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APLICAÇÃO DE INULINASES DE LEVEDURAS ISOLADAS DO SEMI-ÁRIDO PARA A PRODUÇÃO DE CONCENTRADOS DE FRUTOSE

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Dissertação apresentada ao Programa de Pós-graduação em Biotecnologia, da Universidade Estadual de Feira de Santana como requisito parcial para obtenção do título de Mestre em Biotecnologia.

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RESUMO

A enzima inulinase hidrolisa a ligação β-2 da inulina, um polímero linear de frutose, formando xarope de frutose. O uso de inulinases constitui uma forma alternativa de obtenção deste produto. O objetivo deste trabalho foi avaliar a produção, realizar caracterização e aplicação de inulinases produzida por: *Pseudozima* sp CCMB 300, *Kluyveromyces marxianus* CCMB 322 e por um fungo endofítico CCMB 328 isolados na região semi-árida do Brasil. A enzima foi caracterizada cineticamente a natureza da estabilidade térmica foi avaliada assim como o potencial de hidrólise . Os resultados indicam que a inulinase isolada tem potencial para a hidrólise de inulina apresentando taxas de conversão de até 100 % de inulina em frutose.

Palavras-chave: Inulina, inulinase, leveduras, semi-árido, Brasil.

ABSTRACT

Inulinases target the β-2 linkage of inulin, a poliyfructan consisting of linear β-2,1 linked fructose, and hydrolyzes it into fructose. The use of inulinases provides an alternative way to obtain fructose syrup through the hydrolysis of inulin. The objective of this work was to study the production, characterization and application of an inulinase from *Pseudozima* sp CCMB 300, *Kluyveromyces marxianus* (CCMB 322) and fungal endophytes (CCMB 328) isolated from Brazilian semi-arid region. The enzyme was characterized on the kinetic and stability nature and the results suggest that the isolated inulinase has potential for inulin hydrolysis reported rates of up to 100% conversion of inulin to fructose.

Keywords: Inulin, inulinase, yeasts, semi-arid, Brazil

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INTRODUÇÃO

O semi-árido é um ecossistema com enorme potencial de biodiversidade. Apesar da importância do semi-árido para o Brasil, ainda são poucos os investimentos para desenvolver o potencial da região visando ao desenvolvimento científico, tecnológico e sua sustentabilidade. Estudos que visem à elucidação deste potencial são de grande importância tanto do ponto de vista da sustentabilidade quanto do ponto de vista econômico porque novos produtos biotecnológicos podem ser descobertos.

A biotecnologia compreende um vasto conjunto de técnicas que utilizam seres vivos, ou parte deles, para produzir ou modificar produtos, aumentar a produção ou ainda, selecionar micro-organismos para fins específicos. Este projeto tem como tema a aplicação de inulinases de leveduras isoladas do semi-árido para produção de concentrados de frutose.

Devido às aplicações dos micro-organismos, as coleções microbiológicas, como a Coleção de Micro-organismos da Bahia (CCMB), são instituições estratégicas para preservação dos recursos ambientais e para o desenvolvimento científico, por isso é importante buscar informações e realizar estudos utilizando essas coleções.

Neste contexto este trabalho se justifica, pois busca pesquisar o potencial destas leveduras isoladas no semi-árido com o objetivo de desenvolver produtos biotecnológicos, aproveitando a biodiversidade desta região

1 REVISÃO DA LITERATURA

1.1 Inulinase e a produção de xarope de frutose

A frutose é um monossacarídeo amplamente distribuído na natureza e possui um poder adoçante cerca de 70% superior ao da sacarose. Seu uso na indústria de alimentos, de bebidas e na indústria farmacêutica vem crescendo devido ao seu maior poder adoçante, maior solubilidade em água e ausência de problemas de cristalização apresentados pela sacarose (PESSONI et al, 2004; SHARMA& GILL, 2007).

A frutose, isolada pela primeira vez em 1847 da cana-de-açúcar, é uma cetohexose dos grupos dos monossacarídeos, também conhecida por D-frutose ou levulose, sendo considerado o açúcar com o maior poder adoçante encontrado na natureza, cerca de 70% superior à sacarose (PESSONI et al. 2004). A frutose pode ser encontrada em frutas, mel e até nos vegetais e devido ao seu alto poder edulcorante torna possível uma redução calórica na dieta quando comparado com a sacarose (HAULY & MOSCATTO, 2002)

Utilizado como edulcorante, a frutose possui a vantagem de não apresentar o gosto residual amargo como a maioria dos adoçantes artificiais, realçando os sabores e aromas naturais dos alimentos (HAULY & MOSCATTO, 2002).

A frutose é utilizada comercialmente em misturas para bolos, gelatinas e pudins dietéticos, balas, sobremesas geladas, suplementos alimentares, bebidas energéticas e para aumento da funcionalidade do amido, além de ser utilizada também na indústria farmacêutica (LOURENÇO, 2004).

Em 1984, nos EUA, os fabricantes dos refrigerantes Pepsi-cola e Coca-cola, obtiveram sucesso na substituição de sacarose por xarope de frutose em seus produtos (TEAGUE; ARNOLD, 1983). A década de 80 pode ser considerada como a da substituição da sacarose, quando o uso de adoçantes alternativos cresceu rapidamente.

Nesse sentido, o consumo deste açúcar na forma líquida tem aumentado significativamente nos últimos anos, principalmente na indústria. O açúcar líquido apresenta algumas vantagens para utilização neste setor, como facilidade de manuseio e economia no processo. O produto pode estar na forma de xarope de sacarose, xarope de

açúcar invertido (mistura de açúcares simples) ou como xarope de frutose (LOURENÇO, 2004).

Atualmente, estuda-se a aplicação do xarope de frutose para obtenção de um combustível denominado 2-metil-furano, como substituto ao etanol. Este novo combustível apresenta algumas vantagens sobre o etanol, como um rendimento energético cerca de 40% maior, e menor volatilidade que o mesmo (AMORIM, 2007).

O processo convencional de obtenção da frutose é realizado mediante uma transformação enzimática que culmina na isomerização da glicose a frutose a partir do amido, utilizando uma seqüência de três enzimas: alfa-amilase, amiloglicosidase e glicose isomerase (PESSOA-JR; VITOLO, 1999).

Uma alternativa para este processo é a utilização da inulina, como substrato para a produção de concentrados de frutose em apenas uma etapa enzimática (PESSONI et al, 2004). A inulina, que é um polímero de frutose, é um carboidrato do grupo de polissacarídeos classificados como frutanos. É composto por uma cadeia principal de unidades de D-frutose com uma unidade de glicose terminal. A fórmula pode ser descrita como GFn, onde G representa a molécula de glicose, F, a molécula de frutose e n o número de unidades de frutose (GERN et al, 2000).

Santos (1998) apresentou que a produção contínua de a partir da inulina com inulinase imobilizada e verificaram uma conversão de 75% produzindo uma mistura contendo 85% de D-frutose.

1.2 Inulina e suas propriedades

A inulina é considerada um importante carboidrato de reserva em plantas sendo sintetizada por aproximadamente 36.000 espécies, e pode ser encontrada em espécies da família Asterácea como *Cichorium intybus* e *Helianthus tuberosus* e em espécies nativas do cerrado brasileiro, sendo que nestas espécies cerca de 80% da massa seca de seus órgãos subterrâneos são constituídos por inulina. Por isso, este carboidrato torna-se uma valiosa fonte para o estudo da produção de frutose via hidrólise enzimática (CARVALHO, PINTO & FIGUEIREDO-RIBEIRO, 1998; MICHAEL, DAVIDSON & MAKI, 1999).

Além disso a inulina é considerada uma fibra alimentar solúvel, podendo ser utilizada para enriquecer produtos alimentares. Diferentemente de outras fibras, não possui sabores adicionais, além de enriquecer os alimentos sem contribuir muito com a viscosidade. Estas propriedades permitem a formulação de alimentos com alto teor de fibras mantendo a aparência e o gosto das formulações padrões (ROBERFROID, 1991).

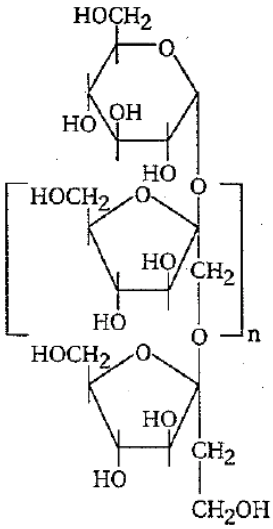
A Tabela 01 apresenta algumas espécies que possuem inulina em sua composição. Dentre estas se destacam a alcachofra, que apresenta 65% em inulina e a chicória, apresentando entre 15-20 % (HAULY & MASCOTTO, 2002).

Tabela 01: Inulina (% do peso fresco) em plantas utilizadas na alimentação humana

Espécie	Parte utilizada	Teor de inulina
Cebola	Bulbo	2-6
Alcachofra Jerusalém	Tubérculo	16-20
Chicória	Raiz	15-20
Alho-porró	Bulbo	3-10
Alho	Bulbo	9-16
Alcachofra	Folhas centrais	3-10
Banana	Fruta	0,3-0,7
Centeio	Cereal	0,5-1
Cevada	Cereal	0,5-1,5
Dente de leão	Folhas	12-15
Yacon	Raiz	3-19
Barba de bode	Folhas	4-11
Trigo	cereal	1-4

Fonte: HAULY & MASCOTTO (2002).

Os alimentos funcionais podem ser classificados como probióticos e prebióticos. Os probióticos são micro-organismos vivos que ingeridos em quantidade e freqüência determinados estabelecem o equilíbrio da microbiota intestinal. Os prebióticos, como a inulina pode ser classificada, são substâncias não-digeríveis pelo organismo humano e estimulam seletivamente a atividade de bactérias benéficas no cólon (ROBERFROID,VAN LOO & GIBSON, 1998).



. Figura 1. Estrutura do trissacarídeo iniciador da série da inulina
(Fonte: OLIVEIRA et AL. (2004).

1.3 A enzima inulinase

Segundo Xiong et al. (2007) inulinases são enzimas que hidrolisam inulina em polímeros de frutanos ou em frutose, podendo ser utilizadas em processos de hidrólise da inulina para obtenção de xarope frutooligossacarídeos e de frutose (JING et al, 2003).

As inulinases são classificadas como 2,1-β-D-frutano-frutanohidrolase (EC 3.2.1.7, endoinulinase) e fructano β-fructosidase (EC 3.2.1.80, exo-inulinase).

A ação de exoinulinase inicia-se na separação frutose da extremidade e se direciona até a molécula final de glicose. Já a endoinulinase inicia a ação enzimático na frutose interna da molécula de inulina (JING, 2003).

Porém é difícil determinar se as enzimas coexistem. Também é difícil separar as duas enzimas completamente por métodos convencionais já que elas possuem propriedades similares (NAGEN, 2004).

Em 1924 a inulinase foi descrita por Pringsheim e Kohn. Inulinases foram inicialmente purificadas a partir de plantas, mas as quantidades obtidas eram insuficientes para serem exploradas comercialmente. Micro-organismos como bactérias, fungos filamentosos e leveduras têm sido descritos como potenciais secretores desta enzima (CRUZ et al, 1998).

Kush et al (1996) afirmou que a ação da inulinase sobre a inulina resulta em uma grande quantidade de D-frutose, com uma pequena quantidade de glicose.

Devido ao sinergismo da ação das duas enzimas, a inulina é hidrolisada completamente até frutose. A hidrólise completa da inulina por estas enzimas pode produzir um concentrado de frutose com aproximadamente com 95% de pureza, em uma única etapa enzimática (SHARMA, GILL, 2007).

Portanto, a hidrólise enzimática da inulina utilizando inulinases isoladas de micro-organismos tem sido considerada como uma promissora tecnologia para obtenção de concentrado de frutose (MAZUTTI et al, 2006).

Vários autores trabalharam com a produção de inulinase a partir de diversos micro-organismos como: *Pichia guilliermondii* (ZHANG T. 2009), *Kluyveromyces marxianus* (PANDEY et al, 1999), *Aspergilus niger*, *Bacillus* sp (Uzunova et al. 2002.), Além disso, outros trabalhos como Ricca et al (2007) caracterizou inulinases

determinando os valores de temperatura e pH ótimos, parâmetros cinéticos, estabilidade térmica e outras características da enzima para diversos micro-organismos.

A hidrólise da inulina também pode ser realizada pelo tratamento com ácidos orgânicos, ou através de catálise heterogênea usando catalisadores sólidos ácidos. No entanto, a abordagem química está associada com algumas desvantagens, como a formação de subprodutos indesejáveis e coloridos, formando compostos, assim como o hidroximetilfurfural, que reduzem o rendimento do produto. Estas desvantagens podem ser superadas se a rota enzimática utilizando inulinases for utilizada (ROCHA, 2006).

Efeitos da temperatura e pH na atividade enzimática

Efeito da temperatura

A temperatura exerce grande influência na atividade enzimática e o ponto ótimo representa o ponto de máxima atividade catalítica. Em temperaturas baixas as enzimas encontram-se muito rígidas e quando se supera um valor considerável a atividade cai bruscamente porque elas se desnaturam (LEHNINGER, 2002).

Pode-se afirmar que a estrutura íntegra das enzimas é determinante para a ação catalítica e que fatores externos podem alterar a conformação protéica e consequentemente interferindo na velocidade das reações. Por isso o controle destes fatores externos são importantes (LEHNINGER, 2002).

A influência da temperatura sobre a cinética da reação enzimática deve ser entendida de duas maneiras: em princípio, aumentos de temperaturas levam à aumentos de velocidades de reação, por aumentar a energia cinética das moléculas componentes do sistema, aumentando a probabilidade de choques efetivos entre elas (STRYER,1996). Porém, temperaturas mais altas levam a desnaturação da enzima, ou seja, à perda de sua estrutura tridimensional por alterarem as ligações que manter esta estrutura, que é essencial para o acoplamento do substrato ao sítio ativo da enzima (STRYER,1996).

As reações catalisadas por enzimas apresentam um comportamento semelhante às reações catalisadas quimicamente. Porém, as enzimas são moléculas protéicas complexas e sua atividade catalítica provém da necessidade de que sua estrutura terciária seja mantida, principalmente por um grande número de ligações não

covalentes, como ligações de hidrogênio, ligações dissulfeto e interações hidrofóbicas (YAHYA, 1998, TÖRNVALL et al 2007).

Em geral, os aumentos de temperatura aceleram reações químicas: a cada 10ºC de aumento, a velocidade de reação se duplica. As reações catalisadas por enzimas seguem esta lei geral. Entretanto, sendo proteínas, a partir de certa temperatura, começam a desnaturar-se pelo calor. (KIELING, 2002).

À medida que a temperatura se eleva o aumento esperado na velocidade, resultante do aumento das colisões entre a enzima e o substrato é contraposto pelo aumento da velocidade de desnaturação (SAID; PIETRO, 2004).

Efeito do pH

A maioria das enzimas apresenta um valor de pH para o qual sua atividade é máxima sendo que, a velocidade de reação diminui à medida que o pH e afasta desse valor ótimo, que é característico para cada enzima. Com freqüência o pH ótimo está próximo da neutralidade (LOURENÇO, 2004).

A influência do pH sobre a catálise enzimática só pode ser compreendida a partir da análise dos grupos dissociáveis presentes nos radicais R dos aminoácidos. De fato, histidina, arginina, lisina glutamato aspartato, cisteína e tirosina têm grupos R que podem ser considerados ácidos fracos de Bronsted, pois são capazes de dissociar, liberando H^+ (LIMA, 2001).

A influência do pH sobre a catálise enzimática é exercida sobre grupos dissociáveis de vários aminoácidos. Alguns desses grupos podem fazer parte dos sítios ativos ou serem importantes na manutenção da estrutura espacial da molécula. Existe uma concentração hidrogeniônica (pH) que propicia um determinado arranjo de grupos protonados e levam a molécula da enzima a uma conformação ideal para exercer seu papel catalítico Esse pH ótimo depende, portanto, do número e tipo de grupos ionizáveis que uma enzima apresenta, ou seja, de sua estrutura primária (RIGEL, 1998).

O sítio ativo pode conter aminoácidos com grupos ionizados que podem variar com o pH e a ionização de aminoácidos que não estão no sítio ativo pode provocar modificações na conformação da enzima.

As enzimas possuem grupos químicos ionizáveis (exemplo: carboxilas –COOH; amino $–NH_2$;tiol –SH; imidazol) nas cadeias laterais de seus aminoácidos. Segundo o pH de meio estes grupos podem ter cargas positiva, negativa ou neutra. Como a

conformação das proteínas depende, em parte das suas cargas elétricas, haverá um pH no qual a conformação será mais adequada para a atividade catalítica. Este é o chamado pH ótimo (MURRAY; GRANNER; MAYES, 2002).. Ligeiras mudanças de pH podem provocar a desnaturação da proteína. Algumas enzimas apresentam variações peculiares. (PAQUES, 2006).

Enzimas e Leveduras

As enzimas, conhecidas industrialmente como biocatalisadores, são em sua maioria proteínas, formadas por longas cadeias de aminoácidos com ligações peptídicas, com exceção de um pequeno grupo de moléculas de RNA com propriedades catalíticas. As enzimas extracelulares são secretadas para fora dos limites da membrana celular. (ZANOTTO, 2003).

Enzimas podem ser consideradas como catalisadores orgânicos produzidas por células vivas, onde exercem as funções vitais de controle dos processos de transformação dos nutrientes em energia e em material celular. (SAXENA, 2003). As enzimas são capazes de atuar em diversas macromoléculas biológicas como as proteínas, carboidratos, lipídeos e ácidos nucléicos, assim como em moléculas menores como os aminoácidos, os açucares e as vitaminas (CARVALHO; CANILHA; SILVA, 2006).

A identificação da atividade de enzimas em micro-organismos, bem como, os estudos relacionados à otimização da produção representam grande interesse industrial. O isolamento de amostras com diferentes potenciais produtores pode representar alternativas para a indústria. Paralelamente, o isolamento de micro-organismos em ambientes naturais pode contribuir com a obtenção de amostras com melhor potencial biotecnológico (LOCK, 2007).

De acordo com Sharma et al. (2001) quase 4000 enzimas são conhecidas, e destas, aproximadamente 200 são utilizadas comercialmente, sendo que a maioria é de origem microbiana.

A utilização de enzimas na indústria biotecnológica tem ganhado destaque por apresentarem uma série de vantagens tais como: custos de produção relativamente baixos, susceptibilidade de produção em larga escala em fermentadores industriais,

características físico-químicas variadas e por representarem um recurso renovável (BAJPAI 2000).

A utilização ampla destas enzimas é conseqüência da elevada especificidade de sua ação como biocatalisadoras, porém, enzimas com o mesmo perfil de atuação sob o substrato, podem apresentar funcionamento ótimo em pH, temperatura e concentração iônica diferentes, o que requer a triagem de enzimas adequadas às condições nas quais serão utilizadas (TOMOTANI; NEVES; VITOLO, 2005; LOWE, 2002).

O uso dos processos microbiológicos aumenta consideravelmente devido a uma ampla variedade de produtos cuja produção envolve direta ou indiretamente a ação de micro-organismos. A aplicação industrial de micro-organismos como as bactérias, leveduras e principalmente os fungos, representa atualmente uma indústria extremamente diversificada e com rendimentos econômicos consideráveis (LOCK, 2007).

As técnicas recentes de biologia molecular, como a engenharia genética, oferecem oportunidades para o aperfeiçoamento de diversos processos de produção e desenvolvimento e pesquisa de novas enzimas (COLWELL, 1997).

As enzimas têm sido os produtos microbianos muito explorados na indústria biotecnológica, sendo utilizadas amplamente no processamento de alimentos, na indústria farmacêutica, na bioquímica, biologia molecular e em diversas aplicações biomédicas (AID, 2004).

REFERÊNCIAS

AMORIM, C. 2007. Frutose é fonte para combustível. Agência brasileira de inteligência.
Disponível em: <http://www.abin.gov.br/modules/articles/article.php?id=335 >. Acesso em: 17 outubro.

BAJPAI, P. 2000. Applications of enzymes in the pulp and paper industry. *Biotechnology Progress.* 15: 147–157.

CRUZ, V.; BELOTE, G.; BELLINE, M.; CRUZ, R. 1998. Production and action pattern of inulinase from *Aspergillus niger*-245: hydrolysis of inulin from several sources. *Critical Reviews in Microbiology.* 29:301-306.

CARVALHO, W.; CANILHA, L.; SILVA, S. 2006. Uso de biocatalisadores imobilizados: uma alternativa para a condução de bioprocessos. *Revista Analytica.* 23: 60-70

CARVALHO, M.; PINTO, M; FIGUEIREDO-RIBEIRO, R. 1998..Inulin production by *Vernonia herbacea* as influenced by mineral fertilization and time of harvest. *Revista Brasileira de Botânica.* 21: 275-280.

COLWELL, R. 1997. Microbial diversity: the importance of exploration and conservation. *Journal of Industrial Microbiology and Biotechnology*. 18:302-307.

DALLA-VECCHIA, R.; NASCIMENTO, M.; SOLDI, V. 2004. Aplicações sintéticas de lípases imobilizadas em polímeros. *Química Nova.* 27:623-630.

GERN, R; JONAS, M.; FURLAN, S.; Ninow, J. 2000. Utilização do extrato de chicória na produção de endo-inulinases por *Paenibacillus* SP. *Revista Saúde e Ambiente*.12: 73-76.

GILL, P.; MANHAS, R.; SINGH, J.; SINGH, P. 2006. Purification and properties of a heat-stable exoinulinase isoform from *Aspergillus fumigatus*. *Journal of Food Engineering.* 76: 369–375.

HAULLY, M.; MASCATTO, J. 2002. A. Inulina e Oligofrutose: uma revisão sobre propriedades funcionais, efeito prebiótico e importância na indústria de alimentos. *Semina: Ciências Exatas e Tecnológica*. 23:105-118.

JING, W. et al. 2003. Production and separation of exo- and endoinulinase from *Aspergillus ficuum*. *Process Biochemistry.* 39:5-11.

LOURENÇO, C. 2004. Desenvolvimento de processo contínuo de obtenção de frutose a partir de sacarose. Dissertação (Mestrado em Engenharia de Alimento). Universidade Estadual de Campinas.

LEHNINGER, A. L. 2002. *Princípios de bioquímica*. 3. ed. São Paulo: Sarvier, p. 278-388.

LIMA, U. 2001. *Biotecnologia industrial***,** São Paulo: Edgard Blücher, v.3

LIU, Y.; LOTERO, E.; GOODWIN, J. 2006. Effect of water on sulfuric acid catalyzed esterification. *Journal of Molecular Catalysis*. 245: 132-140.

LOWE, D. A. 2002. Fungal enzymes. *Fungal Biotechnology*. 4: 681 – 706,

LOCK, L.; CORBELLINI, V.; VALENTE, P.2007 Lipases produced by yeasts: powerful biocatalysts for industrial purpose. *Technology*. 11:8-25.

MAZUTTI, M.; BENDER, J; TREICHEL, H; Di, MARCO.. et al. 2006. Optimization of inulinase production by solid-state fermentation using sugarcane bagasse as substrate. *Enzyme and Microbial Technology***.** 39: 56-59.

MURRAY, R.; GRANNER, D.; MAYES, P. 2002. Bioquímica. 9th ed. São Paulo: Atheneu, p. 354.

NAGEN, R. 2004. Crystal structure of exo-inulinase from *Aspergillus awamori*: the enzyme fold and structural determinants of substrate recognition. *Journal of Molecular Biology.* 344: 471–480.

PESSONI, R.; OLMEDO, P.; CLEMENTE-FILHA, A.; FIGUEIREDO-RIBEIRO, R. 2004. Produção de concentrados de frutose por inulinases de *Penicillium janczewskii* e atividade sobre o nível de glicose plasmática em ratos diabéticos. *Ciência e Tecnologia de Alimentos*. 3: 373-377.

PESSOA JR, A.; VITOLO, M. 1999. Inulinase from *Kluyveromyces marxianus*: culture medium composition and enzyme extraction. *Brazilian Journal Chemistry Engineering*. 16:27-31

PANDEY, A.; SOCCOL, C.; SELVAKUMAR, P.; THOMAZSOCCOL, V. ; KRIEGER, N.; FONTANA, J. 1999. Recent development in microbial inulinases. *Applied Biochemistry Biotechnology***.** 81: 35-52.

PESSONI, R.; FIGUEIREDO-RIBEIRO, R.; BRAGA, M. 1999. Extracellular inulinases from *Penicillium janczewskii*, a fungus isolated from the rhizosphere of *Vernonia herbacea* (Asteraceae). *Journal of Applied Microbiology.* 86: 1024-1032.

PAQUES, W.; MACEDO, A; 2006. Lipases de látex vegetais: propriedades e aplicações industriais. *Química. Nova*. 29:1

ROBERFROID, M. 1993.Dietary fiber, inulin, and oligofructose: a review comparing their physiological effects. *Critical Reviews in Food Science and Nutrition*. 33:103-148.

ROBERFROID, M.; VAN LOO, J.; GIBSON, G. R. 1998. The bifidogenic nature of chicory inulin and its hydrolysis products. *Journal of Nutrition*.128:11-19.

SAID, S.; PIETRO, R. 2004. *Enzimas de interesse Industrial e Biotecnológico* Rio de Janeiro: Eventos.

SANTOS, A. 1998. Síntese de Oligossacarides por inulinase de *Kluyveromyces bulcaricus*. Dissertação (Mestrado em Engenharia de Alimentos). Universidade Estadual de Campinas

SAXENA, R.; SHEORAN, A.; DANDSON, S. 2003. Purification strategies for microbial lipases. *Journal of Microbiological Methods.*52: 1.

SHARMA, R.; CHISTI, Y.; BANERJEE, U. 2001. Production, purification, characterization and applications of lipases. *Biotechnology advances*. 19:627-662.

SHARMA, A.; KAINTH S.; GILL, A. 2006. Inulinase production using garlic (*Allium sativum*) powder as a potential substrate in *Streptomyces* sp. *Journal of Food Engineering* 77;486–491.

STRYER, L. 1996 *Bioquímica.* 4 ed. Rio de Janeiro: Guanabara Koogan

RIGEL,R. Bioquímica. 2. Ed. São Leopoldo:Unisinos, 1998.

SHARMA, A.; GILL P. K. 2007. Purification and characterization of heat-stable exo-inulinase from Streptomyces sp. *Journal of Food Engineering.* 79:1172-1178.

TEAGUE, J.R.; ARNOLD, E.C. 1983.Technology for the production of fructose sweeteners. *Sugar y Azucar*. 78:18-19,

TÖRNVALL, U.; ORELLANA-COCA, C.; HATTI-KAUL, R.; ADLERCREUTZ, D. 2007. Stability of immobilized Candida Antarctica lipase B during chemo-enzymatic epoxidation of fatty acids. *Enzyme and Microbial Technology*. 40: 447-451.

TOMOTANI, E.J., NEVES, L.C.M., VITOLO, M. 2005. Oxidation of glucose to gluconic acid by glucose oxidase in a membrane bioreactor. *Applied Biochemistry and Biotechnology*.121:149-162.

UZUNOVA, K.; VASSILEVA, A.; TONKOVA, A. 2002. Thermostable exo-inulinase production by semicontinuous cultivation of membrane immobilized *Bacillus* sp. *Process Biochemistry.* 37:863-868.

XIONG C, JINHUA W, DONGSHENG L. 2007. Optimization of solid state medium for the production of inulinase by *Kluyveromyces* S 120 using response surface methodology. *Biochemical Engineering Journal.* 34:179-184

WENLING, W.; HUIYING, W.; SHIYUAN, W. 1999. Continuous preparation of fructose syrups from Jerusalem artich oke tuber using immobilized intracellular inulinase from *Kluveromyces sp.* Y-85. Process Biochemistry. 34: 643-646.

ZHANG, T. 2009. Optimization for high-level expression of the *Pichia guilliermondii* recombinant inulinase in *Pichia pastoris* and characterization of the recombinant inulinase. *Process Biochemistry*. 44:1335-1339

YAHYA, A.; ANDERSON, W.; MOO-YONG, M. 1998 *Enzyme and Microbial Technology*. 23:438-441.

ZANOTTO, S.P. 2003. Utilização de enzimas e microrganismos para a obtenção de compostos oticamente ativos. Tese (Doutorado em Biotecnologia), Universidade Federal de Santa Catarina Florianópolis.

PRODUCTION, CHARACTERIZATION AND APPLICATION OF INULINASE FROM *PSEUDOZYMA* SP. CCMB 300.

Abstract

Inulinases target the β-2 linkage of inulin, a poliyfructan consisting of linear β-2,1 linked fructose, and hydrolyzes it into fructose. The use of inulinases provides an alternative way to obtain fructose syrup through the hydrolysis of inulin. The objective of this work was to study the production, characterization and application of an inulinase from *Pseudozima* sp CCMB 300 isolated from Brazilian semi-arid region. The enzyme was characterized on the kinetic and stability nature and the results suggest that the isolated inulinase has potential for inulin hydrolysis.

Keywords: Inulin, inulinase, yeasts, semi-arid, Brazil

2.1 Introduction

Inulinase target the β-2 linkage of inulin, a polyfructan consisting of linear β-2,1 linked fructose, and hydrolyzes it into fructose (Gong, 2008).

Inulin is a naturally occurring polyfructan in plants consisting of linear chains of β (2,1)-linked fructose residues attached to a terminal sucrose molecule. It is produced naturally in over 36,000 plants world wide, including 1200 native grasses and cereals belonging to several genera. In addition, inulin has extensive documented historical human use through the consumption of edible plants and fruits (Sharma; Kainth; Gill, 2006).

Inulinase can be derived from plants and many microorganisms. Dandelion, chicory and Jerusalem artichoke have been proven to contain inulinase that can be purified, but according to Kochhar et al (1999), these sources are not as productive as the microbial one, which seem to be the only source capable of producing enough enzymes for industrial applications. For this reason, in the last three decades, significant efforts have been made to find the best microbial source for the extraction of inulinase (Ricca, et al. 2007).

Conventional fructose production from starch needs at least three enzymatic steps, including α-amylase, amyloglucosidades, and glucose isomerase action, yielding only 45 % fructose solutions. More concentrated fructose solution requires a dedicated chromatographic step. A viable alternative to this process is the hydrolysis of inulin by inulinases. Enzymatic formation of fructose from inulin has a single enzymatic step and yields up to 95 % fructose (Figueiredo-Ribeiro et al., 2007).

The Brazilian semi-arid region represents a large area for bioprospection since naturally occurring microorganisms adapted to a tropical semi-arid environment, with high temperature and low humidity throughout the year, may possess some features of great industrial interest (Uetanabaro and Góes-Neto, 2006).

In this study, we carried out a survey of the production, characterization and application of extracellular inulinases produced by *Pseudozima* sp. from the Brazilian semi-arid region.

2.2 Materials and methods

Chemicals

Inulin, bovine serum albumin, and 3,5-dinitrosalicylic acid were purchased from Sigma Chemical Co. (St Louis, MO, USA). All the other chemicals used were also of high-quality analytical grade.

Microorganisms

The yeast strains *Pseudozyma* sp. CCMB is from Culture Collection of Microorganisms of Bahia (CCMB) of the Universidade Estadual de Feira de Santana, Brazil (Uetanabaro and Góes-Neto 2006).

The yeast strains were maintained in YM agar (3% yeast extract (w/v), 3% malt extract (w/v), 5% peptone (w/v), 10% glucose (w/v), and 20% agar (w/v), pH 6.2).

Inulinase production

The yeast was previously grown on YM agar at 28ºC for 48 h, as described in Oliveira (2007), diluted in sterile distilled water to a concentration of about 10^8 colony-forming units/ml. 10% (v/v) of the diluted growth medium was inoculated in flasks containing (per 1 L) mineral medium ($CaCl_2$, 0.25 g; $(NH_4)_2SO_4$, 3 g; KH_2PO_4, 4.5; $MgSO_4$, 0.25g), supplemented with yeast extract, 1 g; glucose, 10 g; pH 5.0, for fermentation (Patching and Rose 1969). After incubation at 28 ºC for 48 h in an orbital shaker at 150 rpm, the cells were separated by centrifugation at 10,000 g for 10 min at 48 ºC, and the supernatant liquid media was used as the extracellular fraction.

Biomass quantification

The cells, which were previously separated by centrifugation, were used to determine the dry weight at 50ºC.

Enzyme assays

Inulinase activity was measured spectrophotometrically (A_{540}) using the dinitrosalicylic reagent, as reported by Miller (1959). The reaction mixture consisted of 900 µL of 2 % (w/v) inulin in 0.05 M acetate buffer, pH 5.5, and 100 µl culture supernatant. The mixture was incubated for 15 min at 50 ºC. After incubation, 1 mL dinitrosalicylic reagent was added, and the mixture was boiled at 100 ºC for 10 min and cooled with 10 mL distilled water. One unit of enzyme activity of inulinase was defined as the amount of the enzyme that catalyzed the formation of 1 µmol fructose/min.

Protein determination

Total protein determination was performed according to Bradford (1976), using bovine serum albumin as the standard.

Doehlert experimental design for enzyme production

The Doehlert experimental design, with two variables (concentration of glucose and yeast extract) and three replicates at the centre of the domain leading to a total of 9 experiments (Table 1) was used to obtain the knowledge of the effect of glucose and yeast extract concentration on the production of enzyme.

Thus, concentration of glucose was studied in five levels (7.5 to 12.5 g/L) and yeast extract was studied in three levels (0.5 to 1.5 g/L). The experimental errors were evaluated from replication of central point. The experimental data were processed by using the STATISTICA software. All the experiments in this step were carried out in random order.

To estimate the lack of fit of the model to the experimental data an analysis of variance (ANOVA) was performed, using Design Statistics, version 7.0.

Response surface optimization of temperature and pH for inulinase obtained

Response surface modeling was applied to cultures of yeast, to determine the optimum temperature and pH for inulinases obtained. This statistical technique for experimental design has advantages over methods that investigate only one variable at a time.

The experimental design, with two variables (temperature and pH) and three replicates at the centre of the domain leading to a total of 9 experiments (Table 3) was used to obtain the knowledge of the effect of temperature and pH of inulinase activity. The pH was studied in five levels (5 to 9) and temperature was studied in three levels (30 to 70ºC). The experimental errors were evaluated from replication of central point. The experimental data were processed by using the STATISTICA software. All the experiments in this step were carried out in random order.

To estimate the lack of fit of the model to the experimental data an analysis of variance (ANOVA) was performed, using Design Statistica, version 7.0.

Effect of cations on inulinase activity

The effect of Na^+ and K^+ on inulinase activity was studied. The concentrations used were: NaCl (0.05, 0.1, 0.15, 0.2, 0.25 and 0.3 mol/L), KCl (0.05, 0.1, 0.15, 0.2, 0.25 and 0.3 mol/L). The inulinase activity was determined by the standard assay as described previously.

Determination of kinetics parameters

To obtain the parameters of the Michaelis-Menten kinetics of inulinase for the hydrolysis of inulin, K_m and V_{max}, 0.9 µL inulin (1, 1.5, 2.0, 2.5 and 3.0 % (w/v) was added to 100 µL of inulinase in 0.05 M acetate buffer (pH 5.5) and incubated at 50 ºC for 15 mim. The K_m and V_{max} for inulin was determined by the method of Lineweaver-Burk plots.

Doehlert experimental desing for Hydrolysis of inulin

The extent of inulin hydrolysis (%) was calculated as $\frac{\text{amount of fructose released}}{\text{amount of initial total sugars}} \times 100$ (Nakamura et al, 1995). Total sugars were determined by antrone method and reducing sugars were determined by the dinitrosalicylic acid method (Miller, 1959). The Doehlert experimental design, with two variables (concentration of enzyme and fermentation time) and three replicates at the centre of the domain leading to a total of 9 experiments was used to obtain the knowledge of the effect of enzyme concentration and fermentation time on the extent of inulin hydrolysis from a solution 1% (w / v) in citrate buffer 0.05 M.

Thermostability of inulinase

Samples of inulinases in test tubes (selected to be equal in weight, volume and size) were incubated in buffer at different temperatures (50ºC, 60ºC, 70ºC, 80ºC e 90ºC) and for various times (0, 10, 20, 30, 40, 50 and 60 min). After the heating process the tubes were cooled in melting ice and the residual activity measurement was carried out at pH 6.0 and at a temperature of 50ºC.

Statistical analysis

All experiments were carried out in triplicate. One-way analysis of variance was used to compare the specific activity of inulinase among the strains. Where significant differences were detected between strains, the means were compared using Tukey's test. For all statistical analyses, the level of significance was set at 5%, and the analyses were performed using the standard statistical software Statistica 9.0 for windows.

2.3 Results and discussion

Production of inulinases by yeasts

The table 01 shows the Doehlert design applied to optimize the production of inulinase by *Pseudozyma* sp. CCMB 300. The first column describes the number of planning, with C representing the central compound. In the second and third column are the values of glucose concentration and the concentration of yeast extract, respectively. The fourth column represents the enzymatic activity. The last column represents predicted values.

Table 01: Results of the factorial analysis for inulinase production CCMB 300

N°	Concentration of glucose (g/L)	Concentration of yeast extract (g/L)	Experimental values (UA)	Activities predited (UA)
1	7.5 **(-0,5)**	1.5 **(+0,866)**	1.0242	1.0409
2	12.5 **(+0,5)**	1.5 **(+0,866)**	1.7904	1.7736
3	5 **(-1)**	1 **(0)**	1.3198	1.3030
4C	10 **(0)**	1 **(0)**	2.3734	2.3935
4C	10 **(0)**	1 **(0)**	2.4376	2.3935
4C	10 **(0)**	1 **(0)**	2.3697	2.3935
5	15 **(+1)**	1 **(0)**	2.6988	2.7155
6	7.5 **(-0,5)**	0.5 **(-0.866)**	1.7022	1.7189
7	12.5 **(-1)**	0.5 **(-0.866)**	2.4155	2.3987

The response surface graphics (Figures 01 and 02) shows the influence of glucose and yeast extract concentrations in the production of inulinase of CCMB 300. From the analysis of the area chart in Figures 01 and 02 it can be concluded that the production of inulinase by CCMB 300 strains clearly peaks for glucose concentrations in excess of 10 g/L glucose, for the whole range of concentration of yeast extract tested.

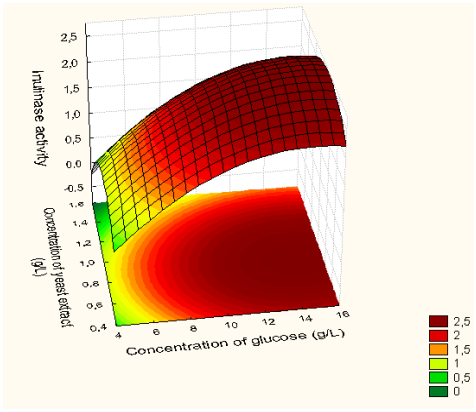


Fig. 01. Response surface for yeast extract concentration versus glucose concentration CCMB 300

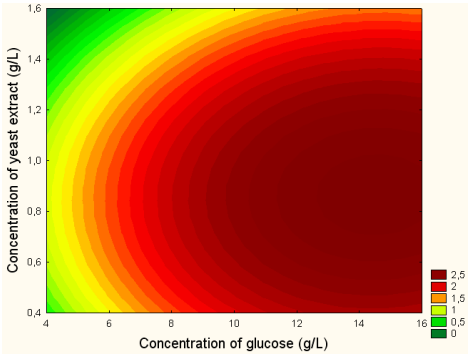


Fig. 02. Level curves for yeast extract concentration versus glucose concentration CCMB 300

Equation 1 illustrates the relation of these two variables and the enzyme activity (UA), where G is the glucose concentration g/L and YE is the yeast extract concentration g/L.

- **(UA) = -2.056 + 0,438(G) – 0.0153(G)2 + 3.758(YE) – 2.258(YE)2+.0106(G) X (YE). (Equation 1)**

Through the derivation of this equation, the media composition, regarding glucose and yeast extract concentrations, which are expected to allow for the production of maximum enzymatic activity, can be obtained. For CCMB 300 the conditions are 14.54 g / L for glucose and 0.86 g / L for yeast extract.

Different nitrogen sources were studied for *Cryptococccus aureus* inulinases. Yeast extract was the better and increased inulinase production (Sheng et al, 2007). According to these authors, vitamins and trace elements present in the yeast extract may enhance inulinase production.

The chart of Paretto (Figure 03) shows that both linearly (L) and quadratically (Q), the variables glucose concentration and yeast extract concentration are significant for the production of inulinase by *Pseudozyma* CCMB 300, because both variables had a p value greater than 0.05

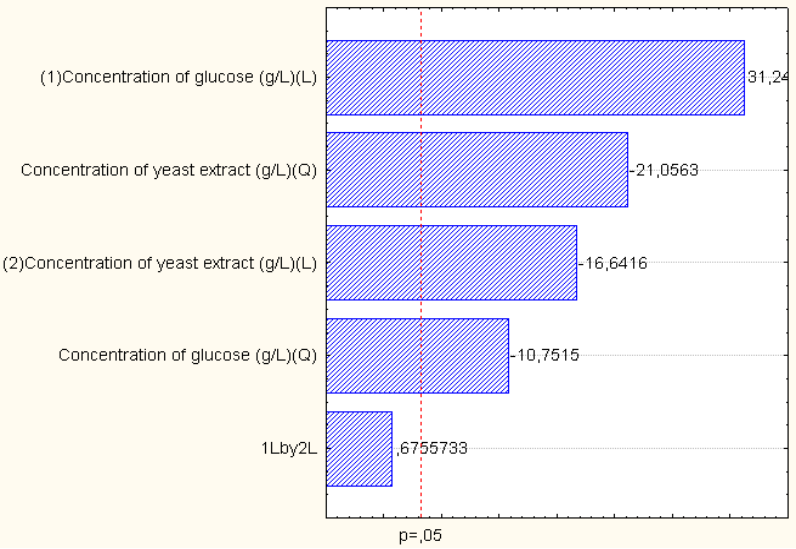


Fig. 03: Pareto Chart for inulinase production CCMB 300

The statistical significance of the regression (Table 02) can be assessed by the ratio of the mean square regression and mean square of the residue and by comparing these sources of variation using the Fisher distribution (F test) Thus, a statistically significant value of this ratio must be greater than the tabulated value for F. It is obtained by the ANOVA table that calculated F (348.30) is higher than the F tabulated (9.01) showing that the function is well suited to the answers.

Table 02 Analysis of variance

Variation source	SQ	gl	MQ	F	Tabulated F (IC de 95%)	R^2
Regression	2.669513	5	0.533903	348.3017	9.01	
Residual	0.004599	3	0.001533			
Lack of Fit	0.001683	1	0.001683	1.155	0.394970	0.99
Pure Error	0.002915	2	0.001458			
Total SQ	2.674111	8				

The table 03 shows the Doehlert design applied to the optimization of temperature and pH inulinase obtained by *Pseudozyma* sp. CCMB 300.

Table 03. Results of the factorial for optimization of temperature and pH CCMB 300

N°	pH	Temperature °C	Experimental values (UA)	Activities predicted (UA)
1	6 (-0.5)	70 (+0.866)	1.5104	1.5743
2	8 (+0.5)	70 (+0.866)	1.9197	1.8557
3	5 (-1)	50 (0)	1.8427	1.7787
4C	7 (0)	50 (0)	2.1973	2.2147
4C	7 (0)	50 (0)	2.2279	2.2147
4C	7 (0)	50 (0)	2.2189	2.2147
5	9 (+1)	50 (0)	2.1971	2.2610
6	6 (-0.5)	30 (-0.866)	1.0458	1.1097
7	8 (+0.5)	30 (-0.866)	1.3744	1.3104

The influence of temperature and pH on the activity of the enzyme was investigated for CCMB 300 by surface response methodology. The results are shown in Figures 04 and 05.

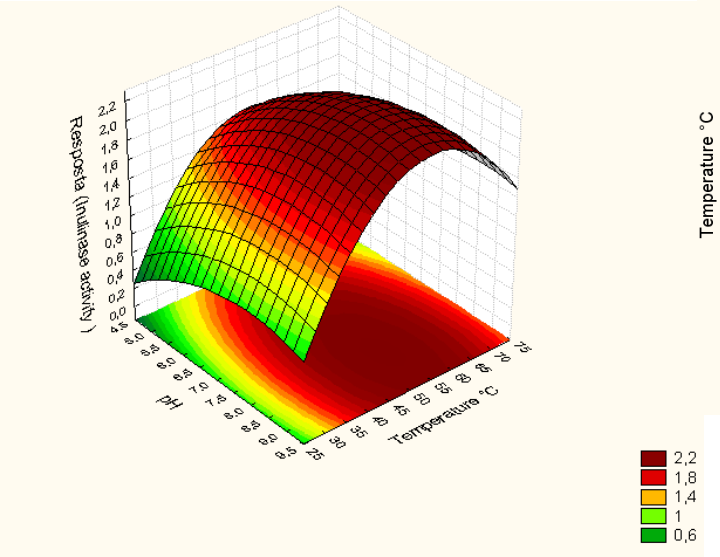


Fig 04. Response surface for pH versus temperature CCMB 300

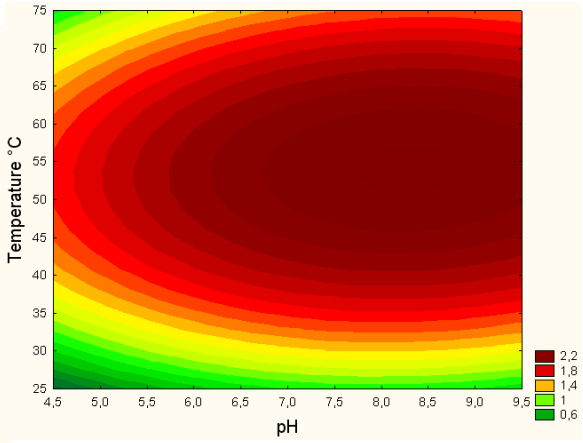


Fig 05. Level curves for pH versus temperature CCMB

The regression model provided for the enzyme activity in relation to pH and temperature in the experimental design is expressed by Equation 2:

(UA) = -5.6887+ 0.7514(P)-0.0486(P)2+0.1814(T) -0.00175(T)2+,00101(P).(T).

(Equation 2)

This equation illustrates the relationship of these two variables with enzyme activity (UA), where: P is the pH and T is the temperature ºC. Through the derivation of this equation, the points of maximum enzymatic activity can be obtained. According to this methodology, the optimal pH and temperature values were of 8.28 and 54°C, respectively.

Many works in the literature deal with the effects of temperature and pH on inulinase activity. It is certain that the response of the enzyme activity to these variables depends mainly on the strain used as a source for enzyme production. The studies of

Pandey et al. (1999) showed that fungal inulinases exhibited an optimum pH between 4.5 and 7.0, yeasts inulinases between 4.4 and 6.5 and bacterial inulinases between 4.8 and 7.0. Inulinases preparations from *A. niger* strains have also been shown to have pH and temperature optima in the ranges of 4.35 to 5.35 and 45 to 60 ºC (Kango, 2008). Information about the effect of temperature and pH on inulinase activity is very important for assessing the feasibility industrial application, and to the development of bioprocesses and to the selection of bioreactors.

The Paretto chart (Figure 06) shows that both linearly and quadratically, temperature and pH are exerting influence on the enzyme activity of inulinase *Pseudozyma* CCMB 300, because both variables had a p value greater than 0.05

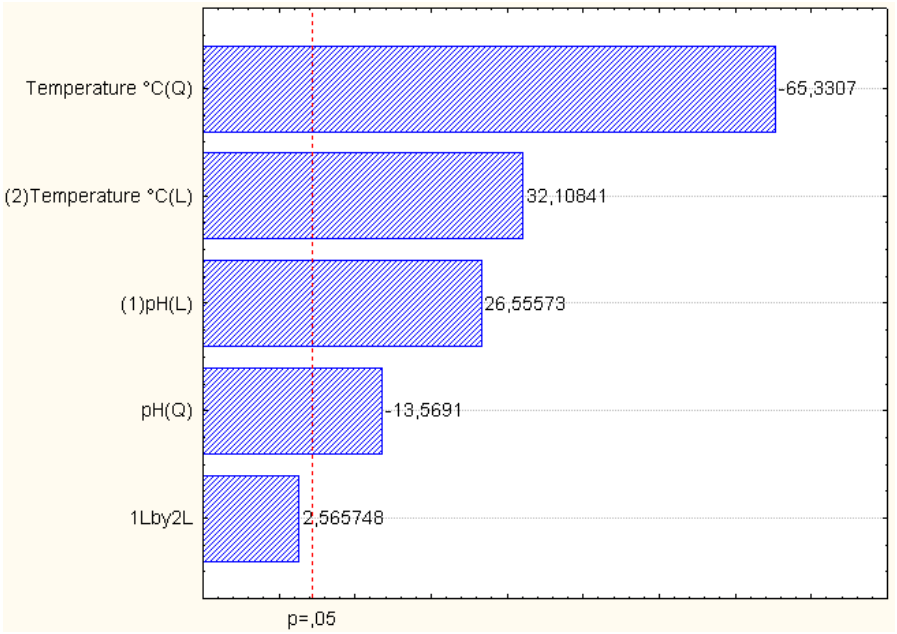


Fig 06: Paretto Chart

The statistical significance (Table 04) of the regression can be assessed by the ratio of the mean square regression and mean square of the residue and by comparing these sources of variation using the Fisher distribution (F test) Thus, a statistically

significant value of this ratio must be greater than the tabulated value for F. It is obtained by the ANOVA table that calculated F (35.67) is higher than the F tabulated (9.01) showing that the function is well suited to the answers.

Table 04 Analysis of variance

Variation source	**SQ**	**gl**	**MQ**	**F**	**Tabulated F (IC de 95%)**	**R^2**
Regression	1.486667	5	0.297333	35.67048	9.01	
Residual	0.025007	3	0.008336			
Lack of Fit	0.024512	1	0.024512	99.111	0.009940	0.98
Pure Error	0.000495	2	0.000247			
Total SQ	1.511674	8				

Effect of cations

The effect of salts NaCl and KCl on the activity of inulinase are shown in Figure 07. The activity was maximal at a concentration of 0.15 mol/L of NaCl and KCl. The concentrations of 0.15 mol/L of NaCl and KCl increased the activity of inulinase from CCMB 300 by approximately 40%. When concentration higher than 0.15 mol/L were used, a decrease in the activity of the enzyme was observed.

The studies of Sheng et al (2007) showed that the addition of 1 mol/L NaCl and KCl, increased the activity of inulinase by 145 % and KCl 142.9 %, a greater increased than CCMB 300.

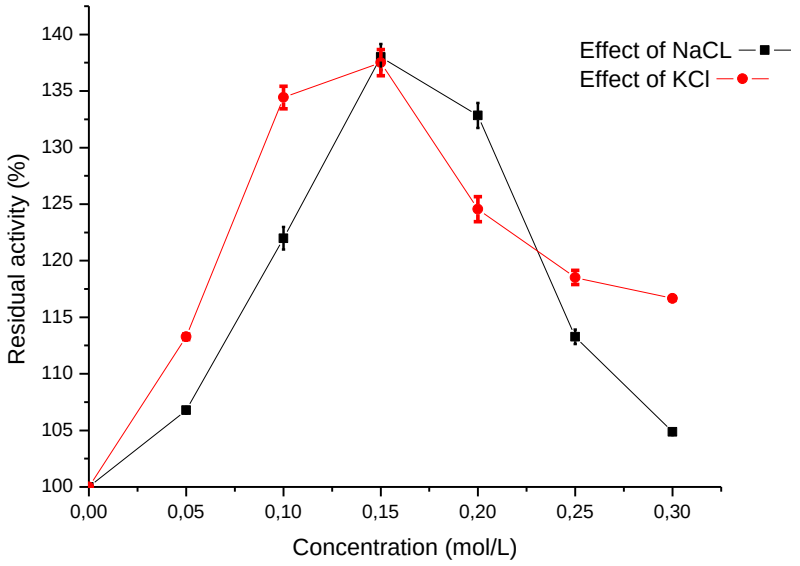


Fig.07. Effect of salts on CCMB 300 inulinase activity

Kinetics Parameters

The Lineweaver-Burk plot showed that the K_m and V_{max} values of the enzyme for inulin were 23 mg/mL and 28.33 µmol/mL.min, respectively (Fig. 08). These results demonstrate that the inulinase from CCMB 300 displayed affinity for inulin and are in agreement with other studies. The K_m value for inulinase from *Aspergillus ficuum* JNPSP was 25.6 mg/mL (Chen et 2009).

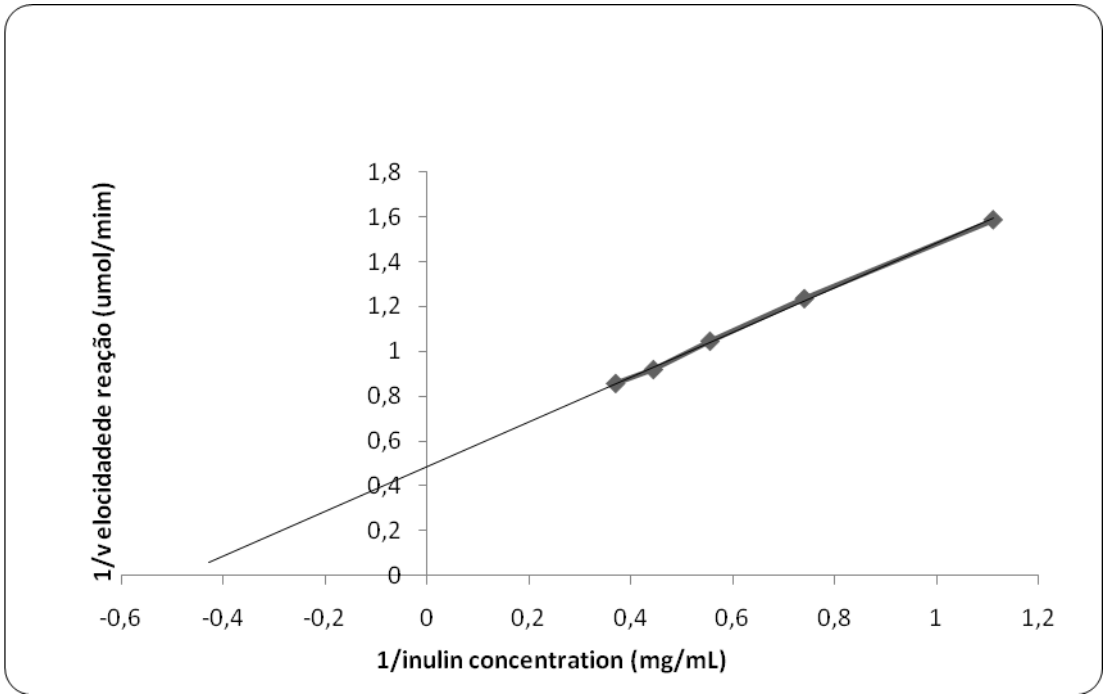


Fig.08. Influence of substrate concentration on the inulinase activity

Thermostability of inulinases

The effect of temperature on the stability of inulinase activity is depicted in Figure 09. A small reduction in inulinase activity at 60ºC, 1.72% after 50 min, was observed. After incubation at 80 ºC for 50 minutes, inulinase still retained 76% of activity. (Sharma; Kainth; Gil, 2006) reported that inulinase produced by S*treptomyces* sp did not show any significant loss activity after treatment at 60 ºC, as CCMB 300, however incubation of inulinase at 80º resulted in a rapid loss of activity (50%).This results suggests the ability of this inulinase by CCMB 300 to endure extreme thermal environments. The thermal stability of enzymes is a very important parameter in process design, as it determines the limits for use and reuse of the enzyme, and therefore process costs.

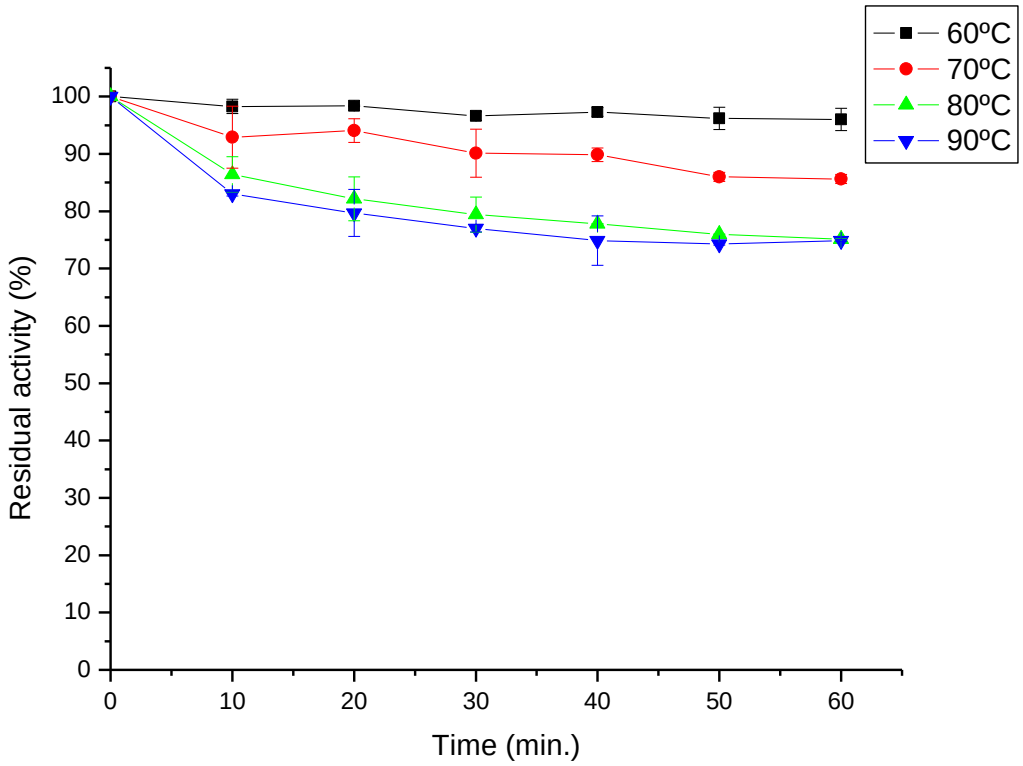


Fig.09 Evaluation of the thermal stability of inulinase from CCMB 300

Hydrolysis of inulin

The Table 05 shows the Doehlert design applied to optimize the hydrolysis in function of time of reaction and enzyme concentration of inulin by *Pseudozyma sp*. CCMB 300 inulinase.

Table 05: Results of the factorial for inulin hydrolysis

N°	Enzyme Concentration (% v/v)	Time (min)	Experimental values (% Hidrolysis)	Response predited (% Hidrolysis)
1	5 **(+0.866)**	4.5 **(-0.5)**	64.543	63.12755
2	5 **(+0.866)**	12.5 **(+0.5)**	49.7088	51.12425
3	3 **(0)**	1 **(-1)**	51.6736	53.06016
4C	3 **(0)**	8 **(0)**	87.0309	87.38700
4C	3 **(0)**	8 **(0)**	88.5784	87.38700
4C	3 **(0)**	8 **(0)**	86.0895	87.38700
5	3 **(0)**	15 **(+15)**	51.0665	49.21775
6	1 **(-0.866)**	4.5 **(-0.5)**	48.6767	47.26125
7	1 **(-0.866)**	12.5 **(+0.5)**	41.6217	43.03715

The hydrolysis was studied in five levels (1 to 12.5 minutes) and enzyme concentration was studied in three levels (1 to 5 %). The experimental data were processed by using the STATISTICA software. All the experiments in this step were carried out in random order.

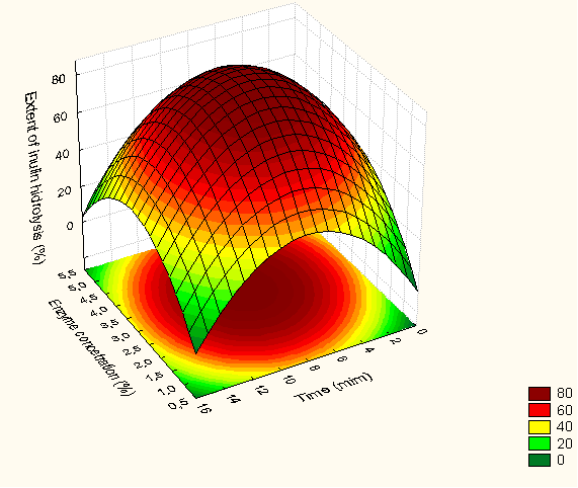


Fig. 10. Response surface for hydrolysis of inulin

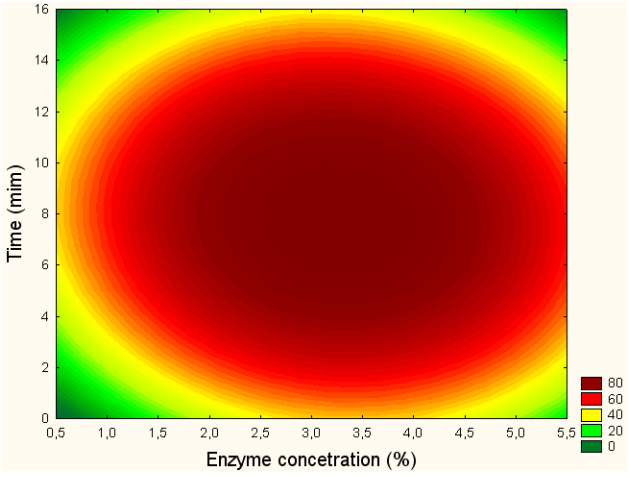


Fig. 11. Level curves for hydrolysis of inulin

Equation 3 illustrates the relation of these two variables with the extent of hydrolysis (% H), where: C is the enzyme concentration (% v/v) and T is the time (min).

$$\% H = -27{,}148 + 41{,}197(C) - 6{,}0227(C)^2 + 12{,}291(T) - 0{,}739(T)^2 - 0{,}2431(C)(T)$$

(Equation 3)

Through the derivation of this equation, the points of maximum enzymatic activity are obtained. In this work the highest productivity, based on the extent of hydrolysis, was observed for 3.26 % of enzyme and 7.7 minutes of incubation. There are few studies of inulin hidrolysys using the same conditions and SRM analysis, as performed in this work.

The Paretto chart (Figure 12) shows that both linearly and quadratic, variables: enzyme concentration and time of hydrolysis are significant for the hydrolysis of inulin Pseudozyma CCMB 300, because both variables had a p value greater than 0.05.

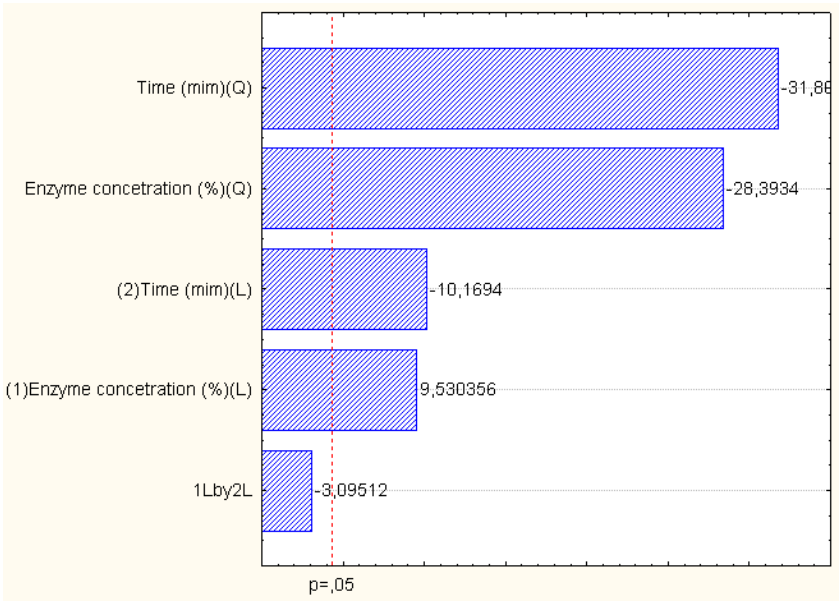


Fig. 12: Pareto Chart for inulin hydrolysis CCMB 300

The statistical significance (Table 06) e of the regression can be assessed by the ratio of the mean square regression and mean square of the residue and by comparing these sources of variation using the Fisher distribution (F test). Thus, a statistically significant value of this ratio must be greater than the tabulated value for F. It is obtained by the ANOVA table that calculated F (103.46) is higher than the F tabulated (9.01) showing that the function is well suited to the answers.

Table 06. Analysis of variance

Variation source	**SQ**	**gl**	**MQ**	**F**	**Tabulated F (IC de 95%)**	**R^2**
Regression	2859.761233	5	571.9522	103.4639	9.01	
Residual	16.584105	3	5.528035			
Lack of Fit	13.426	1	13.426	8.501	0.100253	0.99
Pure Error	3.159	2	1.579			
Total SQ	2876.345	8				

2.4 Conclusion

In this work the production and characterization of the inulinase by *Pseudozyma* sp. CCMB 300 was effectively performed. The enzyme was afterwards used in the hydrolysis of inulin, where a conversion yields of roughly 90% for an initial concentration of inulin of 1% (w/v). Therefore, the inulinase from this yeast is a potential candidate for inulin hydrolysis in the food industry.

Using the response surface methodology it was possible to determine the best media composition for obtaining inulinase from *Pseudozyma* sp. This corresponds to a medium containing 0.86 g / L yeast extract and 14.54 g / L glucose. This statistical method was also used to access the optimum pH (8.28) and temperature (54 ºC for fermentative inulinase production.

The combination of results obtained in this work (enzymatic characterization and hydrolysis of inulin) can provide useful guidelines for the utilization of inulinase from CCMB 300 for fructose production at pilot scales.

This optimization through RMS can be used in future industrial scale fermentation in promoting the rational use of enzyme and substrate in the process.

References

Bradford MN. 1976. A rapide and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of proteindye bidinig. *Analytical Biochemistry*. 72:248-254.

Chen HQ, Chen XM, Li Y, Wang J, Jin ZY et al. 2009. Purification and characterization of exo and endo inulinase from *Aspergillus ficuum* JNSP5 06. *Food Chemistry* 115:1206-1212.

Figueiredo-Ribeiro RCL, Pessoni AB, Braga MR. 2007. Inulinases produced by microbes from the brazilian cerrado: characterization and potential uses. in:. Shiomi N, Benkeblia N, Onodera S. (org.). *Recent advances in fructooligosaccharides*. 18: 339-356.

Gill PK, Manhas RK, Sing P. 2006. Comparative analysis of thermostabilyty of extracellular exoinulinase from *Aspergillus fumigatus* with commercially available (Novozyme) Inulinase. *Bioresource Technology* 97: 355-358.

Gong F, Zhang T, Chi Z, Sheng J, Jing L, Wang X. 2008. Purification and characterization of extracellular inulinase from a marine yeast *Pichia guilliermondii* and inulin hydrolysis by the purified inulinase. *Biotechnology and Bioprocess Engineering* 13: 533-539.

Kango N. 2008. Production of inulinase using tap roots of dandelion (*Taraxacum officinalle*) by *Aspergillus niger*. *Journal of Food Engineering* 85: 473-478.

Kochhar A, Gupta A, Kanur, N. 1999. Purification and immobilization of inulinase from *Aspergillus candidus* for producing fructose. *Journal of the Science of Food and Agriculture* 79: 549-554.

Miller GL, 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry* 3:426-428.

Nakamura T, Ogata Y, Shitara A, Nakamura A, Ohta K.1995. Continuous production of fructose Syrups from inulin by immobilized inulinase from *Aspergillus niger* mutant 817. *Journal of Fermentation and Bioengineering* 80:164-169.

Oliveira RQ. 2007. Bioprospecção de microrganismos leveduriformes produtores de pectinase extracelulares isolados do Semi-árido baiano. *MSc dissertation*, Univ. Estadual de Feira de Santana.

Pandey A, Soccol C, Selvakumar P, Thomazsoccol V, Krieger N, Fontana J. 1999. Recent development in microbial inulinases. *Applied Biochemistry Biotechnology***.** 81: 35-52.

Patching JW, Rose AH. 1969. The effect and control of temperature. *Methods Microbiology* 2: 23-38

Ricca E, Calabro V, Curcio S, Loiro G. 2007. The state of the art in the production of fructose from inulin enzymatic hydrolysis. *Critical Reviews in Biotechnology* 27: 129-145

Sharma A, Kainth S, Gill A. 2006. Inulinase production using garlic (*Allium sativum*) powder as a potential substrate in *Streptomyces* sp. *Journal of Food Engineering* 77;486–491.

Sheng J, Chu Z, Li J, Gao L, Gong, F. 2007. Inulinase production by the marine yeast *Cryptococcus aureus* G7a and inulin hydrolysis by the crude inulinase. *Process Biochemistry* 42: 805–811.

Uetanabaro APT, Góes-Neto A. 2006. Importance of culture collections of microorganisms (CCMs) for the conservation of microbial biotechnological resources of the Brazilian semi-arid region. In: *Towards greater knowledge of the Brazilian semi-arid biodiversity*. 1st ed. Brasília, DF. Ministério da Ciência e Tecnologia, pp 41-43.

PRODUCTION, CHARACTERIZATION AND APPLICATION OF INULINASE FROM *KLUYVEROMYCES MARXIANUS* CCMB 322.

Abstract

Inulinases target the β-2 linkage of inulin, a polyfructan target the β-2 linkage of inulin, a polyfructan consisting of linear β-2,1 linked fructose, and hydrolyzes it into fructose and the use provides an alternative to obtained fructose syrup through hydrolysis of inulin. The objective of this work was to study the production, characterization and applications of an inulinase from *Kluyveromyces marxianus* CCMB 322 isolated from Brazilian semi-arid region. The endeavor was successfully accomplished and the inulinase has displayed potential for inulin hydrolysis.

Keywords: Inulin, Inulinase, yeasts, semi-arid, Brazil

3.1 Introduction

Inulinase (β-2,1 D- fructan fructanohydrolase) , EC 3.2.1.7, target the β-2 linkage of inulin, a polyfructan consisting of linear β-2,1 linked fructose, and hydrolyzes it into fructose (Gong et al, 2008).

Inulinase can be derived from many microorganisms. In the last three decades, significant efforts have been made to find the best microbial source for the extraction of inulinase (Ricca et al, 2007).

Conventional fructose production from starch needs at least three enzymatic steps, including α-amylase, amyloglucosidades, and glucose isomerase action, yielding only 45% fructose solutions. An alternative to this process is the hydrolysis of inulin by inulinases. Enzymatic formation of fructose from inulin has a single enzymatic step and yields up to 95 % fructose (Figueiredo-Ribeiro et al., 2007). The use of microbial inulinases (EC 3.2.1.7), which yield up to 95% pure fructose in a single-step enzymatic reaction is, therefore, a viable alternative for the production of high-fructose syrup (Gill; Manhas; Sing, 2006).

The use of microbial inulinases (EC 3.2.1.7), which yield up to 95% pure fructose in a single-step enzymatic reaction, is a viable alternative for the production of high-fructose syrup (Gill; Manhas; Sing, 2006).

Inulinases have also been used for the production of inulooligosaccharides–low caloric saccharides acting as a growth factor for beneficial microorganisms in the intestinal flora (Skowronek; Fiedurek, 2006).

The Brazilian semi-arid region represents a large area for bioprospection since naturally occurring microorganisms adapted to a tropical semi-arid environment, with high temperature and low humidity throughout the year, may possess some features of great industrial interest (Uetanabaro and Góes-Neto, 2006).

In this study, the survey of the production, characterization and application of extracellular inulinases obtained from *Kluyveromyces marxianus* (CCMB 322) isolated in the Brazilian semi-arid region was carried out.

3.2 Materials and methods

Chemicals

Inulin, bovine serum albumin, and 3,5-dinitrosalicylic acid were purchased from Sigma Chemical Co. (St Louis, MO, USA). All the other chemicals used were also of high-quality analytical grade.

Microorganisms

The yeast strain *Kluyveromyces marxianus* (CCMB 322) is from Culture Collection of Microorganisms of Bahia (CCMB) of the Universidade Estadual de Feira de Santana, Brazil (Uetanabaro and Góes-Neto 2006).

The yeast strains were maintained in YM agar (3% yeast extract (w/v), 3% malt extract (w/v), 5% peptone (w/v), 10% glucose (w/v), and 20% agar (w/v), pH 6.2).

Inulinase production

The yeast was previously grown on YM agar at 28 ºC for 48 h, as described in Oliveira (2007), diluted in sterile distilled water to a concentration of about 10^8 colony-forming units/ml. 10% (v/v) of the diluted growth medium was inoculated in flasks containing containing (per 1 L) mineral medium ($CaCl_2$, 0.25 g; $(NH4)_2SO_4$, 3 g; KH_2PO_4, 4.5; $MgSO_4$, 0.25g), supplemented with yeast extract, 1 g; glucose, 10 g; pH 5.0, for fermentation (Patching and Rose 1969). After incubation at 28ºC for 48 h in an orbital shaker at 150 rpm, the cells were separated by centrifugation at 10,000 g for 10 min at 48 ºC, and the supernatant liquid media was used as the extracellular fraction.

Enzyme assays

Inulinase activity was measured spectrophotometrically (A540) using the dinitrosalicylic reagent, as reported by Miller (1959). The reaction mixture consisted of 900 µL of 2% (w/v) inulin in 0.05 M acetate buffer, pH 5.5, and 100 ml culture supernatant. The mixture was incubated for 15 min at 50ºC. After incubation, 1 mL dinitrosalicylic reagent was added, and the mixture was boiled at 100ºC for 10 min and cooled with 10 mL distilled water. One unit of enzyme activity of inulinase was defined as the amount of the enzyme that catalyzed the formation of 1 µmol fructose/min.

Protein determination

Total protein determination was performed according to Bradford (1976), using bovine serum albumin as the standard.

Doehlert experimental design for enzyme production

The Doehlert experimental design, with two variables (concentration of glicose and yeast extract) and three replicates at the centre of the domain leading to a total of 9 experiments (Table 1) was used to obtain the knowledge of the effect of glucose and yeast extract concentration on the production of enzyme.

Thus, the concentration glucose was studied in 5 levels (7.5 to 12.5 g/L) and yeast extract was studied in three levels (0.5 to 1.5 g/L). The experimental errors were evaluated from replication of central point. The experimental data were processed by

using the STATISTICA software. All the experiments in this step were carried out in random order.

To estimate the lack of fit of the model to the experimental data an analysis of variance (ANOVA) was performed, using Design Statistica, version 7.0.

Response surface optimization of temperature and pH for inulinase obtained

Response surface modeling was applied to cultures of yeast, to determine the optimum temperature and pH for inulinase obtained. This statistical technique for experimental design has advantages over methods that investigate only one variable at a time.

The experimental design, with two variables (temperature and pH) and three replicates at the centre of the domain leading to a total of 9 experiments (Table 1) was used to evaluate the effect of temperature and pH of inulinase activity.

The pH was studied in 5 levels (5 to 9) and temperature was studied in three levels (30 to 70ºC). The experimental errors were evaluated from replication of central point. The experimental data were processed by using the STATISTICA software. All the experiments in this step were carried out in random order.

To estimate the lack of fit of the model to the experimental data an analysis of variance (ANOVA) was performed, using Design Statistica, version 7.0.

Effect of cations oninulinase activity

The effect of Na^+ and K^+ on inulinase activity was studied. The concentrations used were: NaCl (0.05, 0.1, 0.15, 0.2, 0.25 and 0.3 mol/L), KCl (0.05, 0.1, 0.15, 0.2, 0.25

and 0.3 mol/L). The inulinase activity was determined by the standard assay as described previously.

Determination of kinetics Parameters

To obtain the parameters of the Michaelis-Menten kinetics of inulinase for the hydrolysis of inulin, K_m and V_{max}, 0,9 µL inulin (1, 1.5, 2.0, 2.5 and 3.0 % w/v) was added to 100 µL of inulinase in 0.05 M acetate buffer (pH 5.5) and incubated at 50ºC for 15 mim. The K_m and V_{max} for inulin was determined by the method of Lineweaver-Burk plots.

Thermostability of inulinases

Samples of inulinases in test tubes (selected to be equal in weight, volume and size) were incubated in buffer at different temperatures (50ºC, 60ºC, 70Cº, 80ºC and 90ºC) and for various times (0, 10, 20, 30, 40, 50 and 60 min). After the heating process the tubes were cooled in melting ice and the residual activity measurement was carried out at pH 6.0 and at a temperature of 50 ºC.

Doehlert experimental design for hydrolysis of inulin

The extent of inulin hydrolysis (%) was calculated as $\frac{\text{amount of fructose released}}{\text{amount of initial total sugars}} \times 100$ (Nakamura et al, 1995). Total sugars were determined by Antron method and reducing sugars were determined by the dinitrosalicylic acid method. (Miller, 1959).

The Doehlert experimental design, with two variables (concentration of enzyme and fermentation time) and three replicates at the centre of the domain leading to a total of 9 experiments (Table 1) was used to obtain the knowledge of the effect of enzyme concentration and fermentation time on the extents of inulin hydrolysis from a solution 1% (w / v) in citrate buffer 0,05 M.

Statistical analysis

All experiments were carried out in triplicate. One-way analysis of variance was used to compare the specific activity of inulinase among the strains. Where significant differences were detected between strains, the means were compared using Tukey's test. For all statistical analyses, the level of significance was set at 5%, and the analyses were performed using the standard statistical software Statistica 9.0 for windows.

3.3 Results and discussion

Production of inulinases by yeasts

The table 01 shows the Doehlert design applied to optimize the production of inulinase by *Kluyveromyces marxianus* CCMB 322. The first column describes the number of planning, with C representing the central compound. In the second and third column are respectively the values of glucose concentration and the concentration of yeast extract. The fourth column represents the enzymatic activity. The last column presents the predicted values.

Table 01: Results of the factorial analysis for inulinase production CCMB 322

N°	Concentration of glucose (g/L)	Concentration of yeast extract (g/L)	Experimental values (UA)	Activities predicted (UA)
1	7.5 **(-0.5)**	1.5 **(+0.866)**	0.7931	0.8035
2	12.5 **(+0.5)**	1.5 **(+0.866)**	0.6329	0.6224
3	5 **(-1)**	1 **(0)**	0.7937	0.7832
4C	10 **(0)**	1 **(0)**	0.8405	0.8267
4C	10 **(0)**	1 **(0)**	0.8024	0.8267
4C	10 **(0)**	1 **(0)**	0.8372	0.8267
5	15 **(+1)**	1 **(0)**	0.43334	0.4438
6	7.5 **(-0.5)**	0.5 **(-0.866)**	0.3897	0.4001
7	12.5 **(-1)**	0.5 **(-0.866)**	0.2525	0.2420

The response surface graphics below shows the influence of glucose and yeast extract in the production of inulinase of CCMB 322 (Fig 1 and 2).

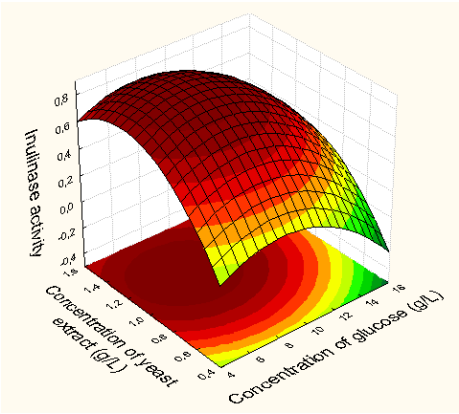


Fig. 01. Response surface for yeast extract concentration versus glucose concentration CCMB 322

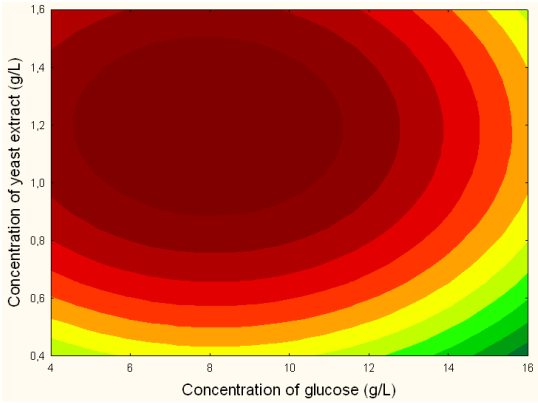


Fig. 02. Level curves for yeast extract concentration versus glucose concentration CCMB 322

From the analysis of the chart it can be verified thet the region where the production of inulinase is optimal lies within concentrations from 4 to 11 g/L of glucose and from 1 to 1.4 g/L of yeast extract for CCMB 322. These regions can be further exploited to obtain an extract with the highest concentration of the enzyme studied under the operational conditions used. Equation 1 illustrates the relationship of these two variables and the enzyme activity (UA), G is the glucose concentration.

$$(UA) = -1{,}1499 + 0{,}1412\,(G) - 0{,}0085(G)^2 + 2{,}4887\,(YE) - 1{,}025\,(YE)^2 - 0{,}0046\,(G)\,x\,(YE) \quad \textbf{(Equation 1)}$$

Through the derivation of this equation, the points of maximum enzymatic activity are obtained, specifically 1.19 g/L for yeast extract and 7.95 g/L for glucose.

Schneider (1996) studied the production of inulinase by *Kluyveromyces marxianus* and reported that the presence of yeast extract is essential for the growth of microorganism and enzyme production. Santos (2002) studied three different culture media to obtain inulinase from *Kluyveromyces marxianus var. bulgaricus* and chose the one that contained 10 g /L yeast extract. The work performed by Singh (2006) on the evaluation of different nitrogen sources for *Kluyveromyces marxianus* YS1 also showed that yeast extract was important for the production of inulinase.

The chart of Paretto (Figure 03) shows that both linearly and quadratically, variables glucose concentration and yeast extract concentration are significant for the production of inulinase from *Kluyveromyces marxianus* CCMB 322, because both variables had a p value greater than 0.05

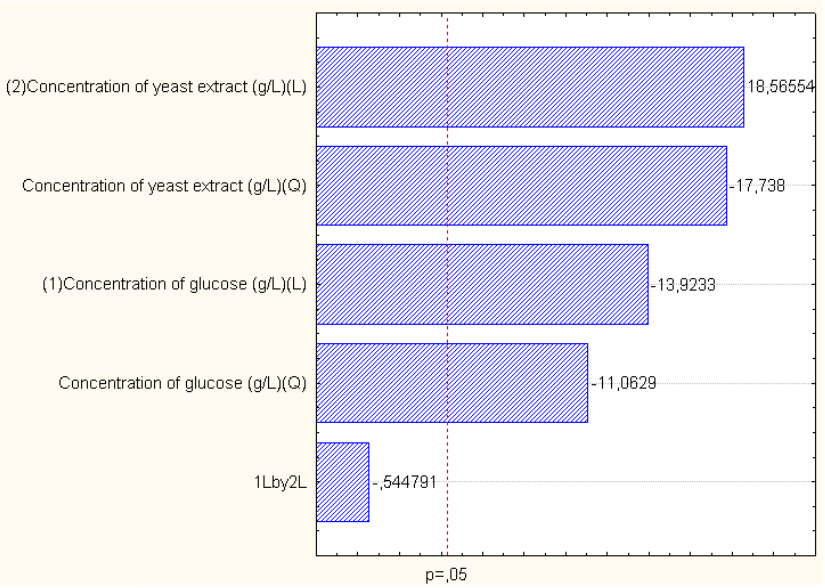


Fig. 03: Pareto Chart for inulinase production CCMB 322

The statistical significance of the regression (Table 02) can be assessed by the ratio of the mean square regression and mean square of the residue and by comparing these sources of variation using the Fisher distribution (F test). Thus, a statistically significant value of this ratio must be greater than the tabulated value for F. The ANOVA table shows that the calculated F (157.17) is higher than the F tabulated (9.01) showing that the function is adequate.

Table 02 Analysis of variance

Variation source	SQ	gl	MQ	F	Tabulated F (IC de 95%)	R^2
Regression	0.406512	5	0.081302	157.17	9.01	
Residual	0.001552	3	0.000517			
Lack of Fit	0.000661	1	0.000661	1.48	0.34	0.99
Pure Error	0.000891	2	0.000446			
Total SQ	0.408064	8				

Response surface optimization of temperature and pH for inulinase obtained

The Table 03 shows the Doehlert design applied to optimization of temperature and pH inulinase by *Kluyveromyces marxianus* CCMB 322.

Table 03. Results of the factorial for optimization of temperature and pH CCMB 322

N°	pH	Temperature °C	Experimental values (UA)	Activities predicted (UA)
1	6 (-0.5)	70 (+0.866)	0.288880	0.216720
2	8 (+0.5)	70 (+0.866)	0.000000	0.072160
3	5 (-1)	50 (0)	0.594200	0.583280
4C	7 (0)	50 (0)	0.512400	0.507900
4C	7 (0)	50 (0)	0.503400	0.507900
4C	7 (0)	50 (0)	0.500200	0.583280
5	9 (+1)	50 (0)	0.150200	0.078040
6	6 (-0.5)	30 (-0.866)	0.505000	0.432840
7	8 (+0.5)	30 (-0.866)	0.000000	0.072160

The influence of temperature and pH on activity of the enzyme was investigated for CCMB 322 by surface response methodology and are shown (Figures 03 and 04).

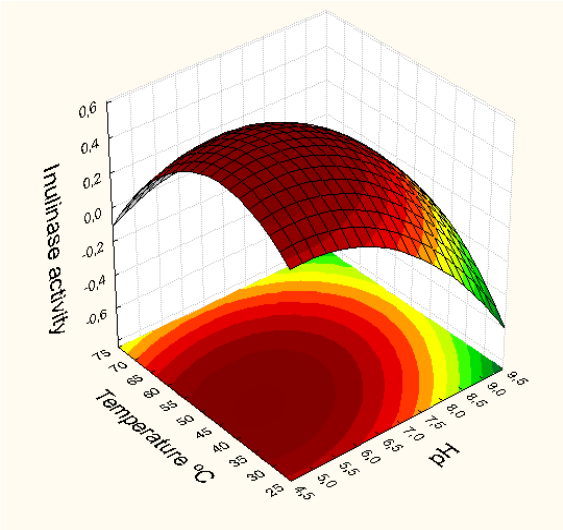


Fig 04. Response surface for pH versus temperature

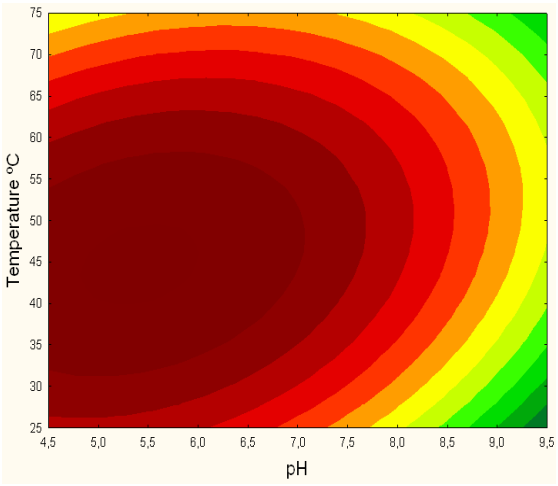


Fig 05. Level curves for pH versus temperature

The regression model provided for the enzyme activity in relation to pH and temperature in the experimental design is expressed by equation 2.

$UA = -1.35552 + 0.3589(P) - 0.0443(P)^2+0.0446(T)-0.0006(T^{2)}+0.0027(P)x(T)$ (Equation 2)

Through the derivation of this equation, the points of maximum enzymatic activity are obtained.

The response surface shows that the critical points for pH and temperature for CCMB 322 were 4.4 and 44.7ºC, respectively. These data are similar to those reported in several studies focused on the inulinase of *Kluyveromyces marxianus*, such as the work by Souza-Mota et al (2005) who selected pH 4.8 and 45 °C as the best conditions for enzyme activity.

The Paretto Chart (Figure 06) shows that the variables temperature and pH are independent for the production of inulinase CCMB 322, because a p value less than 0.05 was obtained.

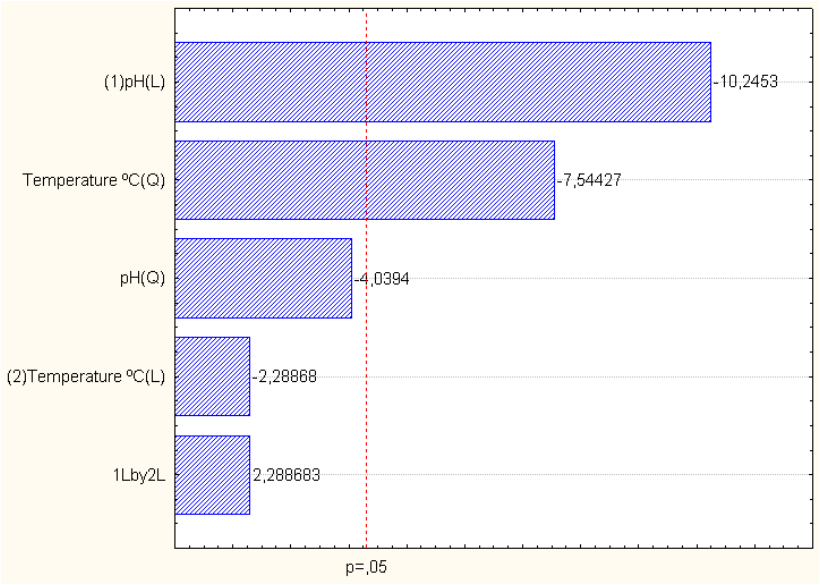


Fig. 06: Pareto Chart for CCMB 322

The statistical significance of the regression (Table 04) can be assessed by the ratio of the mean square regression and mean square of the residue and by comparing these sources of variation using the Fisher distribution (F test). Thus, a statistically significant value of this ratio must be greater than the tabulated value for F. The ANOVA table obtained shows that the calculated F (7.44) is higher than the F tabulated (5.3), therefore validating the function presented.

Table 04 Analysis of variance

Variation source	SQ	gl	MQ	F	Tabulated F (IC 90%)	R^2
Regression	0.4106	5	0.08213	7.4446	5.3	
Residual	0.03309	3	0.01103			
Lack of Fit	0.02863	1	0.02863	12.8469	0.069790	0.92
Pure Error	0.00445	2	0.00222			
Total SQ	0.443762	8				

Effect of cations on inulinase activity

The effect of salts NaCl and KCl on the activity of inulinase are shown in Figure 07. The concentration of 0.15 mol/L of NaCl and KCl increased the activity of inulinase from CCMB 322 by approximately 125%. Concentrations higher than 0.25 mol/L decrease the activity of the enzyme.

The addition of NaCL and KCl 1 mol/L increased the activity of inulinase by 145 % and KCl 142.9 % (Sheng et al, 2007). CCMB 300 showed a smaller increase in activity (40%) in this salt concentration and Shing (2006) showed that KCl increased inulinase activity in 62.2 %.

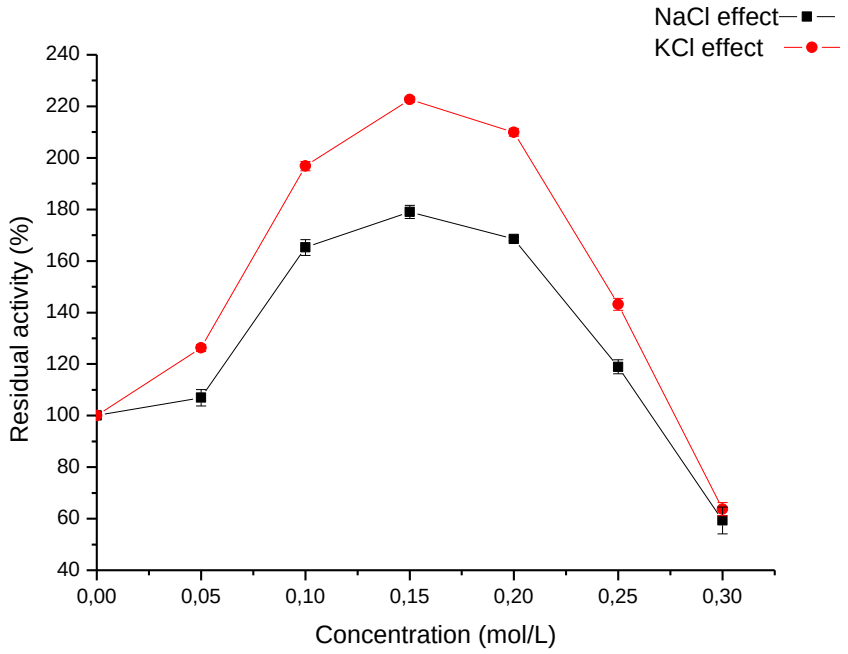


Fig.07. Effect of salts on inulinase activity from CCMB 322

Kinetics Parameters

From the Lineweaver-Burk plot (Figure 08) the K_m (38 mg/mL) and V_{max} (0.26 µmol/mL min) values of the enzyme for inulin were obtained (Figure 08). Data suggest high affinity of the inulinase from CCMB 322 for inulin. The studies of Moriyama (2002) shows that the Km value for inulinase from *Pichia guillirmondii* was 92.6 mg/mL.

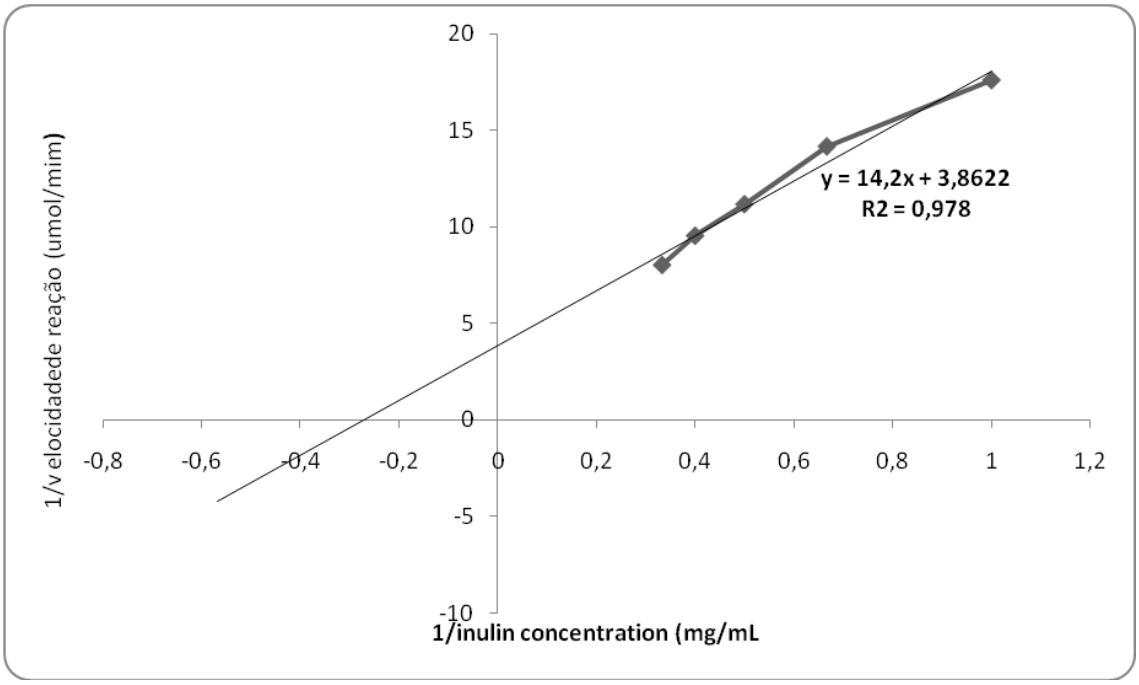


Fig.08. Influence of substrate concentration on the inulinase activity

Thermostability of inulinases

The effect of heat treatment for CCMB 322 is depicted in Figure 09. After 40 min at 60ºC CCMB 322 retained 55.28 % of its original activity. CCMB 322 lost 100% of its activity after 10 min of heat treatment at 70ºC, 80ºC and 90ºC. This results already indicate a low thermal stability by CCMB 322 because the retention of activity was low in 60 and lost all activity at other temperatures.

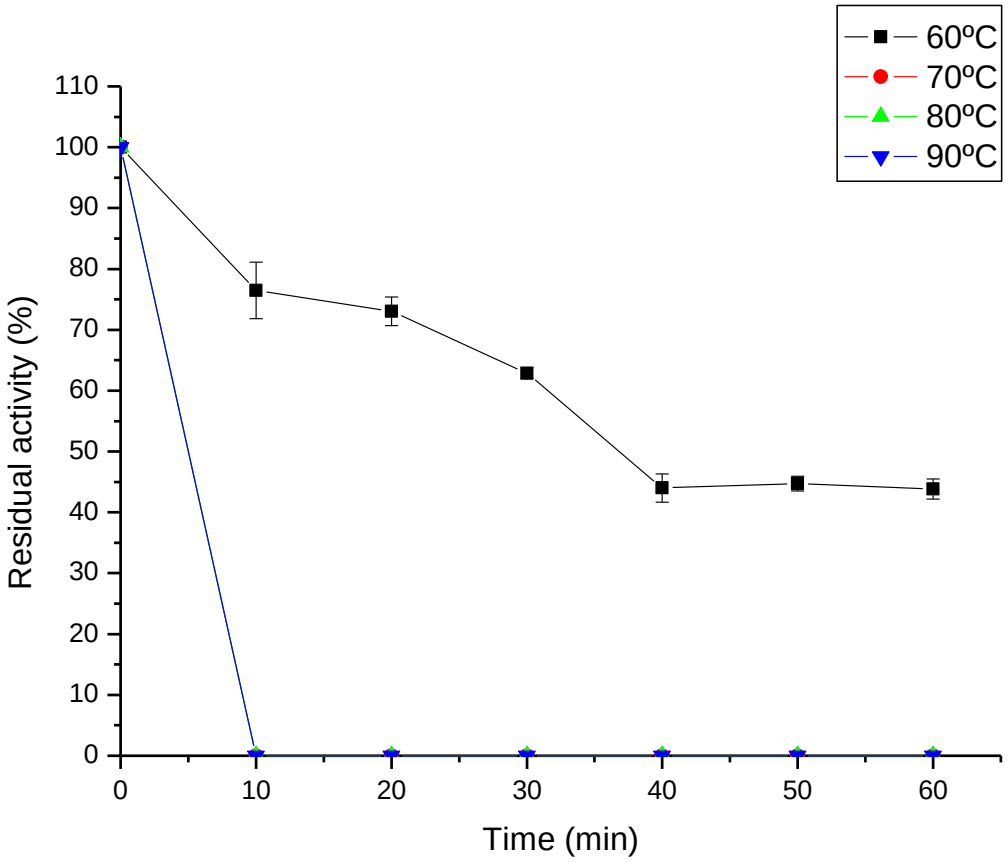


Fig.09 Evaluation of the thermal stability of inulinase from CCMB 322

Hydrolysis of inulin

The table 05 shows the Doehlert design applied to optimize the hydrolysis in function of time of reaction and enzyme concentration of inulin by CCMB 322 inulinase.

Table 05: Results of the factorial for inulin hydrolysis

N°	Enzyme Concentration (%)	Time (minutes)	Experimental values (% Hidrolysis)	Response predited (% Hidrolysis)
1	5 **(+0.866)**	4.5 **(-0.5)**	88.1194	83.14604
2	5 **(+0.866)**	12.5 **(+0.5)**	84.2327	89.20606
3	3 **(0)**	1 **(-1)**	45.5391	50.41096
4C	3 **(0)**	8 **(0)**	98	99.54132
4C	3 **(0)**	8 **(0)**	99	99.54132
4C	3 **(0)**	8 **(0)**	100	99.54132
5	3 **(0)**	15 **(+15)**	66.6678	60.17199
6	1 **(-0.866)**	4.5 **(-0.5)**	72.2412	67.26784
7	1 **(-0.866)**	12.5 **(+0.5)**	52.941	57.91436

The fermentation time was studied in five levels (1 to 12.5 minutes) and enzyme concentration was studied in three levels (1 to 5 %). The experimental errors were evaluated from replication of central point. The experimental data were processed by using the STATISTICA software. All the experiments in this step were carried out in random order.

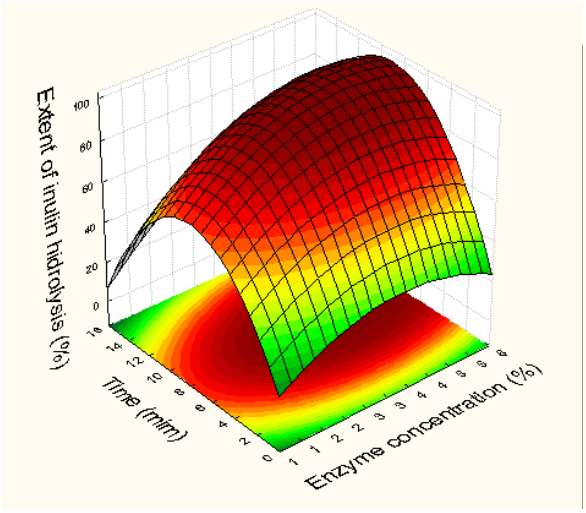


Fig. 10. Response surface for hydrolysis of inulin

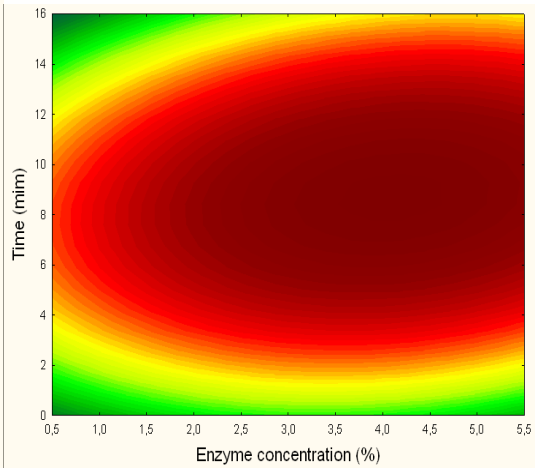


Fig. 11. Level curves for hydrolysis of inulin

.

Equation 3 correlates these two variables with extent of hydrolysis (% H), where: C is the enzyme concentration (%) and T is the time (min).

$$\% H = 6.3905 + 18.0495 (C) - 2.7079 (C)^2 + 13.7011 (T) - 0.9030 (T)^2 - 0.4816 (C)(T)$$

Equation 3

Through the derivation of this equation, the points of maximum enzymatic activity are obtained. From the SRM undertaken, the higher yields observed for the *Kluyveromyces marxianus* (CCMB 322) inulinases were obtained for concentrations of 4.03 mL of enzyme and an incubation time of 8.6 minutes. There are few studies of inulin hydrolyses using the same conditions and SRM analysis, as performed in this work.

The Paretto chart (Figure 12) shows that both linearly and quadratic, variables enzyme concentration and time of hydrolysis are significant for the hydrolysis of inulin *Kluyveromyces marxianus* CCMB 322, because both variables had a p value greater than 0.05

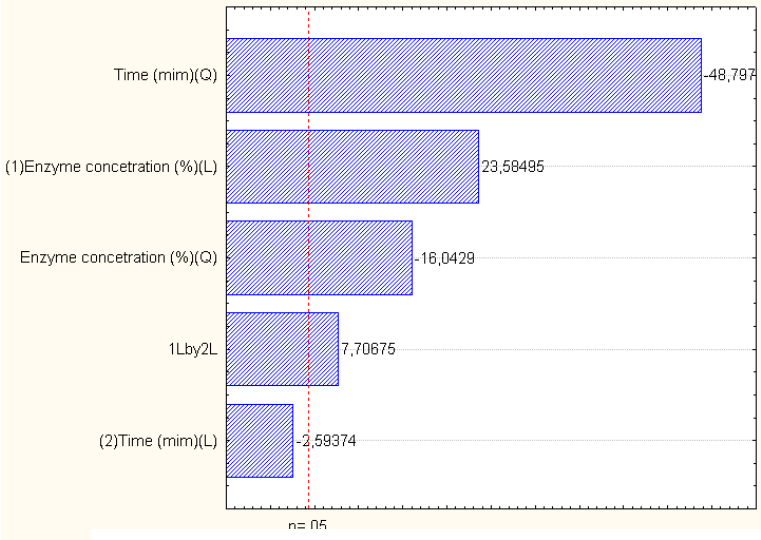


Fig. 12: Pareto Chart for inulin hydrolysis CCMB 322

The statistical significance of the regression (Table 06) can be assessed by the ratio of the mean square regression and mean square of the residue and by comparing these sources of variation using the Fisher distribution (F test). Thus, a statistically significant value of this ratio must be greater than the tabulated value for F. The ANOVA table obtained shows that the calculated F (12.42) is higher than the F tabulated (5.3) , therefore validating the function presented.

Table 06 Analysis of variance

Variation source	**SQ**	**gl**	**MQ**	**F**	**Tabulated F (IC 90%)**	**R^2**
Regression	3473.303	5	694.66067	12.4294	5.3	
Residual	167.666	3	55.888513			
Lack of Fit	165.666	1	165.666	165.666	0.005982	0.95
Pure Error	2.000	2	1.000			
Total SQ	3307.638	8				

3.4 Conclusion

In this work the production and characterization of the inulinase by *Kluyveromyces marxianus* CCMB 322 was effectively performed. The enzyme was afterwards used in the hydrolysis of inulin., where a conversion yield of roughly 100% for an initial concentration of inulin of 1% (w/v). Therefore, the inulinase from this yeast is a potential candidate for inulin hydrolysis in the food industry, although this can be hampered due to its relatively low thermal stability

Using the response surface methodology it was possible to determine the best media conditions for obtaining inulinase from *K. marxianus* CCBM 322. This corresponds to a medium containing to a medium containing 1.2 g / L of yeast extract and 7.8 g / L of glucose. This statistical method was also used to access the optimum pH (4,62) and temperature (43 ºC).

The combination of results obtained in this work can provide useful guidelines for the utilization of inulinase from CCMB 322 for fructose production at pilot scales.

References

Bradford MN. 1976. A rapide and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of proteindye bidinig. *Analytical Biochemistry*. 72:248-254.

Figueiredo-Ribeiro RCL, Pessoni AB, Braga MR. 2007. Inulinases produced by microbes from the brazilian cerrado: characterization and potential uses. in:. Shiomi N, Benkeblia N, Onodera S. (org.). *Recent advances in fructooligosaccharides*. 18: 339-356.

Gill P, Manhas R, Singh J, Singh P. 2006. Purification and properties of a heat-stable exoinulinase isoform from *Aspergillus fumigatus*. *Journal of Food Engineering* 76: 369–375.

Gong F, Zhang T, Chi Z, Sheng J, Jing L, Wang X. 2008. Purification and characterization of extracellular inulinase from a marine yeast *Pichia guilliermondii* and inulin hydrolysis by the purified inulinase. *Biotechnology and Bioprocess Engineering* 13: 533-539.

Kango N. 2008. Production of inulinase using tap roots of dandelion (*Taraxacum officinalle)* by *Aspergillus niger*. *Jornal of Food Engineering* 85: 473-478.

Kochhar A, Gupta A, Kanur N. 1999. Purification and immobilization of inulinase from *Aspergillus candidus* for producing fructose. *Journal of the Science of Food and Agriculture* 79: 549-554.

Miller GL, 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Analytical Chemistry 3:426-428

Moriyama S. 2002 Purification and properties of an extracellular exoinulinase from *Penicillium* sp strain Tn-88 and sequences analysis of coding gene. Biosciences biotechnology biochemistry. 66:1887-1896.

Nakamura T, Ogata Y, Shitara A, Nakamura A, Ohta K.1995. Continuous production of fructose Syrups from inulin by immobilized inulinase from *Aspergillus niger* mutant 817. *Journal of Fermentation and Bioengineering* 80:164-169.

Oliveira RQ. 2007. Bioprospecção de microrganismos leveduriformes produtores de pectinase extracellulares isolados do Semi-árido baiano. MSc dissertation, Univ. Estadual de Feira de Santana.

Patching JW, Rose AH. 1969. The effect and control of temperature. *Methods Microbiology* 2: 23-38

Ricca E, Calabro V, Curcio S, Loiro G. 2007. The state of the art in the production of fructose from inulin enzymatic hydrolysis. *Critical Reviews in Biotechnology* 27: 129-145

Sharma A, Kainth S, Gill P. 2006. Inulinase production using garlic (*Allium sativum*) powder as a potential substrate in *Streptomyces* sp. *Journal of Food Engineering* 77;486–491.

Sheng J, Chu Z, Li J, Gao L, Gong F. 2007. Inulinase production by the marine yeast *Cryptococcus aureus* G7a and inulin hydrolysis by the crude inulinase. *Process Biochemistry* 42: 805–811.

Singh RS, Dhaliwal R, Puri M. 2006. Production of inulinase from *Kluyveromyces marxianus* YS 1 using root extract of *Asparagus racemosus*. Process Biochemistry 41:1703-1707.

Skowronek M, Fiedurek J. 2006. Purification and Properties of Extracellular Endoinulinase from *Aspergillus niger* 20 OSM. *Food Technology and Biotechnology* 44 53–58.

Uetanabaro APT, Góes-Neto A. 2006. Importance of culture collections of microorganisms (CCMs) for the conservation of microbial biotechnological resources of

the Brazilian semi-arid region. In: Towards greater knowledge of the Brazilian semi-arid biodiversity. 1st ed. Brasília, DF. Ministério da Ciência e Tecnologia, pp 41-43.

Singh RS, Rajesh Dhaliwal, Munish Puri. 2006. Production of inulinase from *Kluyveromyces marxianus* YS 1 using root extracts *Asparagus racemosus*. Process Biochemistry 41

Souza-Motta et al. 2005. *Aspergillus niveus* Blochwitz 4128URM: new source for inulinase production. *Brazilian archives of biology and technology.* 48:343-350.

Schneider ALS. Estudo da Produção de Inulinase por *Kluyveromyces marxianus* ATCC 36907. MSc dissertation FEQ-UFSC, 1996.

Santos AMP. Síntese de Oligossacarídeos a partir da Sacarose por Inulinase de MSc dissertation. UNICAMP, 2002.

PRODUCTION, CHARACTERIZATION AND APPLICATION OF INULINASE FROM FUNGAL ENDOPHYTES (CCMB 328)

Abstract

Keywords: Inulin, Inulinase, yeasts, semi-arid, Brazil

Inulinases target the β-2 linkage of inulin, a poliyfructan consisting of linear β-2,1 linked fructose, and hydrolyzes it into fructose. The use of inulinases provides an alternative way to obtain fructose syrup through the hydrolysis of inulin. The objective of this work was to study the production, characterization and application of an inulinase from fungal endophyte CCMB 328 isolated from Brazilian semi-arid region. The enzyme was characterized on the kinetic and stability nature and the results suggest that the isolated inulinase has potential for inulin hydrolysis.

Keywords: Inulin, Inulinase, yeasts, semi-arid, Brazil

4.1 Introduction

Inulinase (β-2,1 D- fructan fructanohydrolase , (EC 3.2.1.7) targets the β-2 linkage of inulin, a poliyfructan consisting of linear β-2,1 linked fructose, and hydrolyzes it into fructose (Gong et al, 2008).

Conventional fructose production from starch needs at least three enzymatic steps, including α-amylase, amyloglucosidades, and glucose isomerase action, yielding only 45 % fructose solutions. More concentrated fructose solution requires a dedicated

chromatographic step. A viable alternative to this process is the hydrolysis of inulin by inulinases. Enzymatic formation of fructose from inulin has a single enzymatic step and yields up to 95 % fructose (Figueiredo-Ribeiro, et al 2007; Gill et al 2006).

Chemical hydrolysis of inulin can be carried out by treatment with organic or mineral acids or through heterogeneous catalysis using solid acidic catalysts. However, the chemical approach is currently associated with some drawbacks, namely formation of unwanted by-products and colored and color forming compounds, which lower product yield and require a more demanding downstream processing. These shortcomings can be overcome if the more specific enzymatic route is used (Rocha et al, 2006).

The Brazilian semi-arid region represents a large area for bioprospection since naturally occurring microorganisms adapted to a tropical semi-arid environment, with high temperature and low humidity throughout the year, may possess some features of great industrial interest (Uetanabaro and Góes-Neto, 2006).

In this study the production, characterization and application of extracellular inulinases produced by (CCMB 328**)** from Brazilian semi-arid region were evaluated.

4.2 Materials and methods

Chemicals

Inulin, bovine serum albumin, and 3,5-dinitrosalicylic acid were purchased from Sigma Chemical Co. (St Louis, MO, USA). All the other chemicals used were also of high-quality analytical grade.

Microorganisms

The yeast strain of fungal endophyte CCMB 328 is from Culture Collection of Microorganisms of Bahia (CCMB) of the Universidade Estadual de Feira de Santana, Brazil (Uetanabaro and Góes-Neto 2006).

The yeast strains were maintained in YM agar (3% yeast extract (w/v), 3% malt extract (w/v), 5% peptone (w/v), 10% glucose (w/v), and 20% agar (w/v), pH 6.2).

Inulinase production

The yeast was previously grown on YM agar at 28 ºC for 48 h, as described in Oliveira (2007), diluted in sterile distilled water to a concentration of about 10^8 colony-forming units/ml. 10% (v/v) of the diluted growth medium was inoculated in flasks containing containing (per 1 L) mineral medium ($CaCl_2$, 0.25 g; $(NH_4)_2SO_4$, 3 g; KH_2PO4, 4.5; $MgSO_4$, 0.25g), supplemented with yeast extract, 1 g; glucose, 10 g; pH 5.0, for fermentation (Patching and Rose 1969). After incubation at 28 ºC for 48 h in an orbital shaker at 150 rpm, the cells were separated by centrifugation at 10,000 g for 10 min at 48 ºC, and the supernatant liquid media was used as the extracellular fraction.

Enzyme assays

Inulinase activity was measured spectrophotometrically (A_{540}) using the dinitrosalicylic reagent, as reported by Miller (1959). The reaction mixture consisted of 900 µL of 2 % (w/v) inulin in 0.05 M acetate buffer, pH 5.5, and 100 µl culture supernatant. The mixture was incubated for 15 min at 50ºC. After incubation, 1 ml dinitrosalicylic reagent was added, and the mixture was boiled at 100 ºC for 10 min and cooled with 10

ml distilled water. One unit of enzyme activity of inulinase was defined as the amount of the enzyme that catalyzed the formation of 1 μmol fructose/min.

Protein determination

Total protein determination was performed according to Bradford (1976), using bovine serum albumin as the standard.

Doehlert experimental design for enzyme production

The Doehlert experimental design, with two variables (concentration of glucose and yeast extract) and three replicates at the centre of the domain leading to a total of 9 experiments (Table 1) was used to obtain the knowledge of the effect of glucose and yeast extract concentration on the production of enzyme.

Thus, concentration glucose was studied in 5 levels (7.5 to 12.5 g/L) and yeast extract was studied in three levels (0.5 to 1.5 g/L). The experimental errors were evaluated from replication of central point. The experimental data were processed by using the STATISTICA software. All the experiments in this step were carried out in random order.

To estimate the lack of fit of the model to the experimental data an analysis of variance (ANOVA) was performed, using Design Statistica, version 7.0.

Response surface optimization of temperature and pH for inulinase obtained

Response surface modeling was applied to cultures of yeast, to determine the optimum temperature and pH for inulinases obtained. This statistical technique for

experimental design has advantages over methods that investigate only one variable at a time.

The experimental design, with two variables (temperature and pH) and three replicates at the centre of the domain leading to a total of 9 experiments (Table 1) was used to obtain the knowledge of the effect of temperature and pH of inulinase activity.

The pH was studied in 5 levels (5 to 9) and temperature was studied in three levels (30 to 70º C). The experimental errors were evaluated from replication of central point. The experimental data were processed by using the STATISTICA software. All the experiments in this step were carried out in random order.

To estimate the lack of fit of the model to the experimental data an analysis of variance (ANOVA) was performed, using Design Statistica, version 7.0.

Effect of cations on inulinase activity

The effect of Na^+ and K^+ on inulinase activity was studied. The concentrations used were: NaCl (0.05, 0.1, 0.15, 0.2, 0.25 and 0.3 mol/L), KCl (0.05, 0.1, 0.15, 0.2, 0.25 and 0.3 mol/L). The inulinase activity was determined by the standard assay as described previously.

Determination of kinetics Parameters

To obtain the parameters of the Michaelis-Menten kinetics (K_m and V_{max}) of inulinase for the hydrolysis of inulin, K_m and V_{max}, 0.9 µL inulin (1, 1.5, 2.0, 2.5 and 3.0 % w/v) was added to 100 µL of inulinase in 0.05 M acetate buffer (pH 5.5) and

incubated at 50 ºC for 15 mim. The K_m and V_{max} for inulin was determined by the method of Lineweaver-Burk plots.

Thermostability of inulinases

Samples of inulinases in test tubes (selected to be equal in weight, volume and size) were incubated in buffer at different temperatures (50ºC, 60ºC, 70Cº, 80º C e 90ºC) and for various times (0, 10, 20, 30, 40, 50 and 60 min). After the heating process the tubes were cooled in melting ice and the residual activity measurement was carried out at pH 6.0 and at a temperature of 50 ºC.

Doehlert experimental design for hydrolysis of inulin

The extent of inulin hydrolysis (%) was calculated as $\frac{\text{amount of fructose released}}{\text{amount of initial total sugars}} \times 100$ (Nakamura et al, 1995). Total sugars were determined by Antron method and reducing sugars were determined by the dinitrosalicylic acid method (Miller, 1959). The Doehlert experimental design, with two variables (concentration of enzyme and fermentation time) and three replicates at the centre of the domain leading to a total of 9 experiments was used to obtain the knowledge of the effect of enzyme concentration and fermentation time on the extent of inulin hydrolysis from a solution 1% (w / v) in citrate buffer 0.05 M.

Statistical analysis

All experiments were carried out in triplicate. One-way analysis of variance was used to compare the specific activity of inulinase among the strains. Where significant differences were detected between strains, the means were compared using Tukey's test. For all statistical analyses, the level of significance was set at 5%, and the analyses were performed using the standard statistical software Statistica 9.0 for windows.

4.3 Results and discussion

Production of inulinases by yeasts

The table 01 shows the Doehlert design applied to optimize the production of inulinase by fungal endophyte. CCMB 328. The first column describes the number of planning, with C representing the central compound. In the second and third column are the values of glucose concentration and the concentration of yeast extract, respectively. The fourth column represents the enzymatic activity. The last column represents predicted values.

Table 01: Results of the factorial analysis for inulinase production CCMB 328

N°	Concentration of glucose (g/L)	Concentration of yeast extract (g/L)	Experimental values (UA)	Activities predited (UA)
1	7.5 **(-0.5)**	1.5 **(+0.866)**	2.473600	2.485039
2	12.5 **(+0.5)**	1.5 **(+0.866)**	2.426600	2.415161
3	5 **(-1)**	1 **(0)**	1.153200	1.141417
4C	10 **(0)**	1 **(0)**	4.006000	4.004029
4C	10 **(0)**	1 **(0)**	4.014800	4.004029
4C	10 **(0)**	1 **(0)**	3.990600	4.004029
5	15 **(+1)**	1 (**0**)	0.847500	0.858596
6	7.5 **(-0.5)**	0.5 **(-0.866)**	1.601300	1.613470
7	12.5 **(-1)**	0.5 **(-0.866)**	1.594900	1.582730

The response surface graphics (Figures 01 and 02) shows the influence of glucose and yeast extract concentrations in the production of inulinase of CCMB 328. From the analysis of the area chart in Figures 01 and 02 it can be concluded that the production of inulinase by CCMB 328 strains clearly peaks for glucose concentrations in excess of 10 g/L glucose, for the whole range of concentration of yeast extract tested.

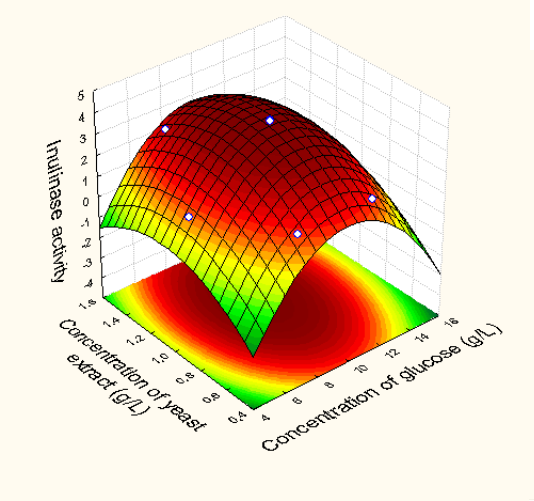


Fig. 01. Response surface for yeast extract concentration versus glucose concentration

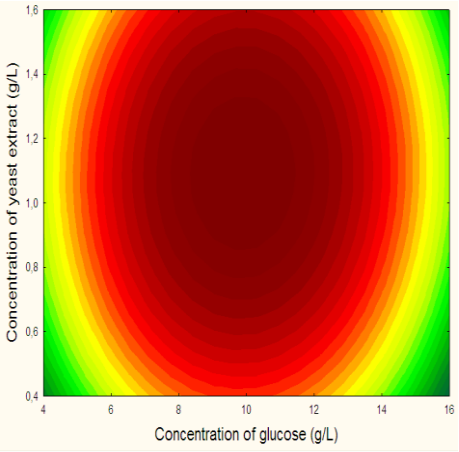


Fig. 02. Level curves for yeast extract concentration versus glucose concentration

Equation 1 illustrates the relation of these two variables and the enzyme activity (UA), where G is the glucose concentration g/L and YE is the yeast extract concentration g/L:

$$\mathbf{UA = -13.6433 + 2.3911(G) - 0.1202\ (G)^2 + 10.7575\ (YE) - 4.9122\ (YE)^2 - 0.00812\ (G).(YE).}$$

Through the derivation of this equation, the media composition, regarding glucose and yeast extract concentrations, which are expected to allow for the production of maximum enzymatic activity, can be obtained. For CCMB 328 the conditions are 9.89 g / L for glucose and 1.09 g / L for yeast extract.

Kalil (2004) using the technique of factorial design and response surface analysis to optimize the culture medium for inulinase production by *Kluyveromices marxianus* According to the results, the best concentration of yeast extract was 10 g / L. Schneider (1996) studied the production of inulinase by *Kluyveromices marxianus* and found that the presence of yeast extract is essential for the growth of microorganism and enzyme production.

The chart of Paretto shows that both linearly and quadratically, variables glucose concentration and yeast extract concentration are significant for the production of inulinase fungal endophyte CCMB 328, because both variables had a P value greater than 0.05

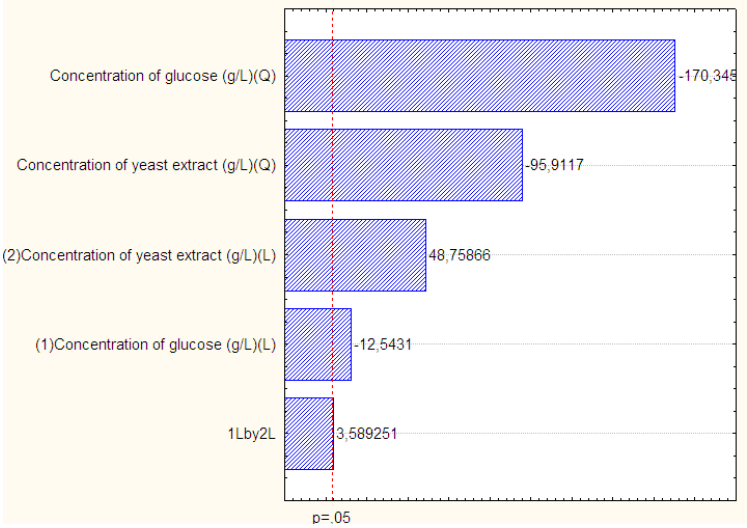


Fig. 03: Pareto Chart for inulinase production CCMB 328

The statistical significance of the regression can be assessed by the ratio of the mean square regression and mean square of the residue and by comparing these sources of

variation using the Fisher distribution (F test) (Table 02). Thus, a statistically significant value of this ratio must be greater than the tabulated value for F. It is obtained by the ANOVA table that calculated F (712.55) is higher than the F tabulated (9.01) showing that the function is well suited to the answers.

Table 02 Analysis of variance

Variation source	SQ	gl	MQ	F	Tabulated F (IC 95%)	R^2
Regression	12.956	5	2.5911402	712.55023	9.01	
Residual	0.011	3	0.0036364			
Lack of Fit	0.01061	1	0.01061	70.71	0.013849	0.99
Pure Error	0.00030	2	0.00015			
Total SQ	12.94509	8				

Response surface optimization of temperature and pH for inulinase obtained

The table 03 shows the Doehlert design applied to the optimization of temperature and pH inulinase obtained by fungal endophyte. CCMB 328.

Table 03. Results of the factorial for optimization of temperature and pH CCMB 328

N°	pH	Temperature °C	Experimental values (UA)	Activities predicted (UA)
1	6 (-0.5)	70 (+0.866)	2.833800	2.843550
2	8 (+0.5)	70 (+0.866)	2.934800	2.925050
3	5 (-1)	50 (0)	2.608000	2.598250
4C	7 (0)	50 (0)	3.210200	3.209333
4C	7 (0)	50 (0)	3,244500	3.209333
4C	7 (0)	50 (0)	3.173300	3.209333
5	9 (+1)	50 (0)	2.717000	2.726750
6	6 (-0.5)	30 (-0.866)	2.906400	2.916150
7	8 (+0.5)	30 (-0.866)	2.972900	2.963150

The influence of temperature and pH on the activity of the enzyme was investigated for CCMB 328 by surface response methodology. The results are shown in Figures 03 and 04.

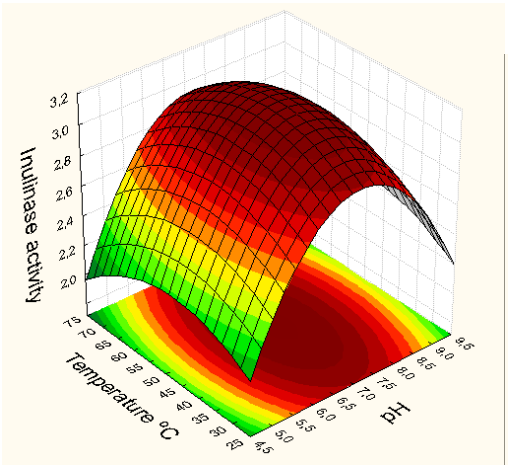


Fig 04. Response surface for pH versus temperature

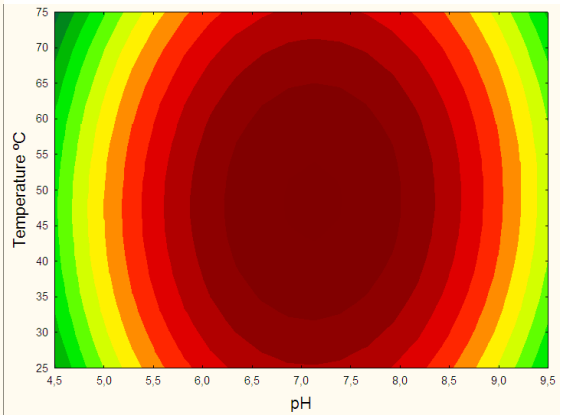


Fig 05. Level curves for pH versus temperature

The regression model provided for the enzyme activity in relation to pH and temperature in the experimental design is expressed by Equation 2:

$$\mathbf{UA = -4.4934+1.9239(pH) -0.1367(pH)^2 + 0.0356(T) - 0.0004(T)^2+0.0004(pH).(T)}$$

This equation illustrates the relationship of these two variables with enzyme activity (UA). Where: P is the pH and T is the temperature ºC. Through the derivation of this equation, the points of maximum enzymatic activity can be obtained. According

to this methodology, the optimal pH and temperature values were of 7.11 and 48.35 °C, respectively.

Regarding to the pH of the enzymatic reaction, although the microbial inulinases are described as stables among the pH 3.5 and 6.5 however ours results indicate a maximum inulinase activity at pH 7.11. In relation to the reaction temperature, it was observed that the the maximum enzymatic activity at 48.35 ºC. And similar values were found in examples of studies described by Cazetta et al (2005).

The Paretto chart (Figure 06) shows that variables temperature concentration and pH are independent for the production of inulinase fungal endophyte CCMB 328, because s had a p value less than 0.05

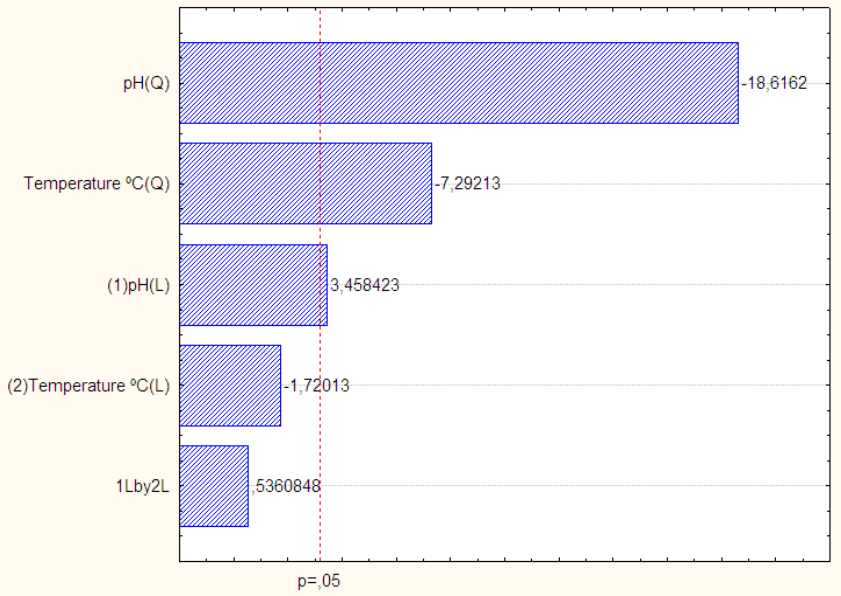


Fig.06: Pareto Chart for pH x temperature surface CCMB 328

The statistical significance of the regression can be assessed by the ratio of the mean square regression and mean square of the residue and by comparing these sources of variation using the Fisher distribution (F test) (Table 04).Thus, a statistically significant value of this ratio must be greater than the tabulated value for F. It is obtained by the ANOVA table that calculated F (75.17) is higher than the F tabulated (9.01) showing that the function is well suited to the answers

Table 04 Analysis of variance

Variation source	SQ	gl	MQ	F	Tabulated F (IC 95%)	R^2
Regression	0.392	5	0.0783983	75.717366	9.01	
Residual	0.003	3	0.0010354			
Lack of Fit	0.000570	1	0.000570	0.4498	0.571487	0.99
Pure Error	0.002536	2	0.001268			
Total SQ	0.391421	8				

Effect of cations on inulinase activity

The effect of salts NaCl and KCl on the activity of inulinase are presented in the Figure 07. The concentration of 0.20 mol/L of NaCl and KCl increased the activity of inulinase from CCMB 328 by approximately 63% and 37% respectively. A concentration higher than 0.20 mol/L decreases the activity of the enzyme. CCMB 322 showed a greater increase in activity (125%) but in a different salt concentration (0.15M)

Our results were similar to those observed by Shing (2006) who reported an increase in enzymatic activity at 62.2% in the presence of KCl. Otha in 2002 using inulinases produced by *Rhizopus* noted that the presence of KCl did not change when the enzyme activity.

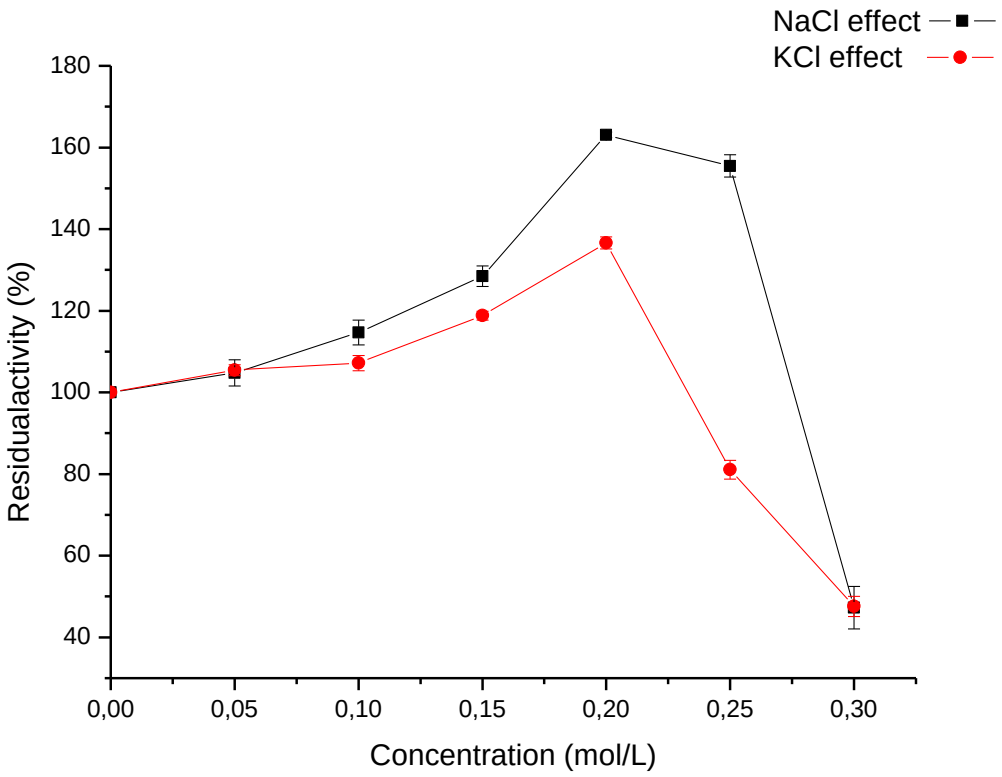


Fig.07. Effect of salts on inulinase activity

Kinetics Parameters

The Lineweaver-Burk plot showed that the K_m and V_{max} values of the enzyme for inulin were 7.53 mg/mL and 2.34 µmol/mL.min, respectively (Fig. 08). Showronek and Fiedurekin (2006) reported km values of inulinase 6.7 g/mL and Vmax 0.0476 mg/mL were similar with our results

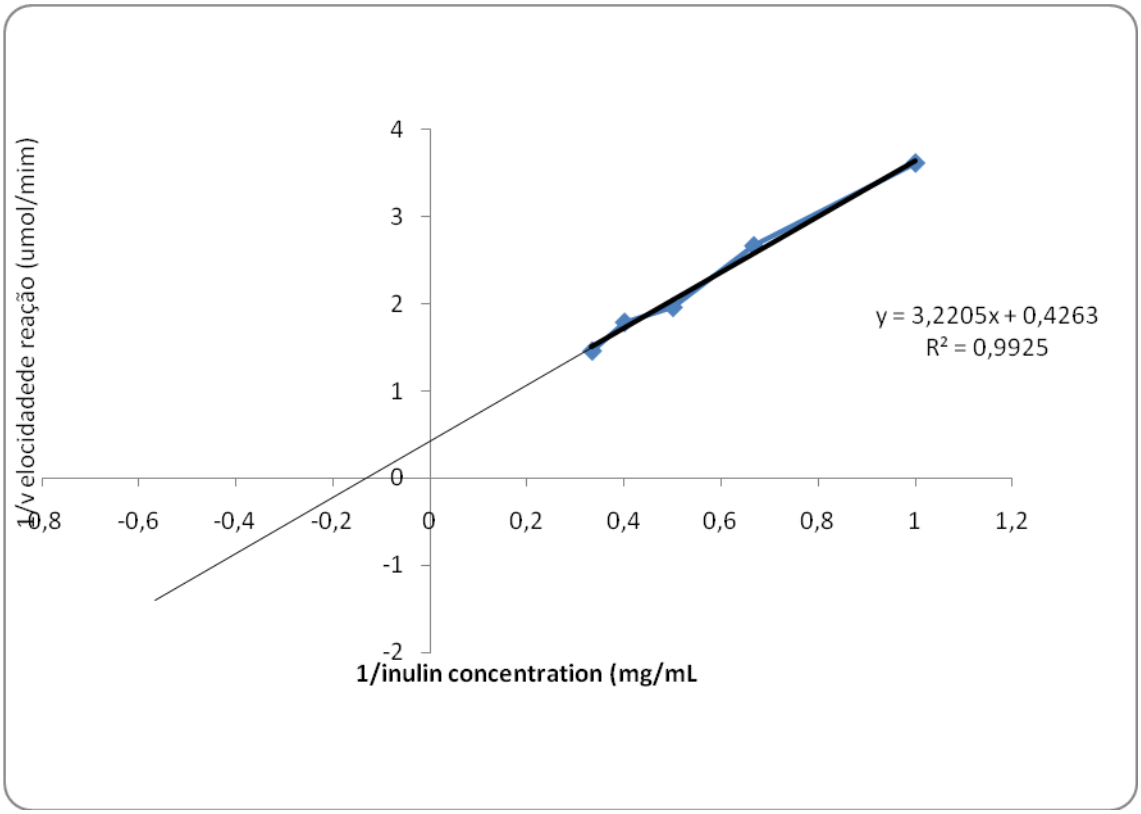


Fig.08. Influence of substrate concentration on the inulinase activity

Thermostability of inulinases

The effect of temperature on the stability of inulase activity is showed in Fig 09. *A* small reduction in inulinase activity at 60 º C after 50 min. retained 86% of activity. After incubation at 80 ºC for 50 minutes, inulinase still retained 60.49% activity.

Wenling et al 1999 reported that inulinase produced by *Kluyveromyces* sp. Y 85 sp retained 65% activity after treatment at 60º. However incubation of inulinase at 80º resulted in a rapid loss of activity (90%). This result suggests the ability of this inulinase by CCMB 328 to endure extreme thermal environments. The thermal stability of enzymes is determines the limits for use and reuse of the enzyme, and therefore process costs.

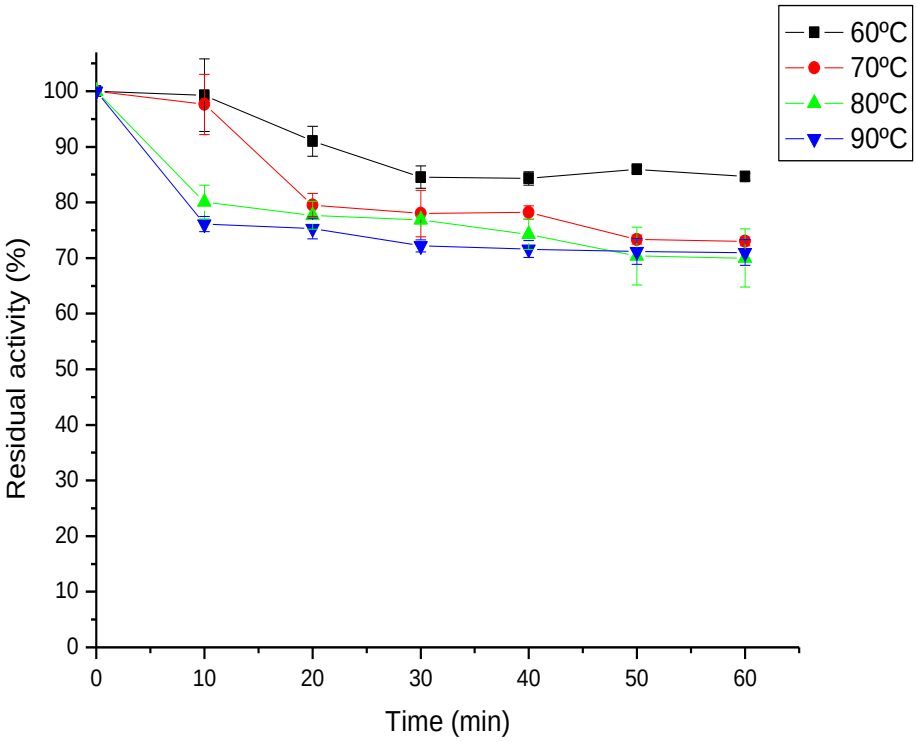


Fig.09 Evaluation of the thermal stability of inulinase from CCMB 328

Hydrolysis of inulin

The table 05 shows the Doehlert design applied to optimize the hydrolysis in function of time of reaction and enzyme concentration of inulin by fungal endophyte CCMB 328 inulinase.

Table 05 Results of the factorial for inulin hydrolysis

N°	Enzyme Concentration (%)	Time (mim)	Experimental values (% Hidrolysis)	Response predited (% Hidrolysis)
1	5 **(+0.866)**	4.5 **(-0.5)**	87.84000	78.35115
2	5 **(+0.866)**	12.5 **(+0.5)**	70.63000	80.11885
3	3 **(0)**	1 **(-1)**	60.40000	37.99520
4C	3 **(0)**	8 **(0)**	69.36000	72.15280
4C	3 **(0)**	8 **(0)**	72.48000	72.15280
4C	3 **(0)**	8 **(0)**	71.52000	72.15280
5	3 **(0)**	15 **(+15)**	62.37000	49.97640
6	1 **(-0.866)**	4.5 **(-0.5)**	42.23000	32.74115
7	1 **(-0.866)**	12.5 **(+0.5)**	25.98000	35.46885

The hydrolysis time was studied in five levels (1 to 12.5 minutes) and enzyme concentration was studied in three levels (1 to 5 %). The experimental errors were evaluated from replication of central point. The experimental data were processed by using the STATISTICA software. All the experiments in this step were carried out in random order.

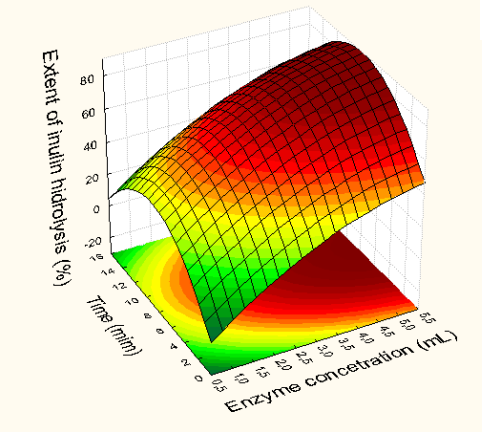


Fig. 10. Response surface for hydrolysis of inulin

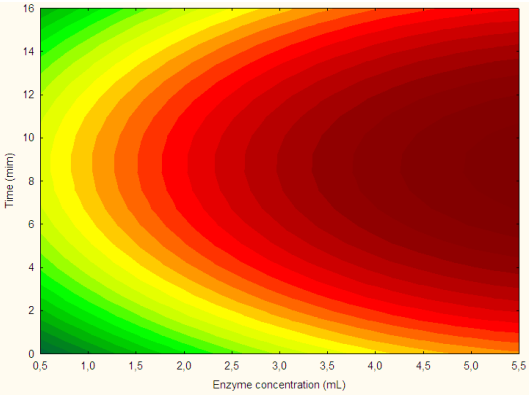


Fig.11. Level curves for hydrolysis of inulin

This equation illustrates the relationship of these two variables with extent of hydrolysis (% H), where: C is the Enzyme Concentration (% v/v) and T is the time (mim). Through the derivation of this equation, the points of maximum enzymatic activity are obtained.

Equation 3 illustrates the relation of these two variables with the extent of hydrolysis (% H), where: C is the enzyme concentration (% v/v) and T is the time (min):

$$\textbf{\% H} = 6.3905 + 18.0495\ \textbf{(C)} - 2.7079\ \textbf{(C)}^2 + 13.7011\ \textbf{(T)} - 0.9030\ \textbf{(T)}^2 - 0.4816\ \textbf{(C)(T)}$$

Through the derivation of this equation, the points of maximum enzymatic activity are obtained. In this work the highest productivity, based on the extent of hydrolysis, was observed for 5.01 % of enzyme and 9.35 minutes of incubation. There are few studies of inulin hydrolyses using the same conditions and SRM analysis, as performed in this work.

The Paretto chart (Figure 12) shows that variables temperature concentration and pH are independent for the production of inulinase CCMB 328, because s had a p value less than 0.05

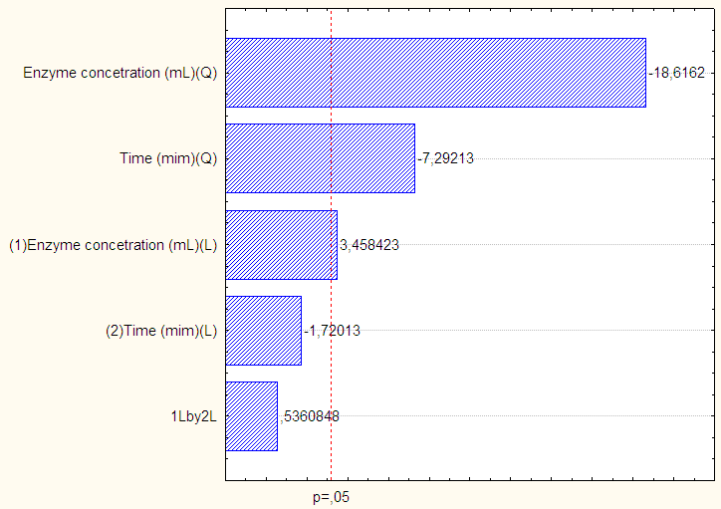


Fig. 12: Pareto Chart for inulin hydrolsis

Table 06 Analysis of variance

Variation source	SQ	gl	MQ	F	Tabulated F (IC 90%)	R^2
Regression	2890.593	5	578.11863	11.168683	5.3	
Residual	155.287	3	51.76247			
Lack of Fit	154.692	1	154.692	519.274		0.94
Pure Error	0.596	2	0.298			
Total SQ	2735.902	8				

The statistical significance of the regression (Table 06). can be assessed by the ratio of the mean square regression and mean square of the residue and by comparing these sources of variation using the Fisher distribution (F test) .Thus, a statistically significant value of this ratio must be greater than the tabulated value for F. It is obtained by the ANOVA table that calculated F (11.16) is higher than the F tabulated (5.3) showing that the function is well suited to the answers.

4.4 Conclusion

In this work the production and characterization of the inulinase by fungal endophyte CCMB 328 was effectively performed. The enzyme was afterwards used in the hydrolysis of inulin, where a conversion yield of roughly 72.46 % for an initial concentration of inulin of 1 % (w/v). Therefore, the inulinase from this yeast is a potential candidate for inulin hydrolysis in the food industry.

Using the response surface methodology it was possible to determine the best media composition for obtaining inulinase from fungal endophyte CCMB 328. This corresponds to a medium containing 1.1 0.86 g / L yeast extract and 10 g / L glucose. This statistical method was also used to access the optimum pH (7.11) and temperature (48.38 ºC) for fermentative inulinase production.

The combination of results obtained in this work (enzymatic characterization and hydrolysis of inulin) can provide useful guidelines for the utilization of inulinase from CCMB 328 for fructose production at pilot scales.

This optimization through RMS can be used in future industrial scale fermentation in promoting the rational use of enzyme and substrate in the process.

References

Bradford MN. 1976. A rapide and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of proteindye bidinig. *Analytical Biochemistry*. 72:248-254.

Cazetta ML, Martins PMM, Monti R, Contiero J. 2005. Yacon (*Polymnia sanchifolia*) extract as a substrate to produce inulinase by *Kluyveromyces marxianus* var. *bulgaricus*. J Food Eng 66:301-305.

Edelman J, Jefford TG. 1964. The metabolism of fructose polymers in plants.4. Beta-fructofuranosidases of tubers of *Helianthus tuberosus* L. *Biochemical Journal* 93: 148-161.

Figueiredo-Ribeiro RCL, Pessoni AB, Braga MR. 2007. Inulinases produced by microbes from the brazilian cerrado: characterization and potential uses. in:. Shiomi N, Benkeblia N, Onodera S. (org.). *Recent advances in fructooligosaccharides*. 18: 339-356.

Gong F, Zhang T, Chi Z, Sheng J, Jing L, Wang X. 2008. Purification and characterization of extracellular inulinase from a marine yeast *Pichia guilliermondii* and inulin hydrolysis by the purified inulinase. *Biotechnology and Bioprocess Engineering* 13: 533-539.

Gill P, Manhas R, Singh J, Singh P. 2006. Purification and properties of a heat-stable exoinulinase isoform from *Aspergillus fumigatus*. *Journal of Food Engineering* 76: 369–375.

Kango N. 2008. Production of inulinase using tap roots of dandelion (*Taraxacum officinalle*) by *Aspergillus niger*. *Jornal of Food Engineering* 85: 473-478.

Kalil SJ, Maugeri F, Rodrigues MI. 2004. Ion exchange expanded bed chromatography for the Purification of an Extracelular Inulinase from *Kluyveromyces marxianus* . Process Biochemistry. 40:581-586

Kochhar A, Gupta AK, Kanur N. 1999. Purification and immobilisation of inulinase from *Aspergillus candidus* for producing fructose. *Journal of the Science of Food and Agriculture* 79: 549-554.

Miller GL. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Analytical Chemistry 3:426-428.

Nakamura T, Ogata Y, Shitara A, Nakamura A, Ohta K.1995. Continuous production of fructose Syrups from inulin by immobilized inulinase from *Aspergillus niger* mutant 817. *Journal of Fermentation and Bioengineering* 80:164-169.

Oliveira RQ. 2007. Bioprospecçãode microrganismos leveduriformes produtores de pectinase extracellulares isolados do Semi-árido baiano. MSc Dissertation, Univ. Estadual de Feira de Santana.

Otha K.2002. Purification and properties of an extracellular inulinase from *Rhizophus* sp. Strain Tn-96. *Journal of bioscience and engineering* 1: 78-80.

Patching JW, Rose AH. 1969. The effect and control of temperature. *Methods Microbiology* 2: 23-38

Ricca E, Calabro V, Curcio S, Loiro G. 2007. The state of the art in the production of fructose from inulin enzymatic hydrolysis. *Critical Reviews in Biotechnology* 27: 129-145.

Rocha JR, Catana R, Ferreira BS, Cabral JMS, Fernandes P. 2004. Design and characterisation of an enzyme system for inulin hydrolysis. Food Chemistry 95:77-82.

Singh RS, Dhaliwal R, Puri M. 2006. Production of inulinase from *Kluyveromyces marxianus* YS 1 using root extract of *Asparagus racemosus*. Process Biochemistry 41:1703-1707.

Sheng J, Chu Z, Li J, Gao L, Gong F. 2007. Inulinase production by the marine yeast *Cryptococcus aureus* G7a and inulin hydrolysis by the crude inulinase. *Process Biochemistry* 42: 805–811.

Skowronek M, Fiedurek J. 2006. Purification and Properties of Extracellular Endoinulinase from *Aspergillus niger* 20 OSM. *Food Technology and Biotechnology* 44 53–58.

Schneider ALS. Estudo da Produção de Inulinase por *Kluyveromyces marxianus* ATCC 36907. MSc dissertation FEQ-UFSC, 1996.

Uetanabaro APT, Góes-Neto A. 2006. Importance of culture collections of microorganisms (CCMs) for the conservation of microbial biotechnological resources of the Brazilian semi-arid region. In: Towards greater knowledge of the Brazilian semi-arid biodiversity. 1st ed. Brasília, DF. Ministério da Ciência e Tecnologia, pp 41-43

Wenling W, Huiying W, Shiyuan W. 1999. Continuous preparation of fructose syrups from Jerusalem artich oke tuber using immobilized intracellular inulinase from *Kluveromyces sp.* Y-85. *Process Biochemistry*. 34: 643-646.

CONSIDERAÇÕES FINAIS

A utilização da metodologia superfície de resposta foi utilizada para identificar as melhores condições de produção e potencializar a atividade hidrolítica de inulinases. Para as leveduras CCMB 300, CCMB 322 e CCMB 328 foi possível prever estas condições de crescimento e produção da enzima avaliando os efeitos do extrato de levedura e da concentração de glicose, a influência do pH e da temperatura, concentrações de enzima e o melhor tempo para hidrólise utilizando 5 % de substrato.

Analisando os resultados da otimização da produção de inulinase observou-se que as três leveduras apresentaram valores próximos de extratos de leveduras no meio de cultura, como melhor condição de crescimento, variando entre 0,86 a 1,19 g/L desta fonte de nitrogênio. Já em relação à concentração de glicose no meio, os resultados apontaram a levedura CCMB 300 com a maior concentração 14,54 g/L.

Avaliando de maneira geral, a CCMB 300 e a CCMB 328 apresentaram resultados melhores em relação à CCMB 322, portanto estas duas representam uma melhor escolha para a introdução em um processo piloto de hidrólise de inulina. Um fator decisivo nesta escolha é o fato estabilidade térmica da CCMB 322 ser verificada baixa em comparação com a CCMB 300 e CCMB 328.

Apesar da CCMB 322 apresentar baixa estabilidade térmica, a mesma poderia ser aproveitada em situações que não exijam temperaturas elevadas porque apresentou a maior taxa de hidrólise da inulina.

Os estudos dos efeitos dos sais na atividade enzimática revelaram-se promissores para o incremento da atividade sugerindo assim tolerância dessas enzimas ao estresse salino.

Um fator a ser considerado neste trabalho está relacionado ao fato da caracterização enzimática obtida com os três microrganismos estudados permitir uma posterior utilização e aplicação em diferentes condições experimentais desejadas e que se ajustadas a cada microrganismo, potencializarão a utilização da enzima e a obtenção de produtos biotecnológicos.

Considerando que os processos industriais sempre buscam por modelos que garantam eficiência nos processos produtivos, os resultados obtidos neste trabalho são de real importância, pois garantem a aplicação da otimização das condições enzimáticas avaliadas em determinados processos industriais. Além disso, identificando uma futura aplicação desta pesquisa no desenvolvimento de uma escala piloto para hidrólise de inulina em biorreatores é perceptível a importância da conservação dos recursos naturais para o desenvolvimento econômico e também da ciência e tecnologia.

Portanto, a combinação dos resultados obtidos neste trabalho (otimização do crescimento, avaliação do pH e temperatura, efeito de sais, estudos cinéticos, estabilidade térmica e hidrólise de inulina) pode fornecer orientações úteis para a utilização da inulinase e dos microrganismos estudados para a produção futura de novos produtos biotecnológicos.