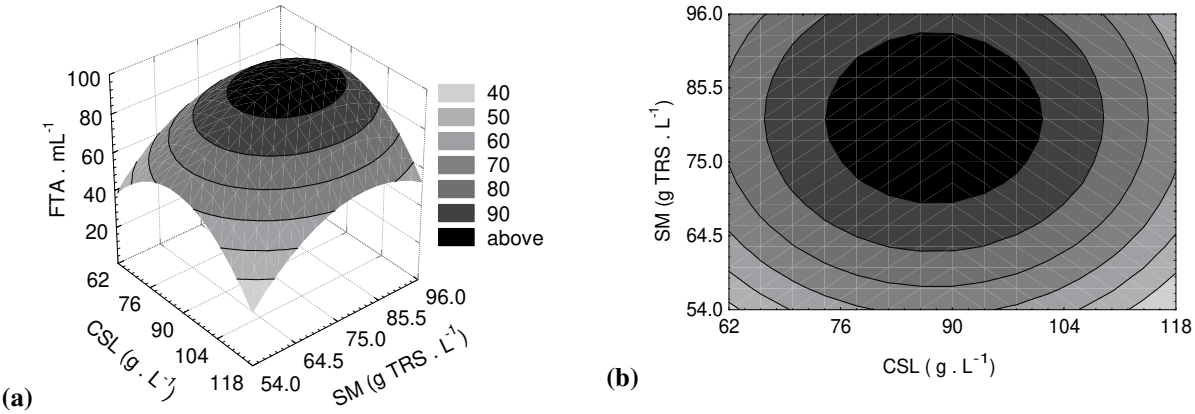

Figure 1: Response surface (a) and contour diagrams (b) for the fructosyl transferase activity as a function of CSL and SM concentrations, according to the third experimental design

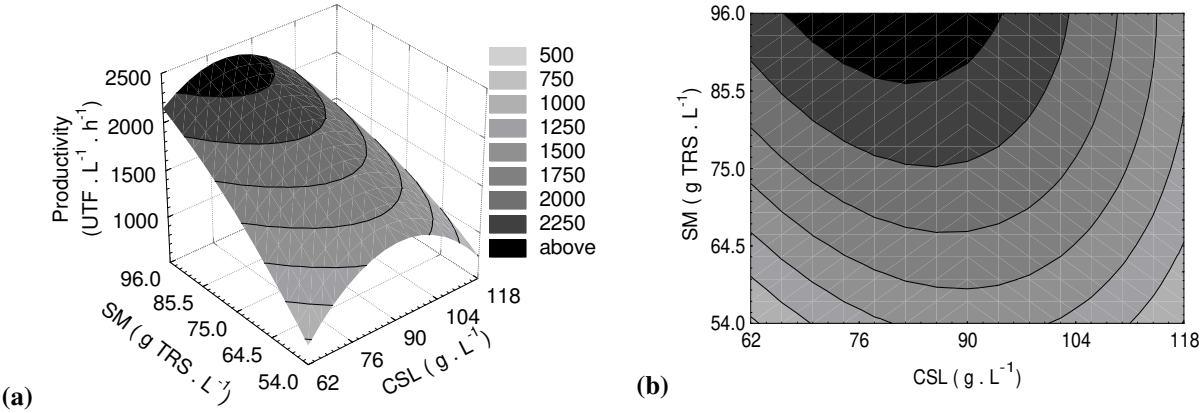

Figure 2: Response surface (a) and contour diagrams (b) for the enzyme productivity as a function of CSL and SM concentrations, according to the third experimental design

Therefore to maximize FTA activity, both variables should be kept at the central levels tested, so the optimised condition determined for the production of the fructosyl transferase enzyme by *Rhodotorula* sp. LEB-V10 was: 250 rpm, 35℃, 9% ± 1% corn steep liquor and 7.5% ± 0.7% of total reducing sugar from sugar cane molasses. The maximum FTA activity predicted in Figure 1 was about 100 FTA.ml^{-1}, approximately 10 times higher than that obtained on a standard synthetic medium. It is noteworthy that to maximize the productivity response, the sugar cane molasses must be kept at the highest

level tested. Considering the FTA yield as the ratio between the maximum FTA activity and the total reducing sugar from sugar cane molasses, the results compiled from all three experimental designs showed that it is better to use lower sugar cane molasses concentrations (Figure 3).

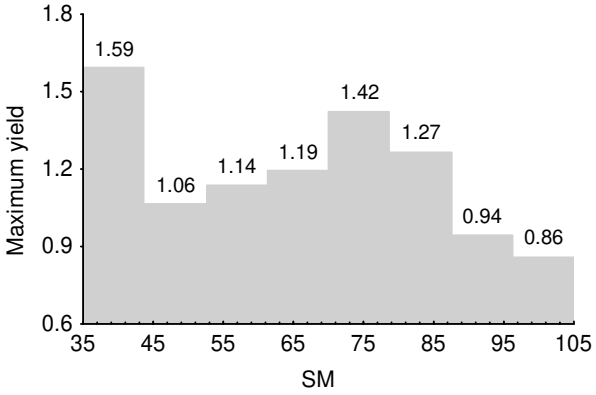

Figure 3: Enzyme activity yields as related to the total reducing sugar concentration from sugar cane molasses.

Four other runs were carried out to confirm this optimum condition (250 rpm, 35℃, 9% corn steep liquor and 7.5% of total reducing sugar from sugar cane molasses), in which the maximum enzyme activity was 94.4 ± 9.2 FTA.ml^{-1}. To minimize production costs (on the energy requirements), another three runs were carried out at 30 ℃, instead of 35℃ (as determined in the first experimental de sign). Under this new condition the maximum enzyme production was 92.4 ± 9.4 FTA.ml^{-1}, and took 36 hours of fermentation.

4. Conclusions

Production, using an alternative medium composition (sugar cane molasses and corn steep liquor) was shown to be an effective enzyme production process, considering both aspects: high production and low costs. On the basis of the experimental design and response surface analysis, the optimum conditions to obtain high fructosyl transferase activity from *Rhodotorula* sp. LEB-V10 were: 250 rpm agitation, temperature of 30-35℃, 9% ± 1% of corn steep liquor and 7.5% ± 0.7% of total reducing sugar from sugar cane molasses. Under these conditions, an enzyme activity of 96 FTA.ml^{-1} was obtained, representing a ten-times increase in activity as compared to the process before optimisation.

Therefore, considering the high FOS production of about 50-60% (Hernalsteens and Maugeri, 2006), and the increase in enzyme production with a simultaneous reduction in

costs, the industrial production of fructosyl transferase by *Rhodotorula* sp. LEB-V10 (Laboratory of Bioprocess Engineering – Unicamp – Brazil) may be regarded as possible and economically attractive.

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ARTIGO 8 :

OPTIMIZATION OF FRUCTO-OLIGOSACCHARIDES BY FRUCTOSYL TRANSFERASE FROM RHODOTORULA SP. LEB-V10

Produção de fructooligossacarídeos por frutosiltranferase de *Rhodotorula* sp. LEB-V10

(Bioresource technology)

Production of fructo-oligosaccharides by fructosyl transferase from Rhodotorula sp.

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Abstract

The increasing demand for fructooligosaccharides as a functional ingredient for use in the food industry has led to a search for novel sources of fructosyl transferase (FTA) enzymes. In this paper a novel strain of *Rhodotorula* (LEB V10), isolated from flowers found in a Brazilian tropical forest, was used in a synthesis reaction for the production of fructooligosaccharides from sucrose. The process was optimised using an experimental design, leading to the determination of the following optimal conditions: 50% sucrose, 50°C (± 1°C), pH 5.0 (± 0.5), and 6.5 units of fructosyl transferase per mililiter (± 0.5). Under these conditions, a 55 to 65% yield was obtained after 24 to 29 hours of reaction. The use of commercial sucrose and sugar cane molasses also led to a high yield, 63% and 52% respectively.

Keywords: *Rhodotorula* sp., factorial design, surface response analysis, optimization, fructooligosaccharides, FOS

1. Introduction

Increasing consumer awareness about how foods contribute to their health has led to the development of a number of functional foods and a large number of functional foods have already been introduced onto the market. Oligosaccharides, in particular, the fructooligosaccharides (FOS), are very popular as functional foods and alternative sweeteners.

Fructooligosaccharides can be obtained from the hydrolysis of inulin or by the action of fructosyl transferase enzymes on sucrose. Fructosyl transferase enzymes have been used and studied for FOS production since the 80s, and the mechanism of their action is now known (Yun, 1996). Many authors have extensively reviewed the fungal and bacterial production of FOS, the best results being achieved with immobilized cells (or mycelia) of *Aspergillus japonicus* (Chien *et al*, 2001), *Aspergillus niger* (L´Hocine *et al*., 2000), *A. oryzae* (Sangeetha *et al*., 2005), *Aureobasidium pullulans* (Sangeetha *et al*, 2004) and *Penicillium citrinum* (Hayashi *et al*., 2000). These microorganisms could produce from 54 to 61% of FOS in a highly concentrated sucrose solution (500 – 700 $g.l^{-1}$), but such isolated enzyme processes were not capable of use in continuous processes, since the enzyme half-lives were only 30 days, one third of the operational stability of the immobilized cells (Yun, 1996).

FOS production by fungal fructosyl transferase produced 1-kestose (GF2) as the major product, nystose (GF3) and fructofuranosyl nystose (GF4) only being synthesized at the end of the reaction time (Hayashi *et al*, 2000). Enzyme production and the FOS synthesis reaction (*A. orizae*) were optimised using an experimental design (Placket Burman) and response surface methodology, the optimal conditions for FOS production being: 600 g. l^{-1} sucrose, pH 5.15 and 18 hours of reaction time (Sangeetha *et al*., 2005).

The screening of yeasts from Brazilian tropical forests resulted in 4 potentially interesting yeast strains, capable of producing high yields of FOS from concentrated sucrose solutions, but only *Rhodotorula* sp. LEB-V10 showed no hydrolytic activity with respect to the FOS synthesized, resulting in a high FOS content at the end of the reaction, with yields greater than 50% (Hernalsteens and Maugeri, 2006a).

The fructooligosaccharides (FOS) have a number of interesting functional properties that make them highly popular, from the prebiotic effect to the control of blood glucose and serum total cholesterol. In view of the great demand for FOS as a food ingredient, the screening and identification of new strains capable of producing them is necessary (Sangeetha *et al*., 2005).

From an economic point of view, yeasts are the most important microorganisms exploited by men, with many technical advantages over filamentous fungi, such as shorter fermentation times, high cell concentration cultures and cell recycling, amongst others. In

this study, experimental designs were used to evaluate the production of FOS by an extra cellular fructosyl transferase from *Rhodotorula* sp. yeast, isolated from flowers found in tropical Brazilian biomes.

2. Materials and methods

2.1. Microorganism and culture conditions

The yeast strain - *Rhodotorula* sp. LEB V10 – was isolated from a Brazilian tropical forest (Hernalsteens and Maugeri, 2006a) and maintained on GYMP agar slants at 4℃ in the Laboratory of Bioprocess Engineering (Faculty of Food Engineering - University of Campinas – Brazil).

The inoculum was prepared by transferring the growth from a fresh slant to 100 ml of a medium containing 2% glucose, 1% yeast extract, 2% peptone and 0.5% KH_2PO_4 (pH 4.5). The cultures were incubated at 30 ℃ in 500 m l baffled-flasks on a rotary shaker at 150 rpm for 24 hours. A 24 hour-old inoculum (10% v/v) was inoculated into the fermentation medium, which containing 9% corn steep liquor and 7% total reducing sugar from sugar cane molasses, and an initial pH of 5.0. These cultures were incubated at 30℃ in a rotary shaker at 250 rpm for 36-48 hours. At the end of the incubation period, the culture broth was centrifuged (4-6℃, 10 min., 6000 G) and the cell free supernatant collected (Hernalsteens *et al*., 2006).

2.2. Crude enzyme preparation

The proteins (and enzymes) from the cell free supernatant were precipitated with 70% ethanol at 0 to 2 ℃, a low agitation speed and slow ethanol addition. The precipitate was re-suspended in sodium acetate buffer (50 mM, pH 4.5) after centrifugation (15 min, 2℃, 6000G). This re-suspended solution was used as the enzyme source.

2.3. Optimisation of FOS production

Factorial design and response surface analysis are important tools in the determination of optimal process conditions. Factorial design shows advantages over the conventional method, which only handles a single parameter per trial. The conventional method does not consider the effect of possible interactions between factors, very important in biological and biochemical process and when agro-industrial by-products and surpluses are used as substrates (Kalil *et al*., 2000; Burkert at al, 2004).

Two factorial designs were carried out. In the first one, a 2^4 factorial design was performed to study the effects of sucrose (S), buffer pH, temperature (T) and enzyme activity (E) on FOS production. Additionally, a 2^3 central composite design (CCD) was employed, in which the temperature, sucrose and enzyme concentrations were studied. FOS production, yield and productivity, as well as the residual concentrations of sucrose and fructose were studied as the responses, in order to obtain the optimised process.

In the first experimental design, 20 runs were carried out, including 16 factorial points and 4 central points. The second design was used to study 3 variables at 5 levels, requiring 17 runs and including 3 central points. The independent variables and their levels were reported, together with their results.

Central points provide additional degrees of freedom for estimating error, which increases the power when testing significance of the effects. All data were treated with the aid of STATISTICA 5.0 (Statsoft Inc., USA).

Other experiments were performed in triplicate to confirm the optimised conditions using commercial sucrose, and in order to determine process behaviour when using real industrial raw materials, triplicate experiments were also carried out using molasses as the sucrose source. The runs with sugar cane molasses were performed considering a total reducing sugar concentration of about 75% (w/w).

The reaction behaviour using different sucrose concentrations was studied with five different sucrose concentrations, from 35 to 55%.

2.4. Enzyme assay

The reaction medium used to determine enzyme activity consisted of 50% (w/v) sucrose in 50 mM sodium acetate buffer, pH 4.5, and 10% (v/v) of an adequately diluted enzyme suspension at 50 ºC. Samples were collected at constant time intervals for 30 minutes of reaction time, and used to quantify glucose using commercial glucose-oxidase kits, and reducing sugars by the Somogi-Nelson method.

Sucrose conversion by fructofuranosidase yields glucose and fructose, but when transfructosilating activity is present, part of the fructose is built into a fructan polymer. By measuring the amounts of glucose and reducing sugars released into the reaction medium, the hydrolytic and transfructosylating activities can thus be assessed (Chen and Liu, 1996). The equation below (eq.1) allows for the determination of the activities by

estimating glucose (G) and reducing sugars (R) in the reaction medium (F = fructose, F' = transferred fructose):

$$R = G + F \Leftrightarrow F = R - G$$

$$F' = G - F \Leftrightarrow F' = 2\,G - R \qquad \text{(eq. 1)}$$

One unit of fructofuranosidase activity (FA) is defined as the amount of enzyme required for the hydrolysis of 1 µmol of sucrose per minute. One unit of transfructosylating activity (FTA) is defined as the amount of enzyme required to transfer one µmol of fructose (F') per minute.

2.5. FOS analysis

The identification and quantification of the oligosaccharides was achieved by high performance liquid chromatography with pulsed amperometric detection (HPLC-PAD) at 22 - 24 ºC, using the GP50 gradient pump, ED40 electrochemical detector and the software PEAKNET, all from DIONEX (USA). Chromatography was performed using a Carbopac PA-100 column equilibrated with 50 mM sodium hydroxide, and the sugars were eluted with a linear gradient of sodium acetate (0 to 200 mM) in 50 mM sodium hydroxide, at a flow rate of 1 ml·min^{-1}.

3. Results and discussion

Table 1 shows the experimental conditions and results for maximum FOS production and yield obtained in the first factorial design. The statistical analysis was performed with data obtained for different reaction times according to when FOS production reached its maximum point.

An estimate of the main effect was obtained by evaluating the difference in process performance caused by a change from the low (-1) to the high (+1) level of the corresponding factor (Haaland, 1989). The estimates of the effects for each variable were determined and reported in Tables 2 and 3 for maximum FOS production and yield, respectively. The statistical parameters t-test and p-value were used to confirm the significance of the factors studied, and, in this case, $p<0.05$ suggested significance at a level of 0.05 or a 95% confidence level.

It can be seen that FOS production was strongly influenced by temperature, and although the enzyme showed optimal activity at around 60ºC (Hernalsteens and Maugeri,

2006b), this temperature was not beneficial for FOS production. On the other hand, an increase in the sucrose or enzyme concentrations or in the pH, exhibited a positive influence. The effects on FOS yield were similar, although in this case, the sucrose concentration was not statistically significant at the 95% confidence level.

Table 1: Coded levels (real values), FOS production and yield for the 1st factorial design

Runs	S (%)	pH	E (FTA.ml^{-1})	T (°C)	FOS (g.l^{-1})	Yield[a] (%)
1	-1 (40)	-1 (4.0)	-1 (1)	-1 (50)	175.6	43.9
2	1 (60)	-1 (4.0)	-1 (1)	-1 (50)	293.4	48.9
3	-1 (40)	1 (5.0)	-1 (1)	-1 (50)	247.4	61.9
4	1 (60)	1 (5.0)	-1 (1)	-1 (50)	313.4	52.2
5	-1 (40)	-1 (4.0)	1 (5)	-1 (50)	217.8	54.5
6	1 (60)	-1 (4.0)	1 (5)	-1 (50)	356.5	59.4
7	-1 (40)	1 (5.0)	1 (5)	-1 (50)	235.7	58.9
8	1 (60)	1 (5.0)	1 (5)	-1 (50)	322.3	53.7
9	-1 (40)	-1 (4.0)	-1 (1)	1 (60)	7.1	1.8
10	1 (60)	-1 (4.0)	-1 (1)	1 (60)	40.6	6.8
11	-1 (40)	1 (5.0)	-1 (1)	1 (60)	112.6	28.1
12	1 (60)	1 (5.0)	-1 (1)	1 (60)	159.8	26.6
13	-1 (40)	-1 (4.0)	1 (5)	1 (60)	13.9	3.5
14	1 (60)	-1 (4.0)	1 (5)	1 (60)	74.3	12.4
15	-1 (40)	1 (5.0)	1 (5)	1 (60)	166.2	41.6
16	1 (60)	1 (5.0)	1 (5)	1 (60)	185.5	30.9
17	0 (50)	0 (4.5)	0 (3)	0 (55)	160.1	32.0
18	0 (50)	0 (4.5	0 (3)	0 (55)	163.8	32.8
19	0 (50)	0 (4.50	0 (3)	0 (55)	150.7	30.1
20	0 (50)	0 (4.5	0 (3)	0 (55)	160.7	32.1

[a] Yield [%] = 100 * FOS [g.l^{-1}] . Sucrose^{-1} [g.l^{-1}].

Table 2: Main effects analysis for maximum FOS production in the first factorial design

Factor	Effect	Std. Error	t-value	p-value
Mean	177.87	1.27	140.14	0.0000 [a]
Sucrose	71.21	2.84	25.09	0.0001 [a]
pH	70.46	2.84	24.83	0.0001 [a]
Enzyme	27.77	2.84	9.79	0.0023 [a]
Temperature	-175.28	2.84	-61.76	0.0000 [a]

[a] Significant factors ($p<0.05$)

Table 3: Main effects analysis for FOS yield in the first factorial design

Factor	Effect	Std. Error	t-value	p-value
Mean	35.61	0.26	140.26	0.0000 [a]
Sucrose	-0.38	0.57	-0.68	0.5464
pH	15.36	0.57	27.07	0.0001 [a]
Enzyme	5.58	0.57	9.82	0.0022 [a]
Temperature	-35.22	0.57	-62.05	0.0000 [a]

[a] Significant factors ($p<0.05$)

A fitted model was accomplished for the first experimental design, although this is not shown: the independent and dependent variables were fitted to the model equation and examined in terms of goodness of fit. ANOVA was used to evaluate the adequacy of the fitted model for both production and yield, and the R-squared values for the fittings were around 0.97, which provided a measure of how much the model could explain the variability in the response observed.

At the end of the reaction time, the residual sucrose concentration was around 10% of the initial value, and the FOS yield reached about 60% in a few of the runs. Concerning the FOS production course, the GF3 and GF4 concentrations started increasing at the end of the reaction, whilst the GF2 content decreased.

On a commercial scale, FOS is produced by immobilized fungal cells from *Aspergillus niger* and *Aureobasidium pullulans* in 60 to 70% sucrose solutions, resulting in 55 to 60% FOS yield (Yun, 1996). Another study on the production of FOS by *Aspergillus*

niger determined the following optimum conditions for synthesis: 50% sucrose, 50°C, 6 FTA per gram of sucrose and 24 hours of reaction (Hidaka *et al*., 1988).

A comparison between the data obtained in the present study and those from the literature shows that, despite the fact that the FOS production process by extra cellular enzymes from *Rhodotorula* sp. LEB-V10 has yet to be optimised, FOS production and yield were similar to those from the literature, although with lower productivity, since 50 hours was required to complete the reaction. Considering the time necessary to achieve this high FOS production, runs one to four, with low enzyme concentrations, required almost 90 hours to complete the reaction, whilst runs 5 to 8, with high enzyme concentrations, required approximately 50 hours. In the latter case, the reaction achieved 70 to 90% of the maximum FOS production after 20 hours, which may be an indication of the process condition if productivity is the main concern.

The second experimental design was done at constant sucrose concentration (50%), since in the previous design, the effect of sucrose concentrations from 40 to 60%, was not significant on FOS yields. On the other hand, on the basis of the first design results, the enzyme concentration, temperature and pH range of variation were increased. Table 4 shows the experimental conditions and results for FOS production, yield and productivity, according to the second factorial design.

The analysis of variance for maximum FOS production (Table 5) was used to evaluate the adequacy of the fitted model. A second order model was established describing the FOS concentration ($g.l^{-1}$) as a function of pH, temperature and enzyme concentration (Equation 2).

$$FOS=288.6+(6.98*E)-(13.2*E^2)-(5.5*pH)-(14.5*T)+(8.2*T^2)-(10.7*pH*T) \quad \text{(Eq. 2)}$$

Based on the F-test the model was predictive, since the calculated F-value was higher than the critical value, despite the high value for the lack of fit. This high lack of fit resulted in a low R-squared value (0.6), indicating that the model could not explain all the variability of the response. The coded model was used to generate response surfaces for the analysis of the effects of the variables on FOS production (Figures 1, 2 and 3).

Table 4: Coded levels (real values) and FOS production, productivity (P) and yield (Y) for the 2^{nd} factorial design

Runs	E (FTA.ml^{-1})	pH	T (℃)	FOS (g.l^{-1})	P[a]	Y[b] (%)
1	-1 (3)	-1 (4.0)	-1 (49)	272.89	3.80	54.6
2	1 (7)	-1 (4.0)	-1 (49)	282.81	3.95	56.6
3	-1 (3)	1 (5.0)	-1 (49)	271.77	3.75	54.4
4	1 (7)	1 (5.0)	-1 (49)	281.85	12.80	56.4
5	-1 (3)	-1 (4.0)	1 (55)	273.81	3.80	54.8
6	1 (7)	-1 (4.0)	1 (55)	287.01	13.05	57.4
7	-1 (3)	1 (5.0)	1 (55)	217.18	3.00	43.4
8	1 (7)	1 (5.0)	1 (55)	255.96	11.65	51.2
9	0 (5)	0 (4.5)	0 (52)	284.86	3.65	57.0
10	0 (5)	0 (4.5)	0 (52)	290.02	3.80	58.0
11	0 (5)	0 (4.5)	0 (52)	294.84	5.75	59.0
12	-1.68 (1.6)	0 (4.5)	0 (52)	264.82	4.20	53.0
13	1.68 (8.4)	0 (4.5)	0 (52)	279.08	7.45	55.8
14	0 (5)	-1.68 (3.5)	0 (52)	299.05	6.35	59.8
15	0 (5)	1.68 (5.5)	0 (52)	331.22	4.25	66.2
16	0 (5)	0 (4.5)	-1.68 (47)	349.80	4.05	70.0
17	0 (5)	0 (4.5)	1.68 (57)	297.54	4.05	59.5

[a] Productivity= P = FOS [g] . l^{-1} . h^{-1}

[b] Yield = Y (%) = 100* FOS [g.l^{-1}].(Sucrose [g/L])$^{-1}$

Table 5: ANOVA for the second design (maximum FOS production)

Source of Variation	Sum of Square	Degree of Freedom	Mean Square	F-ratio
Regression	8867.81	6	1477.97	2.54
Residual	5823.21	10	582.32	
Lack of fit	5751.98	8	718.99	
Pure error	71.23	2	35.62	
Total	14691.02	16	918.19	

*Residual = Lack of fit + Pure error; $F_{0.05,6,10} = 2.4$, R-sqr = 0.60

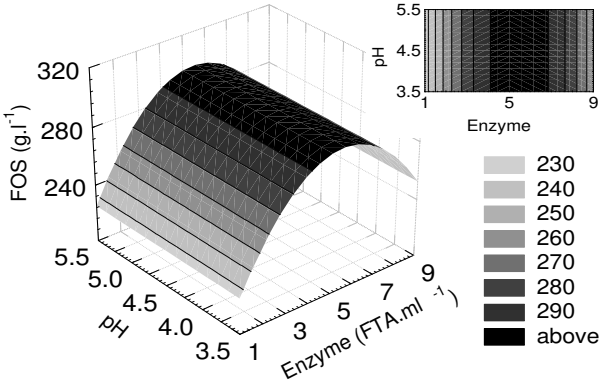

Figure 1: Response surface and contour diagrams for the FOS production as a function of pH and enzyme concentration, according to the second experimental design

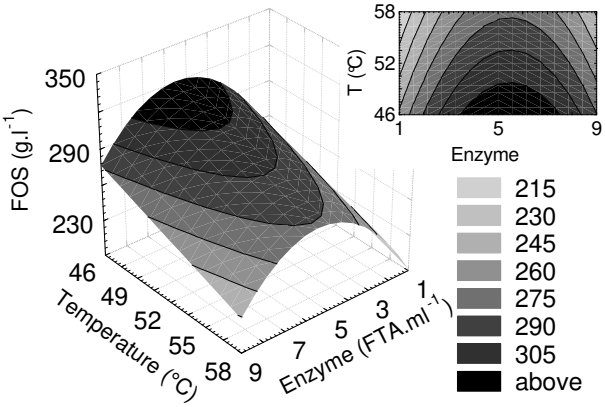

Figure 2: Response surface and contour diagrams for the FOS production as a function of temperature and enzyme concentration, according to the second experimental design

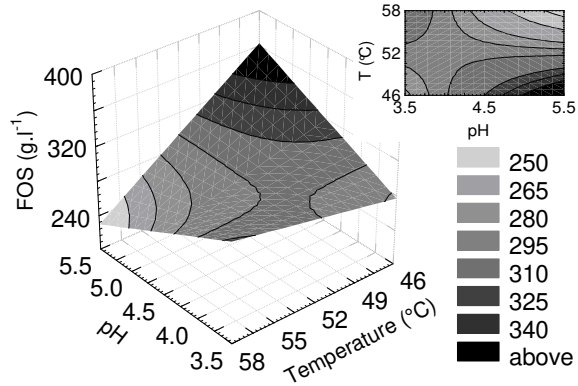

Figure 3: Response surface and contour diagrams for the FOS production as a function of pH and temperature, according to the second experimental design

However, optimisation of the process should be carried out on the basis of the effects of all the factors on FOS yield and productivity. The estimates of the effects for each variable, as well as the interaction between them, were determined and reported in Tables 6 and 7.

Table 6: Main effects analysis for FOS yield from the second factorial design

Factor	Effect	Std. Error	t-value	p-value
Mean	58.0	0.58	100.49	0.0001[a]
Enzyme	2.8	0.54	5.15	0.0358[a]
Enzyme (Q)	-5.1	0.57	-8.96	0.0122[a]
pH	-1.0	0.54	-1.85	0.2051
pH (Q)	0.6	0.57	1.06	0.3995
Temperature	-4.8	0.54	-8.92	0.0123[a]
Temperature (Q)	1.8	0.57	3.10	0.0902
Enzyme x pH	1.3	0.71	1.84	0.2074
Enzyme x Temp.	1.6	0.71	2.26	0.1520
pH x Temp.	-4.3	0.71	-6.08	0.0260[a]

[a] Significant factors (p<0,05)

Table 7: Main effects analysis for FOS productivity from the second factorial design

Factor	Effect	Std. Error	t-value	p-value
Mean	4.11	0.07	61.68	0.0003[a]
Enzyme	3.91	0.06	63.36	0.0002[a]
Enzyme (Q)	0.42	0.07	6.31	0.0242[a]
pH	0.56	0.06	9.07	0.0119[a]
pH (Q)	1.25	0.07	18.92	0.0028[a]
Temperature	0.76	0.06	12.27	0.0066[a]
Temperature (Q)	2.54	0.07	38.34	0.0007[a]
Enzyme x pH	2.08	0.08	25.41	0.0015[a]
Enzyme x Temp.	2.18	0.08	26.64	0.0014[a]
pH x Temp.	-2.75	0.08	-33.68	0.0009[a]

[a] Significant factors (p<0,05)

The only relevant variables with respect to FOS yield were the enzyme concentration (linear and quadratic effect), temperature (linear), and the interaction between pH and temperature. Temperature showed a negative effect as also its interaction with pH, indicating that high yield was achieved at lower temperatures (47 – 49 ℃).

An analysis of the effects of the factors on FOS productivity showed that all of them were significant, and the change from the lower to the higher level led to increasing productivity. The negative effect of the interaction between pH and temperature indicated that although high temperatures and pH are beneficial for the process, a simultaneous increase of both factors decreases productivity.

Process optimisation also included reductions in the residual sucrose concentration (loss of raw material) and residual fructose concentration (minimizing the hydrolytic activity). Tables 8 and 9 show the ANOVA for both results.

Table 8: ANOVA for the second design (residual Fructose)

Source of Variation	Sum of Square	Degree of Freedom	Mean Square	F-ratio
Regression	275.6	2	137.8	6.2
Residual	312.4	14	22.3	
Lack of fit	291.3	12		
Pure error	21.1	2		
Total	588.0	16		

*Residual = Lack of fit + Pure error; $F_{0.05,2,14} = 3.7$, R-sqr = 0,47

The pH had no effect on the residual fructose concentration and the response surface (Figure 4) was constructed considering the effects of temperature and enzyme concentration on the residual fructose concentration, despite the moderately low R-squared value of the model. Observation of the surface and the contour diagram led to the conclusion that to minimize the residual fructose concentration, the synthesis should be carried out at low temperatures and low enzyme concentration.

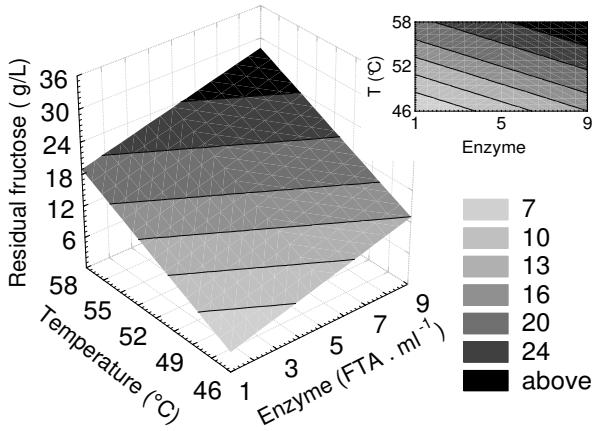

Figure 4: Response surface and contour diagrams for the residual fructose concentration, according to the second experimental design

Table 9: ANOVA for the second design (residual Sucrose)

Source of Variation	Sum of Square	Degree of Freedom	Mean Square	F-ratio
Regression	7638.3	6	1273.0	25.6
Residual	496.3	10	49.6	
Lack of fit	486.6	8		
Pure error	9.7	2		
Total	8134.6	16		

*Residual = Lack of fit + Pure error; $F_{0.05,6,10} = 3.2$, R-sqr = 0.94

For the residual sucrose concentration, ANOVA revealed that the model was predictive, with an F-test value eight times higher than the critical value and also a high R-squared value (0.94). Analysing Figure 5 (response surface and contour diagrams for residual sucrose concentration), a minimum residual sucrose concentration could be achieved by carrying out the synthesis at 51 to 54°C, with 6 to 7 FTA.ml^{-1} and a pH value between 4.7 and 5.0.

Optimisation of FOS synthesis using the extra cellular enzymes from *Rhodotorula* sp. LEB-V10, considered different aspects of the process, such as the total FOS production, yield and productivity and also the residual sucrose and fructose concentrations. An observation of all these factors led to the determination of the following optimal conditions: 50% sucrose, 50°C (± 1°C), pH 5.0 (± 0.5) and 6.5 FTA.ml^{-1} (± 0.5).

Although not all the responses were optimised, conditions leading to a high FOS yield of about 55-70%, with minimal residual sucrose, were obtained, so that the overall process behaviour was quite similar to that using immobilized fungal cells.

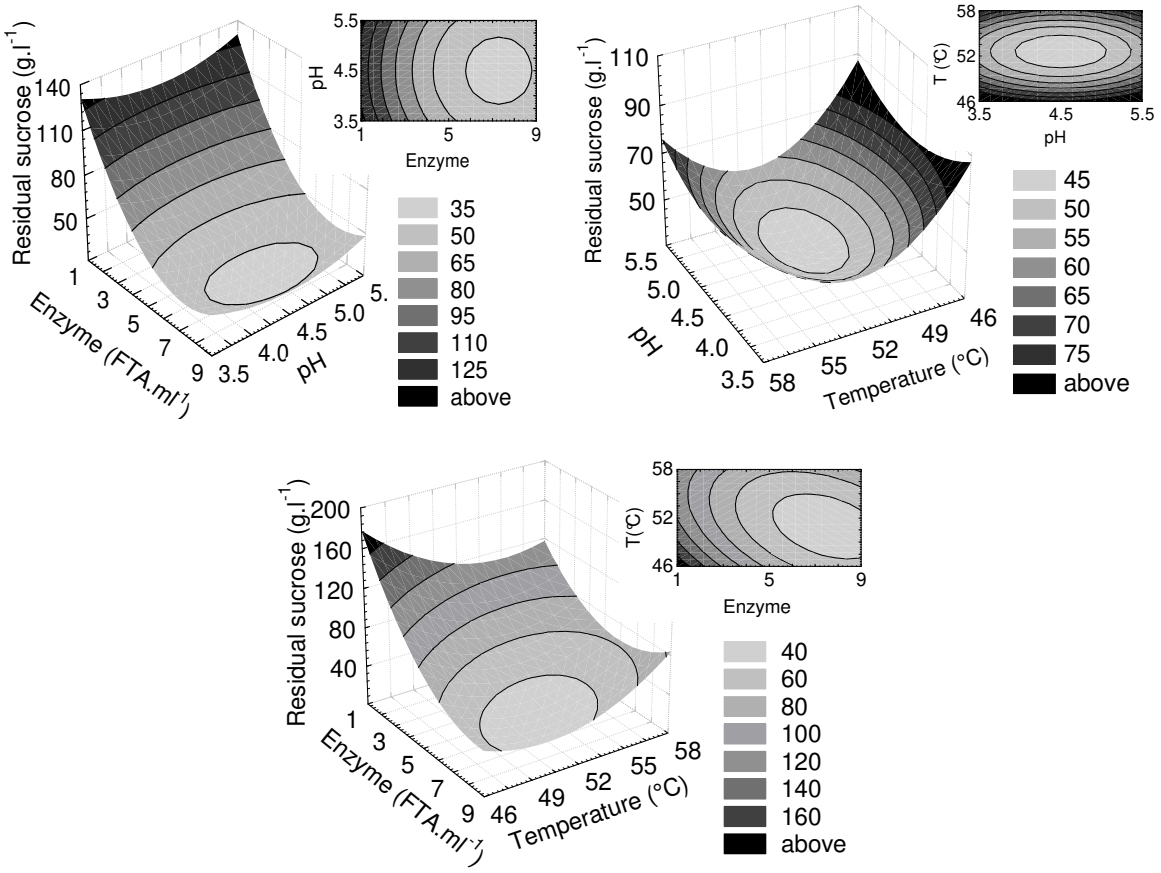

Figure 5: Response surface and contour diagrams for the residual sucrose concentration, according to the second experimental design

To evaluate and confirm the optimum conditions, a triplicate assay under these conditions was performed. The same experiment was carried out using commercial sucrose and sugar cane molasses, so that a Tukey test could be carried out to investigate if the substitution of pure sucrose by such other sources of sucrose, would affect FOS production (Table 10). The use of molasses was advantageous because of its lower cost and lower residual sucrose concentration, obtaining almost the same yield as the reaction with pure sucrose. The use of commercial grade sucrose in substitution of pure sucrose led to an increase in FOS yield, showing no difference between their residual sucrose concentrations.

Table 10: Responses for the assays done under the optimum conditions and the Tukey test

	FOS ($g.l^{-1}$)	Yield (%)	Residual sucrose ($g.l^{-1}$)
Sucrose P.A.	277.7 ± 1.3	55.6 ± 0.3[a]	28.0 ± 6.3[a]
Commercial Sucrose	317.0 ± 7.7	64.1 ± 1.3[b]	26.4 ± 5.9[a]
Sugar cane molasses	260.7 ± 2.7	52.4 ± 0.9[c]	16.0 ± 1.1[b]

[a, b, c] According the Tukey test, the means are significantly different at the 0,05 level.

To determine the relationship between sucrose and FOS production and the other responses, five assays were carried out with different sucrose concentrations, from 35 to 60% (Table 11). The results revealed that the FOS yield was about 56 % (± 1%). Higher concentrations led to higher productivities and residual sucrose concentrations, but the residual fructose concentration was not clearly sucrose concentration dependent.

Table 11: Observed responses at different sucrose concentrations

Sucrose (%)	FOS ($g.l^{-1}$)	Yield (%)	Productivity ($g.l^{-1}.h^{-1}$)	Residual sucrose	Residual fructose
35	190.4	54.4	8.3	17.9	24.9
40	227.2	56.8	8.5	24.5	15.1
45	253.4	56.3	9.4	25.4	14.1
50	277.6	55.5	11.9	29.4	17.7
55	309.7	56.3	10.9	38.6	9.1
60	342.5	57.1	11.4	40.6	14.7

4. Conclusions

Optimisation of the process for FOS production by extra cellular fructosyl transferase from *Rhodotorula* sp. LEB-V10 led to a high yield process using pure sucrose, commercial sucrose or sugar cane molasses. The optimised conditions were shown to be 50% sucrose, 50℃ (± 1℃), pH 5.0 (± 0.5) and 6.5 $FTA.ml^{-1}$ (± 0.5), which led to a 55.6 ± 0.3 yield within 72 hours of reaction. After the first 20 to 24 hours, the FOS production was around 95% of the maximum, but only 25% of the FOS produced was GF3 and GF4. When the production of high GF3 and GF4 concentrations is desired, the reaction should

be sustained up to 60 to 70 hours, when the concentration of GF3 and GF4 reached around 57% of the total FOS content.

Acknowledgments

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