



UNIVERSIDADE ESTADUAL DE FEIRA DE SANTANA
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOTECNOLOGIA



GILDOMAR LIMA VALASQUES JUNIOR

ESTUDO DO POTENCIAL BIOTECNOLÓGICO DE
POLISSACARÍDEOS DE PAREDE CELULAR, PRODUZIDOS POR
LEVEDURAS ISOLADAS DO SEMI-ÁRIDO BAIANO

Feira de Santana, BA
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POLISSACARÍDEOS DE PAREDE CELULAR, PRODUZIDOS POR
LEVEDURAS ISOLADAS DO SEMI-ÁRIDO BAIANO**

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

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Feira de Santana, BA
2014

Ficha Catalográfica - Biblioteca Central Julieta Carteadó - UEFS

V234

Valasques Junior, Gildomar Lima

Estudo do potencial biotecnológico de polissacarídeos de parede celular, produzidos por leveduras isoladas do semi-árido baiano / Gildomar Lima

Valasques Junior. – 2014.

88 f.: il.

Orientador: Sandra Aparecida de Assis

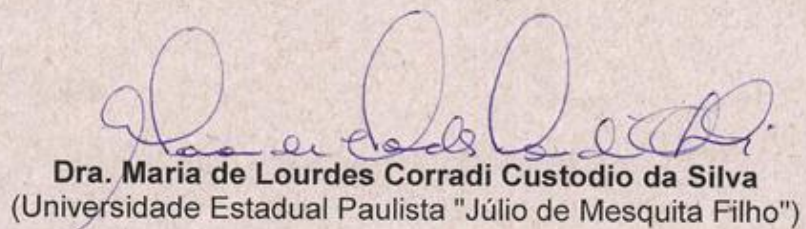
Coorientadoras: Flávia Oliveira de Lima e Elisângela Fabiana Boffo

Tese (doutorado) – Universidade Estadual de Feira de Santana, Programa de Pós-Graduação em Biotecnologia, Feira de Santana, 2014.

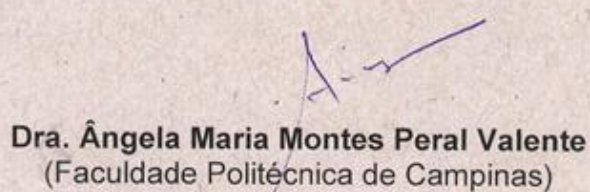
1. Polissacarídeos. 2. Caracterização química. 3. Tratamento de doenças.
4. Atividade anti-inflamatória. I. Assis, Sandra Aparecida de, orient.
II. Lima, Flávia Oliveira de, coorient. III. Boffo, Elisângela Fabiana, coorient.
IV. Universidade Estadual de Feira de Santana. V. Título.

CDU 664.642(814.22)

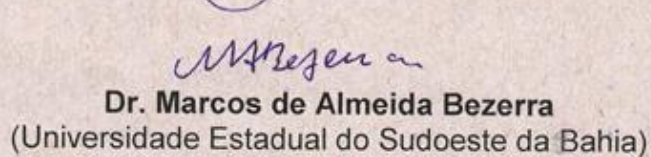
BANCA EXAMINADORA



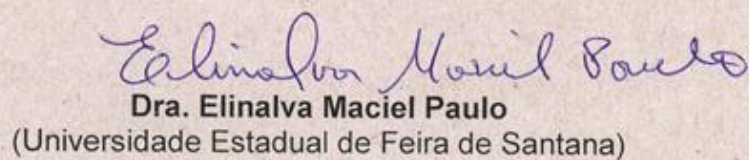
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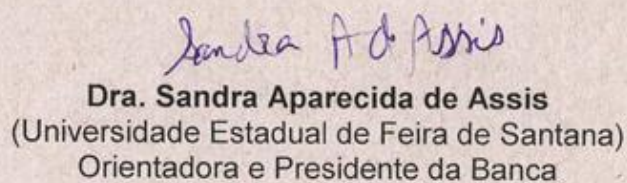
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Feira de Santana – BA
2014

Aos meus pais e demais familiares, a amigos que direta ou indiretamente participaram dessa conquista: a todos eles dedico este importante trabalho, por mim desenvolvido.

AGRADECIMENTOS

Agradeço primeiramente a Deus, nosso pai, que me presenteou com força, coragem e cuidado durante todo o tempo de vida. Agradeço aos meus pais por permitir que tudo isso pudesse estar ocorrendo e aos meus irmãos pelo apoio, o qual me tornou mais forte. Aos meus amigos, que tornaram-se para mim uma família, a qual tive o prazer de escolher, todos que com muita paciência me suportaram durante os períodos de “crises” da difícil tarefa que foi desenvolver um trabalho como esse. Agradeço em especial a aqueles que me acompanharam nas viagens aos laboratórios parceiros localizados em outras cidades, para a realização de procedimentos analíticos.

Agradeço à minha orientadora Prof^a. Sandra Assis por acreditar em meu potencial e pelas orientações e ensinamentos que através deles, fui capaz de produzir esse trabalho. À Professora Elisângela Fabiana Boffo pela dedicação, disponibilidade e atenção em vários momentos cruciais na conclusão do trabalho. Aos colegas de laboratório que nos ajudava, com o desenvolvimento do trabalho de bancada, em especial a Lívia, que durante esse período me ajudou nos experimentos, tornando possível essa conquista. Agradeço também à Professora Flávia Oliveira de Lima pela imensa e crucial participação no trabalho, como também aos colegas do biotério pela essencial colaboração. A todos que direta ou indiretamente participaram de minha vida como acadêmico de doutorado deixo, aqui registrado minha enorme gratidão.

Viva intensamente, desconfie do futuro siga em frente, basta acreditar, vale a pena SONHAR.

Autor desconhecido

RESUMO

Os β -D-glicanos e α -D-mananos são polissacarídeos constituintes estruturais da parede celular de leveduras e fungos, podendo ser encontrados em outros micro-organismos como bactérias, e também, em plantas e cereais. O grau de ramificação e a natureza da ligação (1 $\rightarrow$ 4, 1 $\rightarrow$ 6 e 1 $\rightarrow$ 3) também diferem esses polissacarídeos entre si. Várias aplicações podem ser atribuídas aos glicanos e mananos, a depender do seu tipo, podem atuar como imunomoduladores. Vários trabalhos têm sido publicados, provando a capacidade destes polissacarídeos de estimular macrófagos a produzir citocinas. Outra aplicação desses polissacarídeos está baseada em sua capacidade de causar analgesia, agindo também como antiinflamatório, úteis em vários estados patológicos associados à inflamação. Desta forma, esse trabalho teve como objetivo isolar e caracterizar polissacarídeos de parede celular de quatro fungos diferentes: *Rhodotorula mucilaginosa* (CCMB 33d1), *Aureobasidium pullulans* (CCMB 324), *Pseudozyma* sp. (CCMB 306), *Trichosporonoides* sp. (CCMB 298) e estudar sua aplicação biotecnológica, no tocante à capacidade de suprimir a inflamação “*in vivo*”. As leveduras foram obtidas da Coleção de Cultura de Micro-organismos da Bahia, localizada na Universidade Estadual de Feira de Santana. As melhores condições de pH, temperatura e/ou tempo de extração foram determinadas, aplicando a Metodologia de Superfície de Resposta. Os polissacarídeos extraídos das quatro fontes microbiológicas foram submetidos à análise estrutural pelas Espectroscopias na Região do Infravermelho (FT-IV) e de Ressonância Magnética Nuclear (RMN) e pela Cromatografia Líquida de Alta Eficiência (CLAE). Após as análises dos espectros de IV e RMN, associados ao resultado da composição monomérica obtida por CLAE, após hidrólise ácida, pode-se identificar os polissacarídeos produzidos por *Aureobasidium pullulans* (CCMB 324) e *Pseudozyma* sp. (CCMB 306) como sendo misturas de β -D-glicanos e α -D-mananos, e composição majoritária de β -D-glicanos para os polissacarídeos extraídos da parede celular de *Trichosporonoides* sp. (CCMB 298) e *R. mucilaginosa* (CCMB 33d1). Os ensaios “*in vivo*” de atividade antinociceptiva demonstraram que os polissacarídeos de parede celular extraídos das leveduras isolados do semi-árido nordestino foram capazes de causar analgesia, por provavelmente inibir a produção de mediadores inflamatórios, com exceção do *Trichosporonoides* sp., pois os testes desse último não foram conclusivos. Assim, o semi-árido nordestino demonstrou ser uma fonte promissora de micro-organismos com potencial para a produção de novas biomoléculas capazes de atuar como fonte de possíveis fármacos.

Palavras-chave: Polissacarídeos, Caracterização química, Metodologia Superfície Resposta, atividade anti-inflamatória.

ABSTRACT

The β -D-glucans and α -D-mannans are structural polysaccharides constituting the cell wall of yeasts and fungi which may be found in other microorganisms such as bacteria, and also on plants and cereals. The degree of branching and the nature of connection (1 $\rightarrow$ 4, 1 $\rightarrow$ 6 and 1 $\rightarrow$ 3) these polysaccharides also differ from each other. Several applications can be assigned the glucans and mannans, depending on its type, can act as immunomodulators. Several papers have been published, proving the ability of these polysaccharides stimulate macrophages to produce cytokines. Another application of these polysaccharides is based on its ability to cause analgesia, thus acting as anti-inflammatory, useful in various pathological states associated with inflammation. This study aims to isolate and characterize cell wall polysaccharides of four different yeasts: *R. mucilaginosa* (CCMB 33d1), *Aureobasidium pullulans* (CCMB 324), *Pseudozyma* sp. (CCMB 306), *Trichosporonoides* sp. (CCMB 298) and study their biotechnological applications, regarding the ability to suppress inflammation "in vivo". The yeasts were obtained from the Culture Collection of Microorganisms of Bahia located in the Universidade Estadual de Feira de Santana. The best conditions of pH, temperature and/or extraction time was studied, applying Response Surface Methodology. Polysaccharides extracted from the four fungal sources have been subjected to structural analysis by Infra Red (FT-IR) and Nuclear Magnetic Resonance (NMR) Spectroscopy and High Performance Liquid Chromatography (HPLC). After analysis of the spectra obtained by FT-IR and NMR associated with the result obtained from monomeric composition by HPLC, after acidic hydrolysis, the samples can be identified as mixtures of β -D-glucans and α -D-mannans for *Aureobasidium pullulans* (CCMB 324), *Pseudozyma* sp. (CCMB 306), and the majority of D-glucan composition for the cell wall polysaccharides extracted from *Trichosporonoides* sp. (CCMB 298) and *R. mucilaginosa* (CCMB 33d1). The tests "in vivo" the analgesic activity showed that cell wall polysaccharides extracted from yeast isolated from the semi-arid Northeast were able to cause analgesia, probably by inhibiting the production of inflammatory mediators, except *Trichosporonoides* sp. Because the tests this last was not performed. Thus, the semi-arid Northeast proved to be a promising source of microorganisms with potential to produce new biomolecules capable of acting as a source of potential drugs.

Keywords: Polysaccharides, chemical characterization, Response Surface Methodology, anti-inflammatory activity.

LISTA DE ABREVIATURAS

CCMB: Coleção de Cultura de Micro-organismos da Bahia

CLAE: Cromatografia Líquida de Alta Eficiência

Cm^{-1} : Centímetros recíprocos

D_2O : Água deuterada

DMSO: Dimetilsulfóxido

DP_n: Number-average degree of polymerization

FT-IR: Fourier transform infrared spectroscopy

HPLC: High Performance Liquid Chromatography

HSQC: Heteronuclear Single Quantum Coherence

MHz: MegaHertz

MOR: Morfina

MW_n: Number-average molecular weight

NMR: Nuclear Magnetic Resonance

TFA: Trifluoroacetic acid

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INTRODUÇÃO GERAL

Com o surgimento de novas tecnologias, várias doenças que anteriormente eram consideradas obscuras, atualmente já se têm entendimento mais claro sobre elas. Dentre essas doenças, podem-se incluir alguns tipos de câncer, doenças infecciosas como Hanseníase e tuberculose, doenças inflamatórias com caráter auto-imune, entre outras. Com as novas tecnologias utilizadas para diagnósticos específicos de doenças, surge também a necessidade do desenvolvimento de novas drogas que atuem como objeto terapêutico no tratamento das doenças identificadas. Nesse sentido, a biotecnologia é uma ferramenta útil no desenvolvimento de novas drogas, obtidas a partir de fontes biológicas, tecnologicamente tratadas, e essa ferramenta, atualmente vem sendo explorada na obtenção de novos agentes terapêuticos.

As leveduras são micro-organismos unicelulares, caracteristicamente esféricas ou ovais, que se reproduzem assexuadamente por brotamento ou fissão. No entanto, algumas leveduras produzem brotos que não se separam uns dos outros, formando pseudomicélio. Além disso, alguns fungos exibem dimorfismo, ou seja, podem crescer tanto na forma de fungo filamentoso quanto na forma de levedura. A parede celular é rígida, composta de proteínas, lipídeos e carboidratos (D-glicanos). Essas macromoléculas juntas são responsáveis pela manutenção da integridade física da levedura, fazendo-a suportar diversas condições externas como pH, temperatura e pressão osmótica (Tortora *et al*, 2011).

Os β e α D-glicanos e D-mananos presentes na parede celular das leveduras diferem entre si pelo tipo de ligações (1 $\rightarrow$ 3, 1 $\rightarrow$ 4, 1 $\rightarrow$ 6) e grau de ramificação, características que lhes conferem estruturas específicas e ações biológicas distintas, sendo, portanto, a característica estrutural um fator fundamental para a ação dos β e α glicanos/mananos no sistema imunológico. Esses polissacarídeos podem conter dois tipos de ligações (α e β), como também essas ligações podem ser do tipo 1 $\rightarrow$ 3, 1 $\rightarrow$ 4 ou 1 $\rightarrow$ 6, variando de micro-organismos para micro-organismo produtor. Várias aplicações biotecnológicas têm sido atribuídas aos D-glicanos e D-mananos, principalmente de origem fúngica (Recalde *et al*, 2012; El Enshasy e Hatti-Kaul, 2013).

Vários autores têm atribuído a esses polissacarídeos de parede celular de leveduras diversas atividades farmacológicas, entre elas atividade imunomoduladora, antitumoral, antioxidante, anticoagulante após sulfatação, antiinflamatória,

hipocolesterolemia e analgésica (Baggio et al, 2012; Ruthes et al, 2013; Machova et al, 2014; Vasconcelos et al, 2013; El Enshasy e Hatti-Kaul, 2013). A partir das aplicações biotecnológicas desses biopolímeros, além da diversidade de microrganismos presentes no semi-árido nordestino, entende-se a necessidade de se explorar essa fonte como produtora de biomoléculas capazes de atuar nas diversas doenças que acomete a população mundial.

Nesse contexto, o presente trabalho visou otimizar a extração, caracterizar estruturalmente e estudar os potenciais biotecnológicos de polissacarídeos de parede celular produzidos por leveduras isoladas do semi-árido nordestino, no intuito de verificar a capacidade dos polissacarídeos identificados em tratar doenças.

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CAPÍTULO 1

Optimization of polysaccharides extraction produced from *Aureobasidium pullulans*, structural characterization, and antinociceptive activity

Optimization of polysaccharides extraction produced from *Aureobasidium pullulans*, structural characterization, and antinociceptive activity

Abstract

D-Glucans and D-Mannans are glucose and mannose polymers that consist of β -(1 $\rightarrow$ 3) linked β -D-glucopyranosyl units. They can be found as non-branched β -(1 $\rightarrow$ 3) linked backbones or as β -(1 $\rightarrow$ 3) linked frameworks with β -(1 $\rightarrow$ 6) branches. These polysaccharides are the major structural components of fungal cell walls. They are not found in animals, so that as carbohydrates they can be recognized by the innate immune system of vertebrates. We report a sequential optimization strategy for polysaccharides production by *Aureobasidium pullulans* (CCMB 324), isolated in the Brazilian semi-arid region, through statistically designed experiments. The polysaccharides were characterized by FT-IR, ^1H and ^{13}C NMR spectroscopic techniques and HPLC; and antinociceptive action was examined in experimental protocols on mice. The best yield was of 26% obtained after 3h, of extraction in a temperature of 92°C and pH 13. The analysis results of NMR, FT-IR and HPLC show that the product obtained (poly324) consists of β -(1 $\rightarrow$ 3)-D-Glucan (60%) followed by α -(1 $\rightarrow$ 6)-D-manan (40%) and degree of polymerization of 742. In addition, the antinociceptive effect was evaluated using different experimental tests (acetic acid-induced writhing test, formalin test and tail immersion test). The poly324 showed a potent peripheral antinociceptive effect, possibly by the inhibition of inflammatory mediators.

Keywords: polysaccharides, optimization of extraction, response surface methodology, *Aureobasidium pullulans*, antinociceptive activity.

1. Introduction

D-Glucans and D-mannans are glucose and mannose polymers that consist of β -(1 $\rightarrow$ 3) linked β -D-glucopyranosyl units. They can be found as non-branched β (1 $\rightarrow$ 3) linked backbones or as β -(1 $\rightarrow$ 3) linked frameworks with β -(1 $\rightarrow$ 6) branches. These polymers are the major structural components of fungal cell walls. They are not found in animals, so that as carbohydrates they can be recognized by the innate immune system of vertebrates (Brown and Gordon, 2003; Smiderle et al. 2008).

In vitro experiments showed that polysaccharides can directly activate leukocytes, stimulating their phagocytic, cytotoxic, and antimicrobial activities. In addition, they can stimulate the production of proinflammatory mediators, such as cytokines and chemokines (El Enshasy and Hatti-Kaul, 2013), and have anti-tumor (Mizuno et al., 1990), anti-oxidative (Kozarski et al., 2011), anti-inflammatory (Baggio et al., 2012), and immunomodulating (Zhang et al., 2007) activities.

The mechanisms by which the yeast polysaccharides exhibit their bioactivities are still unknown, although some authors have showed that these molecules can bind to immune cell receptors, and initiate immune reactions, as the production of pro- and anti-inflammatory cytokines (Chen & Seviour, 2007; El Enshasy & Hatti-Kaul, 2013; Tada, et al, 2007).

The affinity ligand-receptor depends mainly on the chemical structure of the ligand, that can be determined by its chemical formulae, conformation, and molecular weight (Zhang et al., 2007).

Yeast polysaccharides vary greatly on these three characteristics, and some studies have shown that different polysaccharide structures may exhibit different biological effects. However, the research data presented up to now are not enough to determine which molecule is responsible for each bioactivity. Therefore, the therapeutic

application of a polysaccharide requires the careful knowledge of its chemical structure (El Enshasy & Hatti-Kaul, 2013).

We report a sequential optimization strategy for polysaccharides (poly324) production by *Aureobasidium pullulans* (CCMB 324), isolated in the Brazilian semi-arid region, through statistical experimental design. Box-Behnken design was used as a response surface matrix to describe the nature of the response surface in the experimental region, to search optimal medium composition for maximizing polysaccharides yield. The polysaccharides were characterized by FT-IR, ^1H and ^{13}C NMR spectroscopic techniques and HPLC; and antinociceptive action was evaluated in experimental protocols on mice.

2. Materials and Methods

2.1 Microorganism

Aureobasidium pullulans (CCMB 324) was obtained from the Collection of Cultures of Microorganisms of Bahia (CCMB, Feira de Santana, Brazil). The microorganism was maintained on YM agar at 4 °C in an incubator (IGO 150 Cell Life – Jouan).

2.2 Culture conditions of *Aureobasidium pullulans* CCMB 324

The strain of *Aureobasidium pullulans* CCMB 324 was incubated in a YM culture medium for activation of 4 days at 30 °C. Cultivation in liquid media was carried out in 250 mL Erlenmeyer flasks containing 100 mL of (g/L): D-glucose, 15; $\text{KH}_2(\text{PO}_4) \cdot 3\text{H}_2\text{O}$, 2.0; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5; $(\text{NH}_4)_2\text{SO}_4 \cdot 7\text{H}_2\text{O}$, 2.0, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.1, pH 5.0. Flasks were inoculated with ten milliliters of cell suspension contend 10^9 colony-forming unit (CFU) of Microorganism. The incubation was performed at 120 rpm for 96

hours in a rotary shaker. After the fermentation, the liquid culture medium, on which *Aureobasidium pullulans* was grown, was filtered and centrifuged (Centrifuge 5804R – Eppendorf, São Paulo, Brazil) at 8,000 g for 15 min at 4°C, and the precipitate, containing the biomass from Microorganism, was used to extract the biopolymers (Dong et al. 2008).

2.3 Extraction of yeast polysaccharides (poly324)

The powder of dry biomass (1g) was extracted several times with 20 volumes of sodium hydroxide solutions at different pHs (11, 12 and 13), temperatures (70, 80 and 90°C) with various extraction times (60, 120, and 180 min.) The suspension was centrifuged at 10.000×g for 10 min. It was added to the supernatant three times of the volume of absolute ethanol, stirred vigorously and left overnight at 4°C. The precipitated polysaccharides were centrifuged at 8000×g for 30 min and the supernatant discarded. The precipitate of crude polysaccharides was dried at 55 °C to a constant weight and the polymer was weighted by a scale (Sartorius ALC-110.4, Germany). The polysaccharides was determined by phenol–sulfuric acid method (Dubois, et.al, 1956). The product yield was measured at the (w/w) % of polysaccharides per unit mass of biomass yeast.

2.4 Experimental design

The temperature (°C), pH and extraction time (minutes) were monitored using Box-Behnken experimental design to polysaccharides extraction. Three repetitions of the central point (c) to a total of fifteen experiments (Table 1) were performed to estimate the possible pure error, and the experimental treatments were varied randomly to detect the presence of possible systematic errors. The temperature was studied at

three levels, ranging from 70 to 90°C, and the time was studied at three time points, ranging from 60 to 180 min, and pH was studied at three levels ranging from 11 to 13 (Table 1). All the calculations and graphics in this work were performed using the software Statistica® version 7.0, and including the analysis of variance (ANOVA) for the responses of this study.

2.5 The number-average molecular weight (MW_n) and degree of polymerization (DP_n)

The MW_n and DP_n of the polysaccharides were determined by the measurement of the reducing sugar value using 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959) and the measurement of total carbohydrates using the phenol–sulfuric acid method (Dubois et al, 1956); $DP_n = [(total\ carbohydrates\ in\ \mu g\ of\ D-glucose)/(reducing\ value\ in\ \mu g\ of\ maltose)] \times 1.9$; $MW_n = [(DP_n) \times 162] + 18$ (Vettori, Franchetti, Contiero, 2012).

2.6. Determination of the protein concentration

Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin (BSA) as a standard.

2.7. Characterization of polysaccharides (poly324) by spectroscopic analysis

The polysaccharides were characterized using a Fourier Transform Infrared Spectrometer (PE IR Spectrum AsciiPeds 1.60, Spectrum One FT-IR Spectrometer). The dried polymer was grinded with potassium bromide (KBr) powder and pressed into pellets for FT-IR spectral measurement in the frequency range of 4000 – 500 cm⁻¹, with 20 scans. In NMR spectroscopic analysis, all spectra were recorded at 343 K on a Varian Inova 500 spectrometer operating at 11.7 T, observing ¹H at 500 MHz and ¹³C at

125 MHz, and using a 5 mm direct probe. The sample was dissolved in D₂O/DMSO (6:1) and transferred to a NMR tube. DMSO was used as a chemical shift reference. D₂O (99.9%) and DMSO (99.9%) were from Cambridge Isotope Laboratories, Inc. (USA).

2.8. Monosaccharide Composition of polysaccharide

The polysaccharide (poly324) (20 mg) was hydrolyzed with 2 M trifluoroacetic acid (TFA) at 100°C for 8 h, followed by evaporation (Ruthes *et al*, 2013). Filtered liquids were analyzed by high-performance liquid chromatography (HPLC) using Amines® HPX-87H (300 mm x 7.8 mm i.d.) (9µm) column (Merck, Darmstadt, Germany) and refractive-index detection; the injection volume was 12 µL. The mobile phase was acetonitrile (MeCN):deionized H₂O (82:18) at a flow rate of 0.4 mL min⁻¹, and the column temperature was maintained at 25°C. For peak identification, an external standard of glucose and mannose was used. Determination of the monosaccharide composition was done in two independent experiments with consistent results.

2.9. Animals

Experiments were conducted using Male Swiss mice (25–30 g) obtained from the State University of Feira de Santana (UEFS). Animals were housed at 22±2°C, under 12 h light-dark cycle, with access to water and food *ad libitum* until use. The experiments were performed after protocol approval by the Animal Ethics Committee of UEFS and were carried out in accordance with the guidelines of International Association for Study of Pain on the use of animals in pain research (ZIMMERMANN, 1983). All efforts were made to minimize the number of animals and intensity of the noxious stimuli used.

2.10. Writing test

The writhes were induced according as previously described (Collier et al., 1968). Mice were pretreated with poly324 (3, 9, and 27 mg/kg), morphine (5mg/kg; reference drug) or saline (control group) by intraperitoneal (i.p.) injection 30 min before i.p. acetic acid administration (0.8 % v/v, 10 mL/kg). After the challenge, the mice were individually placed in a large glass cylinder and the intensity of nociceptive behavior was quantified by counting the total number of writhes occurring between 0 and 30 minutes after the stimulus injection. The writing response consists of a contraction of abdominal muscle together with stretching of the hind limbs. The antinociceptive activity was expressed as the writing scores over 30 min.

2.11. Formalin-induced orofacial nociception

Mice were treated with Poly324 (3, 9 and 27 mg/kg), morphine (5mg/kg; reference drug) or saline (control group) by intraperitoneal (i.p.) injection 30 min before subcutaneous injection of 20 µl of formalin (2%) into the right upper lip. Following injection, the mice were observed from 0 to 5 minutes (neurogenic phase) and from 15 to 40 minutes (inflammatory phase). The nociception score was determined by counting the time that the animal spent rubbing the injected area with fore- or hind-paws during the observation time (Luccarine et al., 2006).

2.12. Tail immersion test

The tail immersion was performed as described (Sewell & Spencer, 1976), with slight modifications. Before the day of the experiment, each animal was habituated to the restraint cylinder for 4 consecutive days (20 min per day). On the experimental day, mice were placed in the restraint cylinder and the tail tip (2 cm) was immersed in a

water bath at $48^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$. The latency for the tail withdrawal reflex was measured before (baseline) and after treatments. To prevent tissue damage of the tail, the cut-off time was 10 s. Mice were then randomly selected to perform in one of experimental groups: control (saline, i.p.), Poly324 (9, 27 and 81 mg/kg, i.p.) and morphine (5mg/kg, s.c.; reference drug) and tested for antinociception at 0.5, 1, 3 and 5 h following the administration of the drugs. Antinociception of each mouse was calculated according to the following formula:

$$\text{Antinociception index} = [(\text{test latency} - \text{baseline latency}) / (10 - \text{baseline latency})] \times 100$$

2.13. Drugs and dilutions

The following drugs and reagents were used: morphine (Cristália, Brazil), formaldehyde and acetic acid (Merck, Germany). All drugs were dissolved in sterile 0.9% (w/v) NaCl (saline).

2.14. Statistical analysis

The data are presented as means $\pm$ standard error of the mean (SEM) of measurements made on 6-7 animals in each group. Statistical comparisons between groups were made using one-way analysis of variance (one-way ANOVA) followed by the Bonferroni's test, repeated measures of two-way ANOVA with Bonferroni's test, as appropriate. Statistical differences were considered to be significant at $P < 0.05$.

3. Results

3.1 Optimization Extraction of polysaccharide Poly324

A 15-run Box-Behnken design with three factor, including three replicates at the centre point, was used for fitting a second-order response surface. The three centre point runs were added to provide as a measure of process stability and inherent variability. The considerable variation in the polysaccharide yield from the *Aureobasidium pullulans* CCMB 324 under different conditions was shown in Table 1.

Table 1. Box-Behnken design with experimental and predicted values mass of poli324

(g)

Run	pH	Temperature (°C)	Time (h)	Mass of poly324 (g)		
				Predicted	Experimental	Difference
1	11 (-1)	90 (+1)	2 (0)	0.121	0.092	-0.029
2	12 (0)	90 (+1)	1 (-1)	0.080	0.102	0.022
3	12 (0)	90 (+1)	3 (+1)	0.208	0.210	0.002
4	13 (+1)	90 (+1)	2 (0)	0.209	0.216	0.003
5	11 (-1)	80 (0)	1 (-1)	0.067	0.076	0.009
6	11 (-1)	80 (0)	3 (+1)	0.065	0.093	0.020
7C	12 (0)	80 (0)	2 (0)	0.200	0.200	0.000
8C	12 (0)	80 (0)	2 (0)	0.200	0.190	-0.010
9C	12 (0)	80 (0)	2 (0)	0.200	0.210	0.010
10	13 (+1)	80 (0)	1 (-1)	0.068	0.041	-0.027
11	13 (+1)	80 (0)	3 (+1)	0.238	0.230	-0.008
12	11 (-1)	70 (-1)	2 (0)	0.084	0.078	-0.006
13	12 (0)	70 (-1)	1 (-1)	0.086	0.084	-0.002
14	12 (0)	70 (-1)	3 (+1)	0.124	0.103	-0.021
15	13 (+1)	70 (-1)	2 (0)	0.170	0.200	0.030

(C): central point; coded values are presented in the parentheses

The model adequacy was checked by an F-test and the determination coefficient R^2 . The analysis of variance (Table 2) showed that this regression model was highly significant ($P < 0.05$) with F-value of 6.70.

Table 2. Analysis of variances in the regression model for optimization of polysaccharide Poly324 of *Aureobasidium pullulans*.

Source of variations	SS	Df	MS	Fcal	Ftab	Rsqr
Regression	0.011126	9	0.0012362	6.70	4.77	0.9236
Residual	0.000746	5	0.0001493			
lack of fit	0.004500	3	0.0015000	15.00	9.16	
Pure error	0.000200	2	0.0001000			
Total error	0.061537	14				

SS - Sum of square; Df - Degree of freedom; MS - Mean square; Fcal - calculated F value; Ftab - tabulated F value; Rsqr - R-square.

The fitness of the model was further confirmed by a satisfactory value of determination coefficient, which was calculated to be 0.9236, indicating that 92.36% of the variability in the response could be predicted by the model. Furthermore, the predicted polysaccharides mass by the final quadratic model, along with the corresponding values observed, were given in Table 1.

The polynomial model for polysaccharides extraction was regressed by considering only the significant term and shown as below:

$$\text{Poly324 (g)} = -5.501(\pm 0.980) + 0.772(\pm 0.131)(\text{pH}) - 0.428(\pm 0.075)(\text{H}) - 0.034(\pm 0.005)(\text{pH})^2 - 0.056(\pm 0.005)(\text{H})^2 + 0.043(\pm 0.005)(\text{pH}) * (\text{H}) + 0.002(\pm 0.001)(\text{pH}) * (\text{T})$$

(T): Temperature (°C); (H): Time (Hours)

From the results of table 1, we can construct three-dimensional graphics, relating the variables in question (Figure 1). The optimum conditions for extraction of polysaccharides can be obtained from the optimal point coordinator of the surface, and are 13, 92°C and 3h to pH, temperature and time respectively.

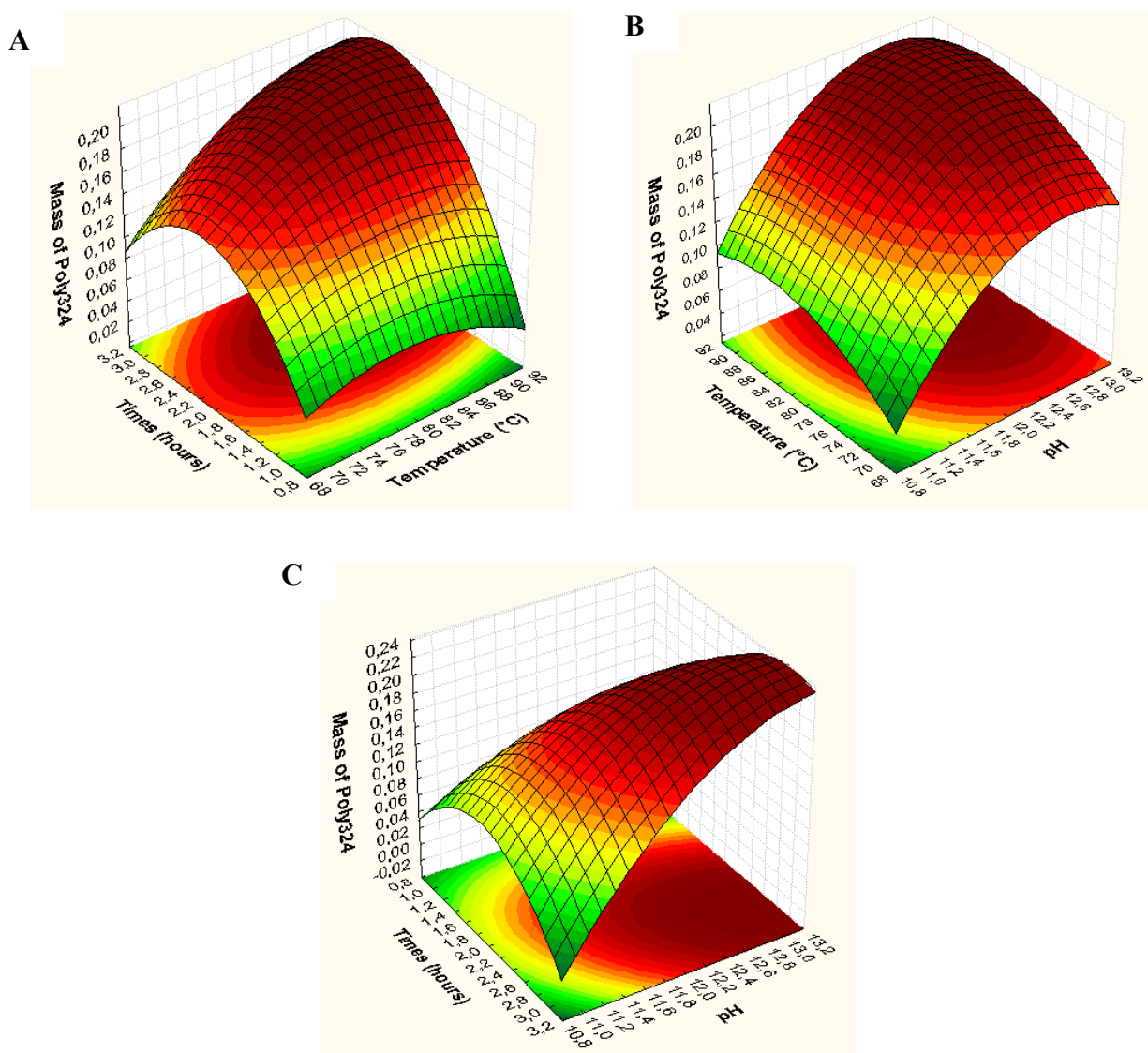


Figure 1. Response surface plots for optimization of the extraction of polysaccharide poly324. (A): Temperature and Times fixed level of pH. (B): pH and Temperature fixed level of Times. (C): pH and Times fixed level of Temperature.

3.2 Characterization of poly324 by spectroscopic analysis and HPLC

The analysis of the infrared spectra of biomass obtained was performed to identify the main functional groups. Figure 2 shows a signal at 3268 cm^{-1} assigned to the hydroxyl stretching vibration of the polysaccharide. The FT-IR spectrum showed peaks at 1049 cm^{-1} , which is due to the stretching of anomeric C_1H group vibration (Yu, 2002) suggested for β -D-glucan and α -D-mannan gum pellets (Figure 2).

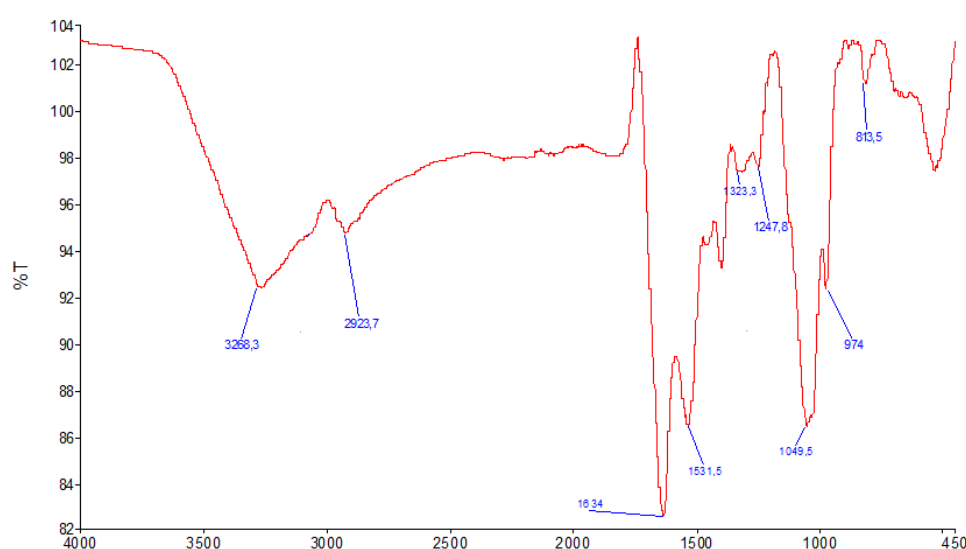


Figure 2: FT-IR spectrum of poly324 produced from *Aureobasidium pullulans*. The dried polymer was grinded with potassium bromide (KBr) powder and pressed into pellets for FT-IR spectral measurement in the frequency range of $4000 - 500\text{ cm}^{-1}$, with 20 scans.

Absorption at 1247 cm^{-1} may be related to the vibration of the axial deformation of the C-O-C group present in a ring of six carbon atoms, characteristic of β -D-glucans (Luo et al, 2008; Yu, 2002) and α -D-mannan (Machová et al, 2014). It is also important to note that the spectra showed absorption peak at 974 cm^{-1} that is an indicative of β -linked glycosidic bonds. The intense absorption in the region between 1634 cm^{-1} is related to the presence of functional groups corresponding to proteins, confirmed by the

technique described by Bradford (1956). The MWn and DPn of the polysaccharide were 120.2 and 742 kDa, respectively.

The ^{13}C and gHSQC NMR spectra (Figure 3) reveal signals characteristic of polysaccharides.

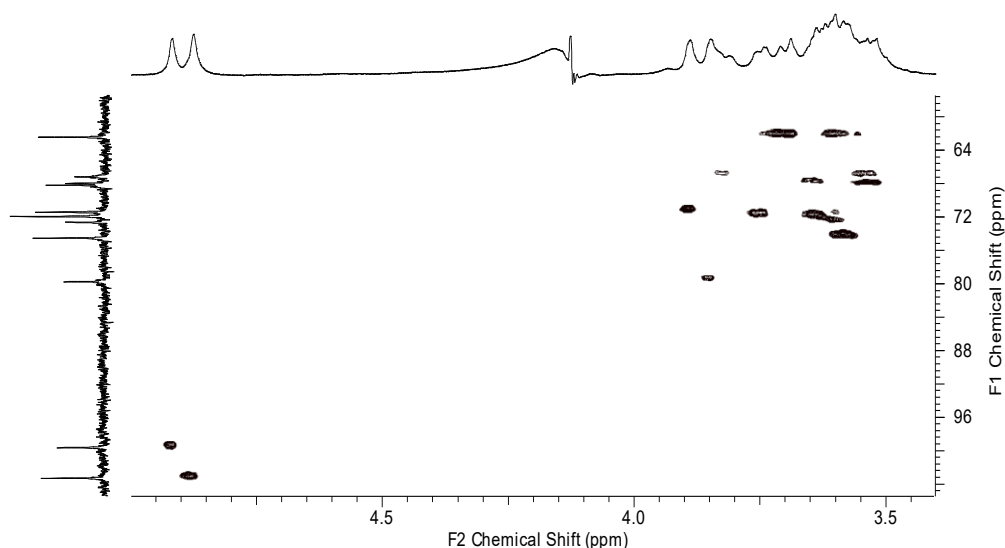


Figure 3. gHSQC spectrum of poly324 from *Aureobasidium pullulans*. gHSQC experiments were performed in $\text{D}_2\text{O}/\text{DMSO}$ (6:1) at 343K, operating at 11.7 T, observing ^1H at 500 MHz and ^{13}C at 125 MHz (chemical shifts are expressed in δ ppm).

The Table 3 shows the signals of the carbons and hydrogens bonds present in poly324.

Table 3. Chemical shifts (ppm) of ^1H and ^{13}C NMR signals for poly324

Sugar residue	$^1\text{H}/^{13}\text{C}$					
	1	2	3	4	5	6a 6b
$\rightarrow 3\text{)}\text{-}\beta\text{-D-Glc-(1}\rightarrow$	4.87 103.0	3.89 71.9	3.86 80.0	3.54 68.2	3.64 72.0	3.70 62.4 3.60
$\rightarrow 6\text{)}\text{-}\alpha\text{-D-Man-(1}\rightarrow$	4.94 99.7	3.58 72.6	3.61 74.5	3.56 67.2	3.75 71.4	3.82 67.9 3.54

The six signals at a $\delta\text{C}/\text{H}$ 103.0/4.87, 71.9/3.89, 80.0/3.86, 68.2/3.54, 72/3.64 and 62.4/3.70 and 3.60 were characteristic of a $\beta\text{-(1}\rightarrow 3\text{)-D-glucan}$ and attributed to C-1,

C-2, C-3, C-4, C-5, and C-6, respectively, by comparison with data from the literature (Wu, 2005; Majumder et al., 2009). The six signals at a δC 99.7/4.94, 72.6/3.58, 74.5/3.61, 67.2/3.56, 71.4/3.75 and 67.9/3.65 and 3.54 were characteristic of a α -(1 $\rightarrow$ 6)-D-mannan and attributed to C-1, C-2, C-3, C-4, C-5, and C-6, respectively, by comparison with data from the literature (Yu, 2002; Luo et al., 2008; Ahmad et al., 2010; Wu, 2005; Majumder et al., 2009).

The results of the HPLC analysis obtained after hydrolysis of poly324 is shown in Figure 4. The retention time of standards of glucose and mannose were equivalent to 13.48 and 14.33 min, respectively. The monomer composition demonstrates the presence of mannose and glucose after hydrolysis with TFA. Quantitative analysis of individual peaks associated spectrometric analysis to suggest that the majority of poly324 consists of β -(1 $\rightarrow$ 3)-D-Glucan (60%) followed by α -(1 $\rightarrow$ 6)-D-mannan (40%).

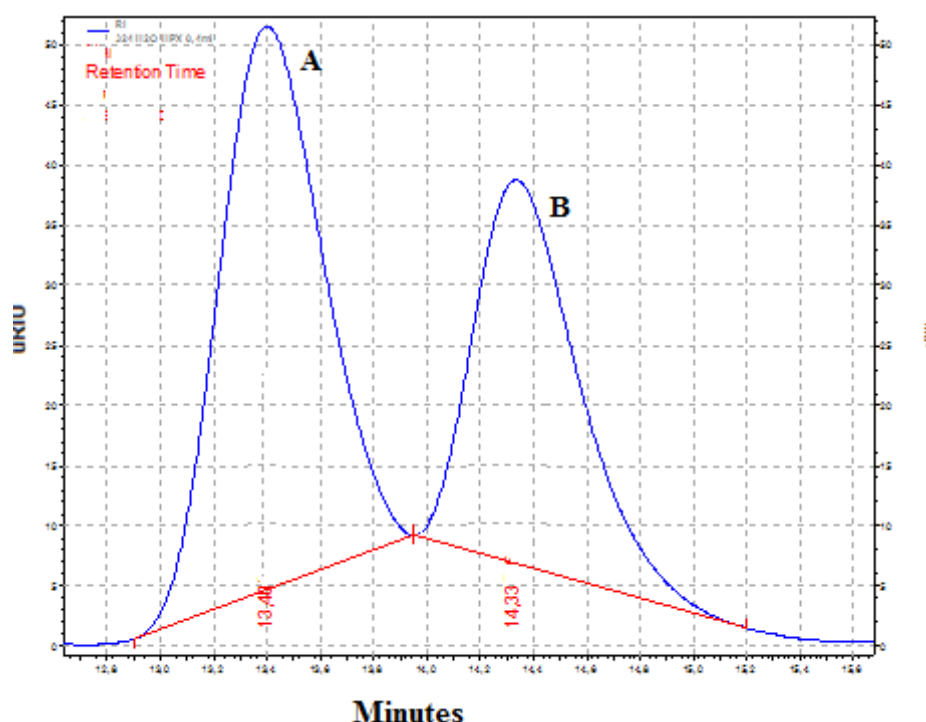


Figure 4. HPLC analysis of Poly324 after acid hydrolysis with TFA. (A): Glucose. (B): Mannose. The injection volume was 12 μ L. The mobile phase was acetonitrile (MeCN):deionized H_2O (82 : 18) at a flow rate of 0.4 mL min $^{-1}$, and the column temperature was maintained at 25°C.

3.3 Antinociceptive effect of poly324

The writhing test was initially used to evaluate the antinociceptive effect of the poly324. Intraperitoneal injection of poly324 (3-27mg/kg), 30 min before of the acetic acid administration, produced a significant ($p < 0.05$) and dose-related inhibition of the writhing response (Figure 5). Morphine (5 mg/kg), a standard analgesic used as positive control, also produced a significant inhibition of acetic acid-induced writhing response ($p < 0.0001$).

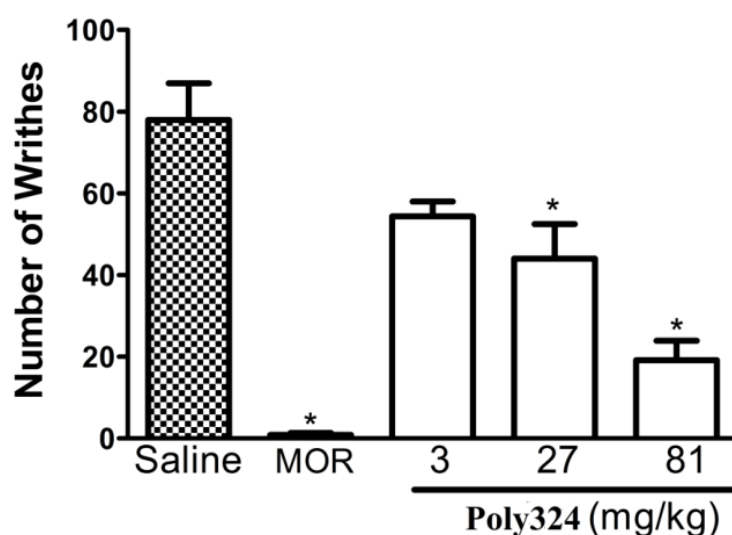


Figure 5. Effects of treatment with poly324 on acid acetic-induced writhing response. Mice were treated with poly324 (3-27 mg/kg, i.p.) or saline (control group) 30 min before acetic acid. Morphine (MOR; 5mg/kg) was the reference drug. Results are presented as means $\pm$ S.E.M of 6 mice per group. *Significantly different from control group ($p < 0.05$), ANOVA followed by Bonferroni's test.

The effect of poly324 on the formalin-induced orofacial nociception is showed in the Figure 6.

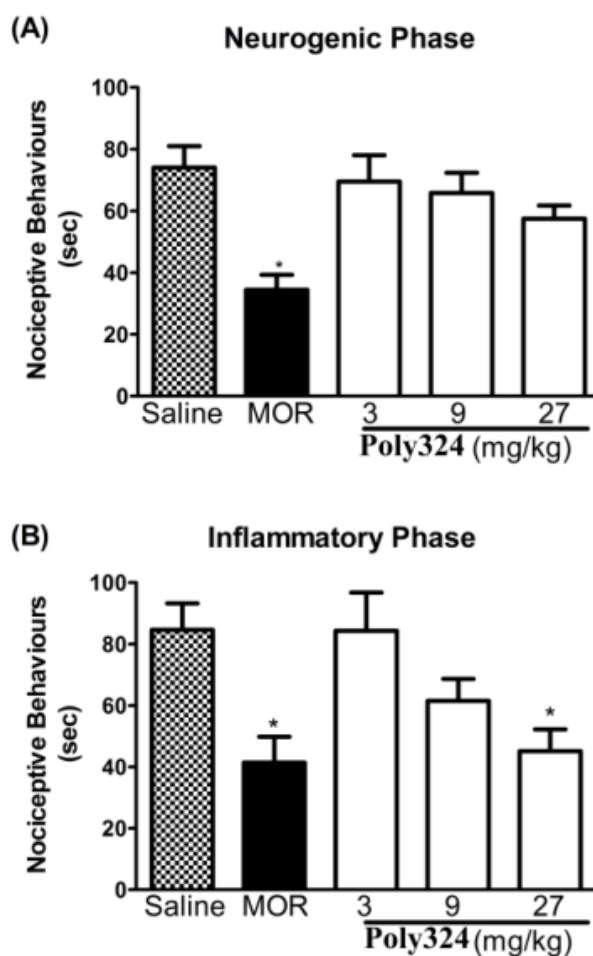


Figure 6. Effects of treatment with poly324 on the formalin-induced orofacial nociception. Panels (A) and (B) represent effects of poly324 on the neurogenic and inflammatory phases of formalin test, respectively. Mice were treated with poly324 (3-27 mg/kg, i.p.) or saline (control group) 30 min before of the injection 2% formalin in the right upper lip. Morphine (MOR 5 mg/kg) was the reference drug. Results are presented as means $\pm$ SEM of 6-7 mice per group. *Significantly different from control group ($p < 0.05$), ANOVA followed by Bonferroni's test.

The treatment with poly324 (27 mg/kg) 30 min before of the formalin injection showed activity only in the inflammatory phase of test ($p < 0.005$). Morphine (5 mg/kg) caused antinociceptive effects in both the neurogenic and inflammatory phases of the formalin test ($p < 0.005$).

The treatment with poly324 (9, 27 and 81 mg/kg) did not alter the latency response to the immersion test. In contrast, the administration of morphine (5mg/kg) caused a significant increase in the latency response ($p < 0.001$) (Figure 6).

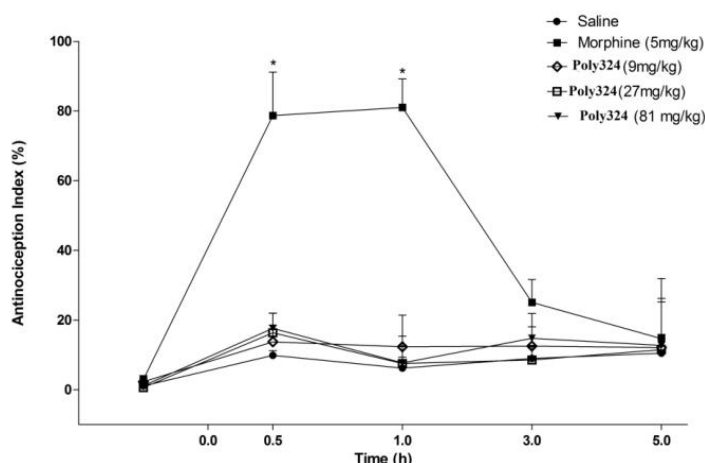


Figure 7. Effects of the poly324 treatment on tail immersion test. The figure show data of tail withdrawal reflex latencies represented as antinociception %. Thermal nociceptive threshold was evaluated before and up 5h following administration of poly324 (9, 27 and 81 mg/kg) or saline. Morphine (5 mg/kg, was the reference drug. Data are expressed as means $\pm$ S.E.M.; $n = 6$ mice per group. *Significantly different from control group ($p < 0.001$). Two-way ANOVA followed by the Bonferroni's test.

4. Discussion

Several polysaccharides have been isolated from several species of fungi and has shown various biological activities (Yoshida et al, 2005; Ishibashi et al, 2004; Luo et al, 2008). Particularly D-mannans and D-glucans have been responsible for presenting antioxidant, antinociceptive, immunomodulatory and anticoagulant activity. Among the isolated D-mannans and D-glucans, those that link the type β -(1 $\rightarrow$ 3) and α -(1 $\rightarrow$ 6) seem to be most active (El Enshasy & Hatti-Kaul, 2013; Baggio et al., 2012; Zhang et al., 2007). These polysaccharides may vary according to the degree of polymerization and

molecular weight. This differentiation gives different powers relating besides several biological activity (Manners, 1976). The isolated Poly324 the *Aerobasidium pullulan* CCMB 324 is composed of a mixture containing 60% β -(1 $\rightarrow$ 3)-D-glucan and 40% α -(1 $\rightarrow$ 6)-D-mannan. D-mannans and D-glucans are components of cell wall of yeasts and other fungi, and have different compositions, and can be soluble or insoluble. The cell wall polysaccharides that have high solubility in alkali have attracted great interest because they can be administered intravenously, and have better oral absorption, thus, may have greater potential for use as drugs (Wang & Zhang, 2009; Bohn and BeMille, 1995; Chakraborty, 2006).

Due to the variability of extraction described by several authors, this study applied a statistical methodology in order to find the optimum conditions for extraction of Poly324, and the pH, temperature and extraction time elected for the study variables. The yield was higher than that described by Dong (2008) and Li (2012), which, under the proposed conditions in their studies, they obtained a yield of 16% and 10% respectively, while satisfying the conditions proposed in this study were obtained about 26% yield.

There are few reports in the literature on the antinociceptive activity of mannans isolated, but Ruthes *et al.* (2013) describes activity for antinociceptive Fucomannogalactan. Thus, the poly324 produced by the *Aureobasidium pullulans*, has the potential to inhibit nociception, which was confirmed in studies discussed in the same study.

Fungi differents produce a wide variety of D-glucan and D-mannan with vast differences in proportion and arrangement of their glycosidic linkages. Theses structural differences alter biological activity and potency (El Enshasy & Hatti- Kaul, 2013; Noss

et al, 2013; Machová et al, 2014). Therefore, we evaluated the antinociceptive effect of poly324 produced by *Aureobasidium pullulans* on different models of nociception.

The acetic acid-induced writhing test is a screening tool for assessment of the analgesic properties of new drugs (Collier et al., 1968). Therefore, the ability of inhibition of acetic acid-induced writhing response by the treatment with poly324 suggests the first sign of its antinociceptive action.

The formalin orofacial nociception is a well-established model to investigate the analgesic potential of substances on facial pain. The injection of formalin in the orofacial region induces a biphasic nociception (Luccarine et al., 2006). In the first phase, the neurogenic pain is caused by direct activation of the nociceptive fibers, while the second phase is characterized as inflammatory pain and associated with the release of mediators such as histamine, serotonin, bradykinin and prostaglandins. In the present study, the results demonstrate that the administration of poly324 reduced the nociceptive responses in the second phase of the formalin test. These results suggest that antinociceptive effect of poly324 probably is due the inhibition of the inflammatory mediators, since it suppressed only the second phase of nociceptive response.

The thermal hypernociception analyzed in the immersion test in mice is considered to involve spinal nociceptive mechanisms, and used for the screening of central analgesic drugs (Le Bars et al., 2001). The fact that the treatment with poly324 does not increase the reaction time when compared to control group (saline) in the immersion test, indicate that poly324 does not present spinal antinociceptive mechanisms.

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CAPÍTULO 2

Optimization for polysaccharides extraction from *Pseudozyma sp*, structural characterization, and antinociceptive activity

Optimization for polysaccharides extraction from *Pseudozyma sp*, structural characterization, anti-inflammatory and antinociceptive activity

Abstract

D-Glucans and D-mannans are polymers of glucose and mannose that are widely distributed throughout the biosphere. Specifically, β -D-glucan and α -D-mannans found in the cell walls of plants, bacteria and fungi are well known to possess significant and wide biological and physiological activities, including antitumor, antioxidant, anticoagulant, antifungal and immunomodulating activity. We report a sequential optimization strategy for polysaccharides production by *Pseudozyma sp.* (CCMB 306), isolated in the Brazilian semi-arid region, through statistically designed experiments. The polysaccharides were characterized by FT-IR, ^1H and ^{13}C NMR spectroscopic techniques and HPLC; and antinociceptive action was examined in experimental protocols on mice. The best yield was of 25.6% obtained after 2,3h, of extraction in a temperature of 84°C and pH 14. The analysis results of NMR and FT-IR show that the product obtained is suggest that the majority of poly306 consists of β -(1 $\rightarrow$ 3)-D-Glucan (55%) followed by α -(1 $\rightarrow$ 6)-D-Manan (45%) and degree of polymerization of 837. In addition, the antinociceptive effect was evaluated using different experimental tests (acetic acid-induced writhing test, formalin test and tail immersion test). The Poly306 showed a potent peripheral antinociceptive effect, possibly by the inhibition of inflammatory mediators.

Keywords: polysaccharides, optimization, response surface methodology, yeast, antinociceptive activity.

1. Introduction

D-Glucans and D-mannans are polymers of glucose and mannose that are widely distributed throughout the biosphere. Specifically, β -D-glucan and α -D-manans found in the cell walls of plants, bacteria and fungi are well known to possess significant and wide biological and physiological activities, including antitumor (Zhang *et al*, 2007), antinociceptive (Baggio *et al*, 2012), inflammatory (Ruthes *et al*, 2013), antioxidant (Machova *et al*, 2014b), anticoagulant (Vasconcelos *et al*, 2013), antifungal and immunomodulating activity (El Enshasy and Hatti-Kaul, 2013).

Some studies have shown that those extracted molecules of fungal can have other effects, such as anti-inflammatory and antinociceptive activity (Le Bars *et al*, 2001). These can occur due to a decrease in the concentration of interleukins, interferon and tumor necrosis factor and inhibition of inflammatory cells, such as lymphocytes and macrophages. However, it is not possible to attribute a relation between structure and activity because most of the investigations were carried out with crude polysaccharide extracts (Baggio *et al*, 2010; Ruthes *et al*, 2013).

Among nociception models, the abdominal contortions test induced by acetic acid is described as typical for measuring visceral inflammatory pain. Although with a low specificity, the method is sensitive, having been used as a screening tool for evaluating analgesic or anti-inflammatory properties of novel agents (Ruthes *et al*, 2013; Carbonero, 2008).

We report a sequential optimization strategy for polysaccharides production by *Pseudozyma sp.* (CCMB 306), isolated in the Brazilian semi-arid region, through statistically designed experiments. Box-Behnken design was used to describe the nature of the response surface in the experimental region, to search optimal medium composition for maximizing polysaccharides yield. The polysaccharides (poly306) were

characterized by FT-IR, ^1H and ^{13}C NMR spectroscopic techniques and HPLC; antinociceptive action was examined in experimental protocols on mice.

2. Materials and Methods

2.1 Microorganism

The *Pseudozyma sp* (CCMB 306) was obtained from the Collection of Cultures of Microorganisms of Bahia (CCMB, Feira de Santana, Brazil). The microorganism was maintained on YM agar at 4 °C in an incubator (IGO 150 Cell Life – Jouan).

2.2 Culture conditions of *Pseudozyma sp* CCMB 306

The strain of *Pseudozyma sp*. CCMB 306 was incubated in a YM culture medium for activation of 4 days at 30 °C. Cultivation in liquid media was carried out in 250 mL Erlenmeyer flasks containing 100 mL of (g/L): D-glucose, 15; $\text{KH}_2(\text{PO}_4) \cdot 3\text{H}_2\text{O}$, 2.0; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5; $(\text{NH}_4)_2\text{SO}_4 \cdot 7\text{H}_2\text{O}$, 2.0, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.1, pH 5.0.. Flasks were inoculated with ten milliliters of cell suspension contend 10^9 UFC of microorganism. The incubation was performed at 120 rpm for 96 hours in a rotary shaker. After the fermentation, the liquid culture medium, on which microorganism. was grown, was filtered and centrifuged (Centrifuge 5804R –Eppendorf, São Paulo, Brazil) at 8,000 g for 15 min at 4°C, and the precipitate, containing the biomass from microorganism, was used to extract the biopolymers (Dong et al. 2008).

2.3 Extraction of yeast polysaccharides (poly306)

The powder of dry biomass (0,5g) was extracted several times with 20 volumes of sodium hydroxide solutions at different pHs (11, 12 and 13), temperatures (70, 80 and 90°C) with various extraction times (60, 120, and 180 min.) The suspension was

centrifuged at $10.000\times g$ for 10 min. It was added to the supernatant three times of the volume of absolute ethanol, stirred vigorously and left overnight at 4°C . The precipitated polysaccharides were centrifuged at $8000\times g$ for 30 min and the supernatant discarded. The precipitate of crude polysaccharides was dried at 55°C to a constant weight and the polymer was weighted by a scale (Sartorius ALC-110.4, Germany). The polysaccharides were determined by phenol– sulfuric acid method (Dubois, et.al, 1956). The product yield was measured at the grams (g) of polysaccharides per unit mass of biomass yeast.

2.4 Experimental design

The temperature ($^{\circ}\text{C}$), pH and extraction time (minutes) were monitored using Box-Behnken experimental design to polysaccharides extraction. Three repetitions of the central point (c) to a total of nine experiments (Table 1) were performed to estimate the possible pure error, and the experimental treatments were varied randomly to detect the presence of possible systematic errors. The temperature was studied at three levels, ranging from 70 to 90°C , and the time was studied at three time points, ranging from 60 to 180 min, And pH was studied at three levels ranging from 11 to 13 (Table 1). All the calculations and graphics in this work were performed using the software Statistica® version 7.0, and including the analysis of variance (ANOVA) for the responses of this study.

2.5 The number-average molecular weight (MW_n) and degree of polymerization (DP_n)

The MW_n and DP_n of the polysaccharides were determined by the measurement of the reducing sugar value using 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959)

and the measurement of total carbohydrates using the phenol–sulfuric acid method (Dubois et al, 1956); $DP_n = [(total\ carbohydrates\ in\ \mu g\ of\ D-glucose)/(reducing\ value\ in\ \mu g\ of\ maltose)] \times 1.9$; $MW_n = [(DP_n) \times 162] + 18$ (Vettori, Franchetti, Contiero, 2012).

2.6. Determination of the protein concentration

Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin (BSA) as a standard.

2.7. Characterization of poly306 by spectroscopic analysis

The polysaccharide was characterized using a Fourier Transform Infrared Spectrometer (PE IR Spectrum AsciiPeds 1.60, Spectrum One FT-IR Spectrometer). The dried polymer was grinded with potassium bromide (KBr) powder and pressed into pellets for FT-IR spectral measurement in the frequency range of $4000-500\text{ cm}^{-1}$, with 20 scans. In NMR spectroscopic analysis, all spectra were recorded at 343 K on a Varian Inova 500 spectrometer operating at 11.7 T, observing ^1H at 500 MHz and ^{13}C at 125 MHz, and using direct probe. The samples was dissolved in $\text{D}_2\text{O}/\text{DMSO}$ (6:1) and transferred to NMR tube. DMSO was used as a chemical shift reference. D_2O (99.9%) and DMSO (99.9%) were from Cambridge Isotope Laboratories, Inc. (USA).

2.8. Monosaccharide Composition of polysaccharide

The polysaccharide (poly306) (20 mg) was hydrolyzed with 2 M TFA at 100°C for 8 h, followed by evaporation (Ruthes *et al*, 2013). Filtered liquids were analyzed by high-performance liquid chromatography (HPLC) using Amines® HPX-87H (300 mm x 7.8 mm i.d.) ($9\mu\text{m}$) column (Merck, Darmstadt, Germany) and refractive-index

detection; the injection volume was 12 μL . The mobile phase was acetonitrile (MeCN): deionized H_2O (82 : 18) at a flow rate of 0.4 mL min^{-1} , and the column temperature was maintained at 25°C . For peak identification, an external standard of glucose and mannose was used. Determination of the monosaccharide composition was done in two independent experiments with consistent results.

2.9 Pharmacological studies

2.9.1 Animals

Male Swiss mice (25–30 g) obtained from the State University of Feira de Santana (UEFS), were housed in a temperature-controlled rooms ($22 \pm 2^\circ\text{C}$), under 12:12 h light-dark cycle, with access to water and food *ad libitum* until use. Animal care and handling procedures were conducted in accordance with the guidelines of International Association for Study of Pain on the use of animals in pain research (Zimmermann, 1983) and performed after protocol approval by the Animal Ethics Committee of UEFS. All efforts were made to minimize the number of animals used and their discomfort.

2.9.2 Drugs and dilutions

The following materials were obtained from the sources indicated. Indomethacin was purchased from Sigma Chemical Company (St. Louis, MO, USA), carrageenan from FMC Corporation (Philadelphia, PA), diazepam from Cristália (São Paulo, Brazil) and formaldehyde and acetic acid from Merck (Darmstadt, Germany). Indomethacin was dissolved in Tris HCl 0.1 M pH 8.0 plus physiological saline. Remaining drugs were dissolved in physiological saline.

2.9.3 Writing test

Mice were pretreated with poly306 (3, 10, and 30 mg/kg), indomethacin (10mg/kg; reference drug) or saline (control group) by intraperitoneal (i.p.) injection 30 min before i.p. acetic acid administration (0.8 % v/v, 10 ml/kg). After the challenge, the mice were individually placed in a large glass cylinder and the intensity of nociceptive behavior was quantified by counting the total number of writhes occurring between 0 and 30 minutes after the stimulus injection (Collier et al., 1968). The writing response consists of a contraction of abdominal muscle together with stretching of the hind limbs. The antinociceptive activity was expressed as the writing scores over 30 min.

2.9.4 Formalin test

Mice were pretreated with Poly306 (10, 30 and 90 mg/kg), indomethacin (10mg/kg; reference drug) or saline (control group) by intraperitoneal (i.p.) injection 30 min before injection under surface of the right hind paw of 20 µl of formalin 2.5% (0,92% formaldehyde) made up in saline. Following injection, the mice were observed from 0 to 5 minutes (early phase) and from 15 to 30 minutes (late phase). The nociception score was determined by counting the time that the animal spent licking, biting and shaking the injected limb during the observation time (Dubuisson & Dennis, 1977).

2.9.5 Open field test

Mice were treated with diazepam (10mg/kg; reference drug), Poly306 (90mg/kg) or saline (control group) and 30 min afterward were placed individually in a wooden box (40x60x50 cm) with the floor divided into 12 squares. The number of squares crossed with the four paws was measured for a period of 3 min.

2.9.6 Paw oedema test

The mice were treated with Poly306 (30 and 90 mg/kg) or saline (control group) by intraperitoneal (i.p.) injection 30 min before intraplantar administration of carrageenan (100µg/paw). Dexamethasone (2mg/kg, s.c) was reference drug. The volume of the mice paw was measured with plethysmometer (Ugo Basile, Italy) before (V_o) the injection of carrageenan and 3h after (V_t), as described previously (Winter et al., 1962). The amount of paw swelling was determined for each mouse and the difference between V_t and V_o was taken as the oedema value (oedema mm³/paw).

2.9.7 Statistical analysis

The data are presented as means $\pm$ standard error of the mean (SEM) of measurements made on 6-7 animals in each group. Statistical comparisons between groups were made using one-way analysis of variance (one-way ANOVA) followed by the Tukey's test. Statistical differences were considered to be significant at $P < 0.05$.

3. Results

3.1 Extraction of polysaccharide poly306

A 15-run Box-Behnken design with three factor, including three replicates at the centre point, was used for fitting a second-order response surface. The three centre point runs were added to provide as a measure of process stability and evaluate inherent variability. The considerable variation in the polysaccharide yield from the *Pseudozyma* sp CCMB 306 under different conditions was shown in Table 1.

Table 1. Box-Behnken design with experimental and predicted values mass of polysaccharide poly306 (g)

Run	pH	Temperature (°C)	Time (h)	Mass of poly306 (g)		
				Predicted	Experimental	Difference
1	11 (-1)	90 (+1)	2 (0)	0.047000	0.050500	-0.003500
2	12 (0)	90 (+1)	1 (-1)	0.044000	0.044625	-0.000625
3	12 (0)	90 (+1)	3 (+1)	0.054000	0.060125	-0.006125
4	13 (+1)	90 (+1)	2 (0)	0.120000	0.109750	0.010250
5	11 (-1)	80 (0)	1 (-1)	0.035000	0.030875	0.004125
6	11 (-1)	80 (0)	3 (+1)	0.042000	0.032375	0.009625
7C	12 (0)	80 (0)	2 (0)	0.110000	0.098333	0.011667
8C	12 (0)	80 (0)	2 (0)	0.095000	0.098333	-0.003333
9C	12 (0)	80 (0)	2 (0)	0.090000	0.098333	-0.008333
10	13 (+1)	80 (0)	1 (-1)	0.060000	0.069625	-0.009625
11	13 (+1)	80 (0)	3 (+1)	0.092000	0.096125	-0.004125
12	11 (-1)	70 (-1)	2 (0)	0.039000	0.049250	-0.010250
13	12 (0)	70 (-1)	1 (-1)	0.043000	0.036875	0.006125
14	12 (0)	70 (-1)	3 (+1)	0.050000	0.049375	0.000625
15	13 (+1)	70 (-1)	2 (0)	0.096000	0.092500	0.003500

(C): central point; coded values are presented in the parentheses

The model adequacy was checked by an F-test and the determination coefficient R^2 . The analysis of variance (Table 2) showed that this regression model was highly significant ($P < 0.05$) with F-value of 8,281. The value of 1.63 for lack of fit implies that it is not significant comparing to the pure error.

Table 2. Analysis of variances in the regression model for optimization of poly306

Source of variations	SS	Df	MS	Fcal	Ftab	Rsqr
Regression	0.011126	9	0.001236	8.281	3.31	0.9371
Residual	0.000746	5	0.000149			
Lack of fit	0.000530	3	0.000177	1.630	9.16	
Pure error	0.000217	2	0.000108			
Total error	0.011872	14				

SS - Sum of square; Df - Degree of freedom; MS - Mean square; Fcal - calculated F value; Ftab - tabulated F value; Rsqr - R-square.

The fitness of the model was further confirmed by a satisfactory value of determination coefficient, which was calculated to be 0.9371, indicating that 93.71% of

the variability in the response could be predicted by the model. Furthermore, the predicted poly306 mass by the final quadratic model, along with the corresponding values observed, were given in Table 1.

The polynomial model for poly306 extraction was regressed by considering only the significant term sand shown as below:

$$\text{Poly306 (g)} = -1.8465(\pm 1.0206) + 0.1411(\pm 0.1369)(\text{pH}) - 0.0637(\pm 0.0782)(\text{H}) - 0.0067(\pm 0.0054)(\text{pH})^2 - 0.0344(\pm 0.0054)(\text{H})^2 + 0.0004(\pm 0.0005)(\text{pH}) * (\text{H}) + 0.0063(\pm 0.0052)(\text{pH}) * (\text{H})$$

(T): Temperature (°C); (H): Time (Hours)

From the results of Table 1, it is shown three-dimensional graphics, relating the variables in question (Figure 1). The optimum conditions for extraction of poly306 can be obtained from the optimal point of the model, and are 14, 84°C and 2.3h to pH, temperature and time respectively.

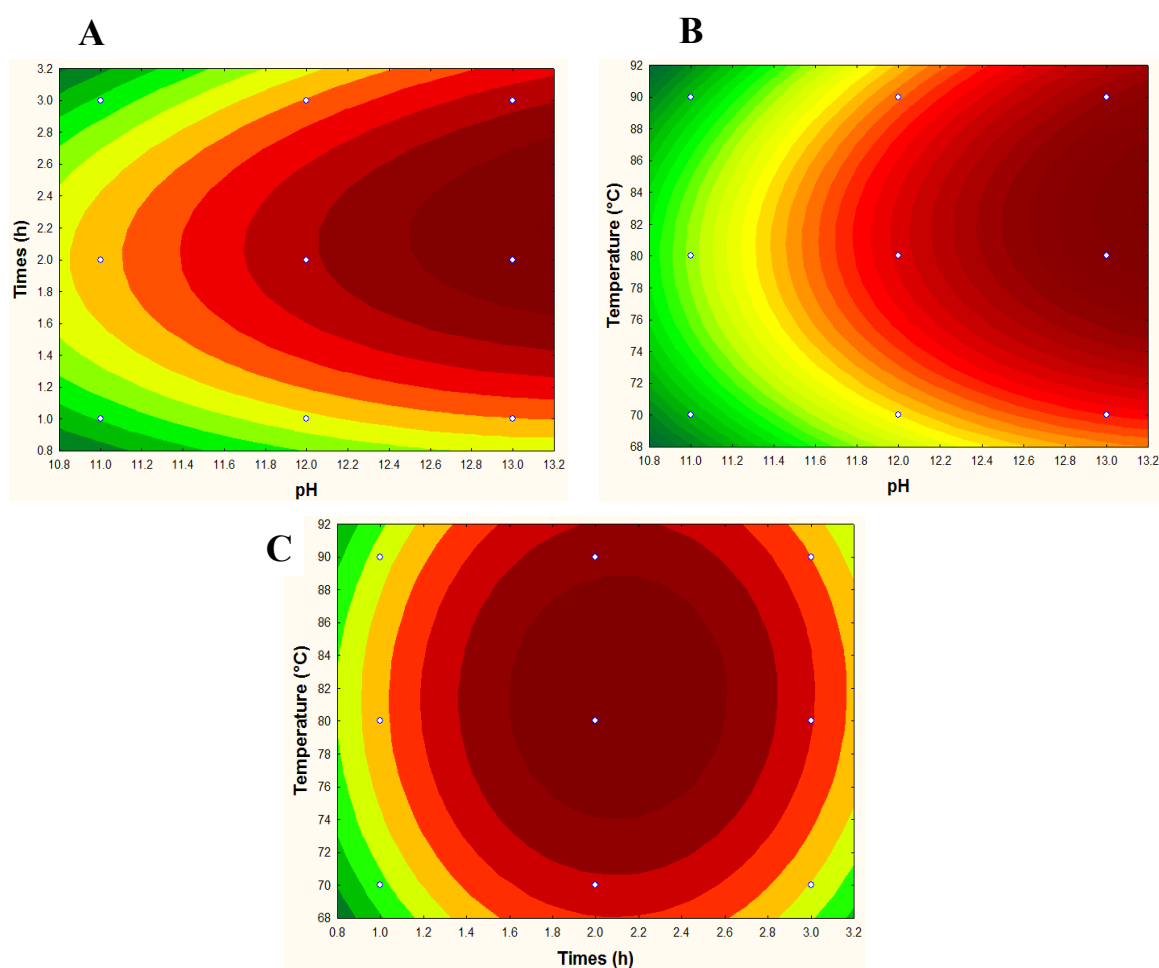


Figure 1. Contour curve plots for the extraction of polysaccharide poly306. (A): pH and Time fixed level of Temperature. (B): pH and Temperature fixed level of Time. (C): Time and Temperature fixed level of pH.

3.2 Characterization of poly306 by spectroscopic analysis and HPLC

The analysis of the infrared spectra of biomass obtained was performed to identify the main functional groups. Figure 2 shows a signal at 3265 cm^{-1} assigned to the hydroxyl stretching vibration of the polysaccharide. The FT-IR spectrum showed peaks at 1055 cm^{-1} , which is due to the stretching of anomeric C_1H group vibration (Yu, 2002) suggested for β -D-glucan gum pellets (Figure 2).

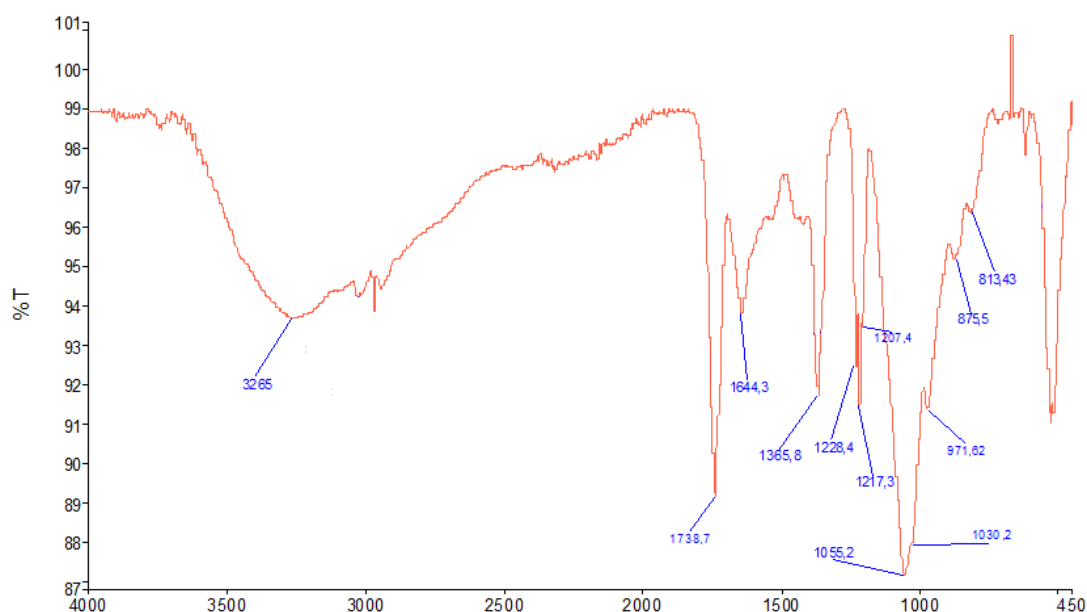


Figure 2: FT-IR spectrum of poly306 produced from *Pseudozyma* sp. The dried polymer was grinded with potassium bromide (KBr) powder and pressed into pellets for FT-IR spectral measurement in the frequency range of 4000 – 500 cm^{-1} , with 20 scans.

Absorption at 1055cm^{-1} may be related to the vibration of the axial deformation of the C-O-C group present in a ring of six carbon atoms, characteristic of β -D-glucan (Luo et al, 2008; Yu, 2002) and α -D-mannan (Machová et al, 2014). It is also important to note that the spectra showed absorption peak at 971 cm^{-1} that is an indicative of β -linked glycosidic bonds. The intense absorption in the region between 1738cm^{-1} is related to the presence of functional groups corresponding to proteins, confirmed by the technique described by Bradford (1976). The MWn and DPn of the polysaccharides were 135.6kDa and 837 respectively.

The gHSQC spectrum of poly306 (Figure 3) reveals signals characteristic of polysaccharide.

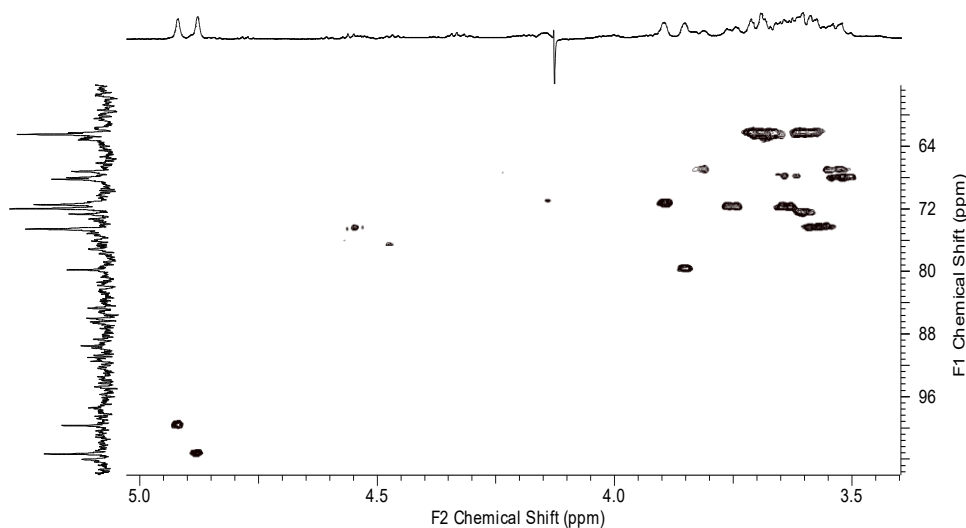


Figure 3. gHSQC spectrum of poly306 from *Pseudozyma sp.* gHSQC experiments were performed in D₂O/ DMSO (6:1) at 343K, operating at 11.7 T, observing ¹H at 500 MHz and ¹³C at 125 MHz (chemical shifts are expressed in δ ppm).

The Table 3 shows the signals of the carbons and hydrogens bonds present in poly306.

Table 3. Chemical shifts (ppm) of ¹H and ¹³C NMR signals for poly306

Sugar residue	¹ H/ ¹³ C						
	1	2	3	4	5	6a	6b
→3)-β-D-Glc-(1→	4.87	3.90	3.85	3.53	3.63	3.69	3.60
	103.3	72.7	80.0	68.2	71.4	62.4	
→6)-α-D-Man-(1→	4.92	3.59	3.58	3.54	3.76	3.80	3.62
	99.7	73.3	74.5	68	71.9	67.2	

The six signals at a δ C/H 103.3/4.87, 72.7/3.90, 80.0/3.85, 68.2/3.53, 71.4/3.63 and 62.4/3.69 and 3.60 were characteristic of a β -(1→3)-D-glucan and attributed to C-1, C-2, C-3, C-4, C-5, and C-6, respectively, by comparison with data from the literature (Wu, 2005; Majumder et al, 2009). The six signals at a δ C 99.7/4.92, 73.3/3.59, 74.5/3.58, 68.0/3.54, 71.9/3.76 and 67.2/3.64 and 6.62 were characteristic of a α -(1→6)-D-mannan and attributed to C-1, C-2, C-3, C-4, C-5, and C-6, respectively, by

comparison with data from the literature (Yu, 2002; Luo et al, 2008; Ahmad et al, 2010; Wu, 2005; Majumder et al, 2009; Smiderle et al, 2008).

The results of the HPLC analysis obtained after hydrolysis of poly306 is shown in Figure 4. The retention time standard of glucose and mannose were equivalent to 13.39 and 14.30 min respectively. The monomer composition demonstrates the presence of mannose and glucose after hydrolysis with TFA. Quantitative analysis of individual peaks associated spectrometric analysis to suggest that the majority of poly306 consists of β -(1 $\rightarrow$ 3)-D-Glucan (55%) followed by α -(1 $\rightarrow$ 6)-D-Mannan (45%).

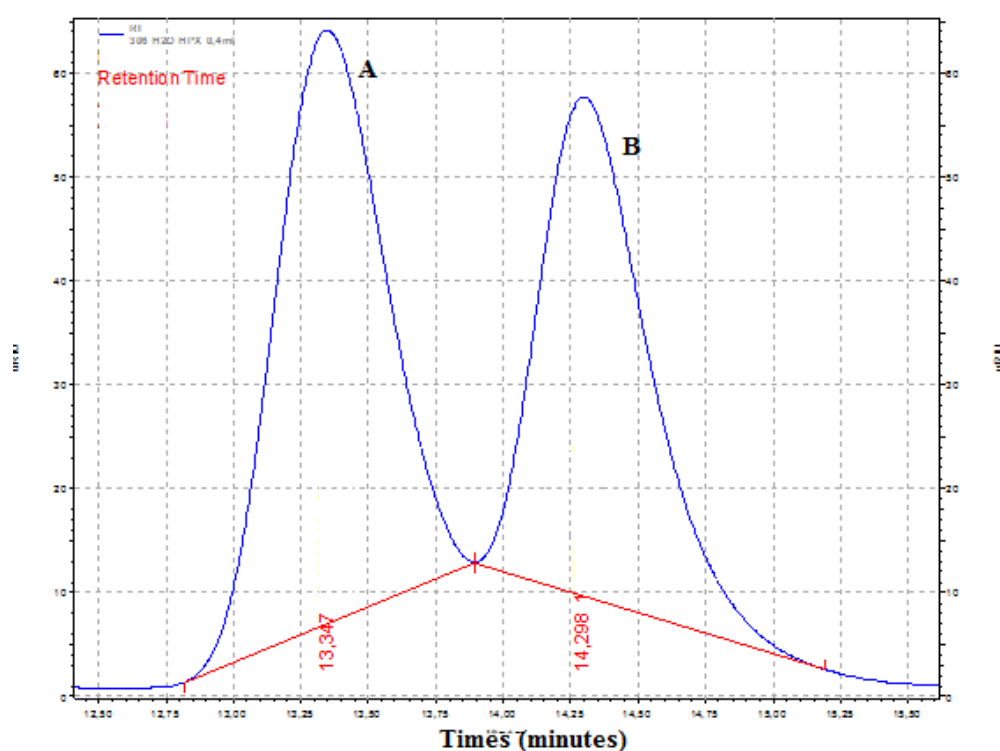


Figure 4. HPLC analysis of Poly306 after acid hydrolysis with TFA. (A): Glucose. (B): Mannose. The injection volume was 12 μ L. The mobile phase was acetonitrile (MeCN): deionized H₂O (82 : 18) at a flow rate of 0.4 mL min⁻¹, and the column temperature was maintained at 25°C.

3.3 Poly306 decreased acid acetic-induced writhing response

The writhing test was initially used to evaluate the antinociceptive effect of the poly306. The dose of 3 mg/kg of poly306 did not inhibit significantly the writhing response. On the other hand, the doses of 10 and 30 mg/kg inhibited the writhing response significantly ($p < 0.05$) (Figure 5). Indomethacin (10 mg/kg), a standard NSAID used as reference drug, also produced a significant inhibition of acetic acid-induced writhing response.

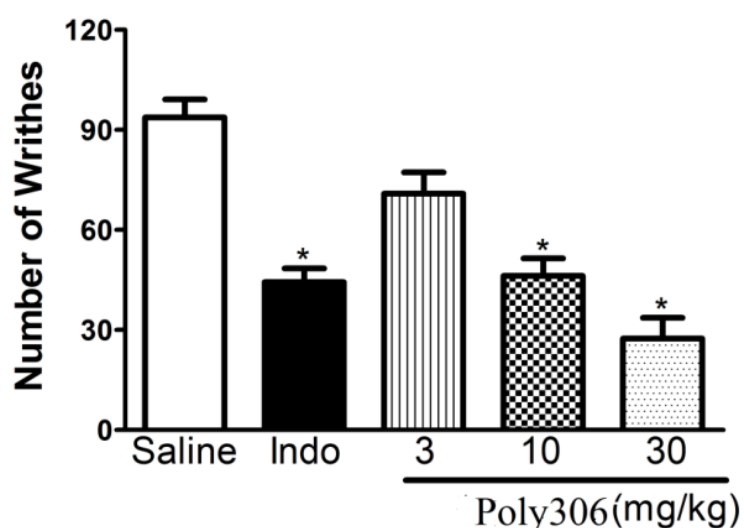


Figure 5. Effects of treatment with poly306 on acid acetic-induced writhing response. Mice were treated with poly306 (3-30 mg/kg, i.p.) or saline (control group) 30 min before acetic acid. Indomethacin (Indo; 10 mg/kg, i.p.) was the reference drug. Results are presented as means $\pm$ SEM of 6 mice per group. *Significantly different from control group ($p < 0.05$), ANOVA followed by Tukey's test.

3.4 Poly306 decreased formalin-induced nociception

The antinociceptive property of poly306 was confirmed in the formalin test. Injection of formalin in control animals induced a biphasic nociceptive behavior, with the early phase ranging from 0 to 5 minutes (Figure 6A) and the late phase from 15 to

30 minutes (Figure 6B) after the injection. In the formalin model, the dose of 10 and 30 mg/kg of poly306 did not significantly inhibit the nociceptive response. On the other hand, the treatment with poly306 (90 mg/kg) or indomethacin (10mg/kg) significantly inhibited the late phase of the formalin test ($p<0.05$).

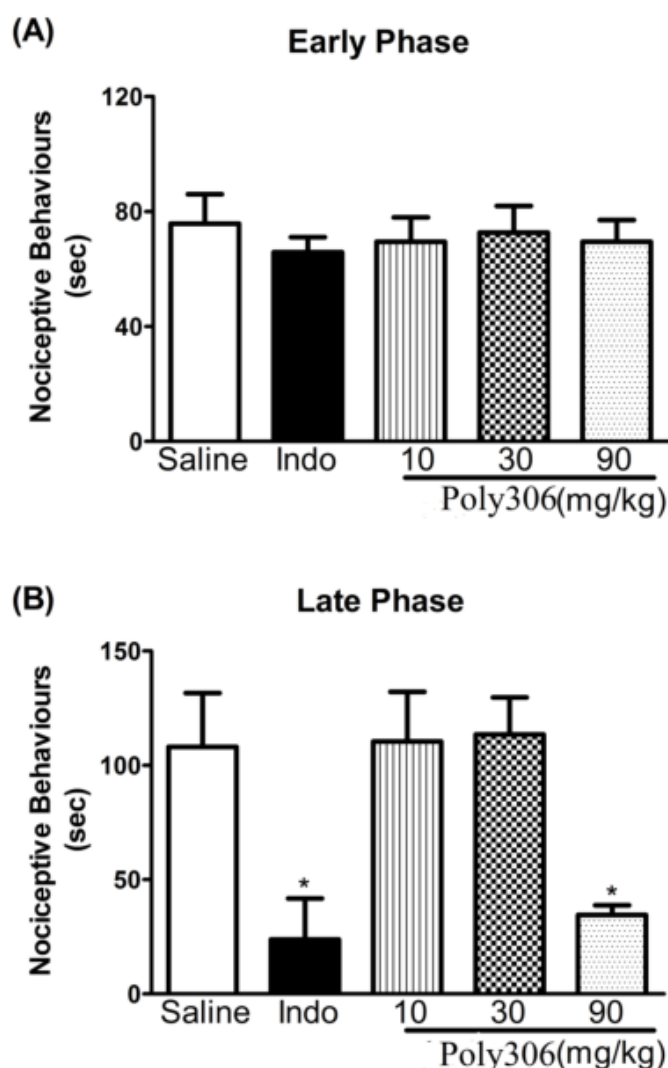


Figure 6. Effects of treatment with poly306 on the formalin-induced nociception. Panels (A) and (B) represent effects of poly306 on the early and late phases of formalin test, respectively. Mice were treated with poly306 (10-90 mg/kg, i.p.) or saline (control group) 30 min before formalin. Indomethacin (Indo; 10mg/kg, i.p.) was the reference drug. Results are presented as means $\pm$ SEM of 6-7 mice per group. *Significantly different from control group ($p<0.05$), ANOVA followed by Tukey's test.

3.5 Poly306 did not change the mice's locomotor activity

The open field test was used to exclude the possibility that the antinociceptive effect of poly306 could be resultant from non-specific disturbances in the locomotor activity of the mice. The treatment with 90mg/kg of poly306 (53.2 ± 6.1) did not changed significant locomotor activity of animals when compared to control mice (64.5 ± 4.7). As expected, diazepam (10mg/kg) treatment significantly decreased the response evaluated (10.5 ± 3.6).

3.6 Poly306 decreased carrageenan-induced oedema paw

The anti-inflammatory activity of poly306 was evaluated with the paw oedema test in mice. The Figure 6 shows that the treatment with poly306 (30 and 90 mg/kg) 30 min before carrageenan injection reduced significantly the oedema paw ($p < 0.05$). Dexamethasone (2 mg/kg), used as reference drug, also produced a significant antiedematogenic effect (figure 7).

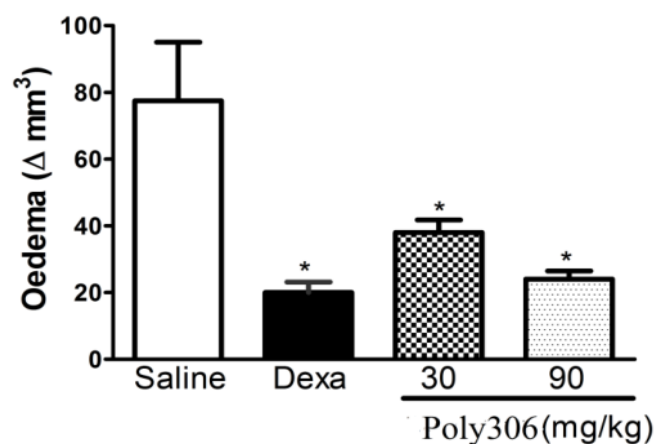


Figure 7. Effects of treatment with poly306 on carrageenan-induced oedema paw. Mice were treated with poly306 (30 and 90 mg/kg, i.p.) or saline (control group) 30 min before carrageenan. Dexamethasone (Dexa; 2mg/kg, s.c.) was the reference drug. Results are presented as means $\pm$ SEM of 6 mice per group. *Significantly different from control group ($p < 0.05$), ANOVA followed by Tukey's test.

4. Discussion

Different pH, temperature and extraction time, are applied by many authors in the extraction of fungus polysaccharide (Yoshida et al, 2005; Ishibashi et al, 2004; Luo et al, 2008; Tada et al, 2007, Li, et al, 2012 and Seviour et al, 1992). The pH conditions studied (11-13), temperature (70-90°C) and time (1 to 3 hours) were used based on the procedures used in the extraction of polysaccharide in general. From the model it could be applied to obtain the optimum conditions equivalent the 14, 84°C and 2,3h to pH, temperature and time respectively. The estimated by the model obtained, maximum yield was equivalent to 25,6%. These values of optimized extraction conditions were validated by flask culture study in triplicate sets of experiments and the maximum polysaccharide production obtained was $24 \pm 1,8\%$ which was in good agreement with the predicted value. This is higher than the actual yield obtained by other authors (Yoshida et al, 2005; Ishibashi et al, 2004; Luo et al, 2008; Tada et al, 2007 and Li, et al, 2012);

The analysis results FT-IR, gHSQC and HPLC (Figures 2, 3 and 4) show that the product obtained were typical of polysaccharide with β -(1 $\rightarrow$ 3) and α -(1 $\rightarrow$ 6) linkages based on the peak assignments from the literature and degree of polymerization of 837. (Yu, 2002; Luo et al, 2008; Ahmad et al, 2010; Wu, 2005; Majumder et al, 2009). Peak intensities of the ^{13}C and ^1H NMR have been previously used to determine the abundance of a particular linkage in the polysaccharide (Uzochukwu et al., 2002). The intensities of characteristic peaks of 103.3, 99.7 ppm and 4,91 and 4.87 ppm indicated the presence of equal abundance β -(1 $\rightarrow$ 3) linkages along with α -(1 $\rightarrow$ 6) linkages characteristic of β -(1 $\rightarrow$ 3)-D-Glucan followed by α -(1 $\rightarrow$ 6)-D-Mannan (Komura et al, 2010; Uzochukwu et al., 2002; Lacchine et al, 2006; Leung et al, 2006).

As studies have shown that the biological activity of β -D-glucan depends on their structure, solubility, molecular weight and degree of branching (Lindesquist, 2005; Tada et al, 2007; Chem et al, 2007), we evaluated the analgesic and anti-inflammatory properties of poly306 produced by the *Pseudozyma sp.*

The writhing test is widely used as a screening tool for the assessment of new antinociceptive and anti-inflammatory substances (Collier et al., 1968). The ability of poly306 in inhibited acetic acid-induced writhing suggests its antinociceptive potential. However, as this test has low specificity (Le Bars et al., 2001), we confirmed the antinociceptive effect of poly306 using the formalin test. This test has two distinct phases of pain: the early phase (neurogenic pain) characterized by direct stimulation of nociceptive afferent endings, and late phase (inflammatory pain) characterized by peripheral inflammation with a release of inflammatory mediators (Hunskar & Hole, 1987). In this study, the treatment with poly306 induced antinociceptive activity only in the late phase of formalin, we suggest that its antinociceptive effect is due, at least part, to an anti-inflammatory action. The treatment with poly306, at dose therapeutic, did not affect the locomotor activity of the mice as tested in the open field test. This confirms the antinociceptive action of poly306 showed by the nociceptive tests. To confirm the hypothesis of anti-inflammatory action of poly306, we evaluated the effects of poly306 on the carrageenan induced paw oedema in mice. Carrageenan-induced paw oedema is widely used to test new anti-inflammatory drugs. The inflammation caused by intraplantar injection of carrageenan involves the release of several inflammatory mediators, which increased blood supply and accumulation of leukocytes, resulting in oedema (Morris et al 2003; Nunes et al., 2009). The treatment with poly306 reduced significantly the carrageenan induced paw oedema, probably through inhibition of inflammatory mediators.

Various authors have demonstrated antioxidant (Machová, et al, 2012; Machová, et al, 2014b), antiviral (Recalde et al, 2012) and immunomodulatory (Tester and Al-Ghazzewi, 2013) activity of D-Mannans, however, has been rarely reported to the D-mannans isolated antinociceptive activity. Ruthes, et al, (2013) showed that cell wall polysaccharides of *Amanita muscaria* showed antinociceptive activity, and its membership includes the Fucomannogalactan. The present study shows that cell appears polysaccharides containing D-glucan and D-mannan isolated from *Pseudozyma* sp. exhibit antinociceptive activity, so the data contained in the literature differentiating and contributing to the discovery of new biological activities assigned to the fungal cell wall polysaccharides containing D-glucan and D-mannan.

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CAPÍTULO 3

Extraction Optimization of water-soluble polysaccharides from *Trichosporonoides* sp. by Response Surface Methodology and structural characterization

Extraction Optimization of water-soluble polysaccharides from *Trichosporonoides* sp. (CCMB 298) by Response Surface Methodology and structural characterization

Abstract

β -D-glucans are the most commonly used term for homopolysaccharides that has β -D-linkages in the backbone, and may also possess β -D-glucosidic linkages at position 6 in different, often repeating units (branches). In nature, β -D-glucans are widespread and are found in plants, algae, bacteria, yeast and mushrooms. β -D-glucans possess differences in molecular weights and degree of branching. Many biologically active polysaccharides have been isolated from various fungi and some of them are now used in clinics. The response surface methodology (RSM) is a collection of mathematical and statistical techniques for designing experiments, building models, evaluating the effects of factor and searching optimum condition of factors for desirable responses. We report a sequential optimization strategy for D-glucan production by *Trichosporonoides* sp. CCMB 298 isolated in the Brazilian semi-arid region, through statistically designed experiments. The polysaccharides (*poly298*) was characterized by FT-IR, ^1H and ^{13}C NMR spectroscopic techniques. The best yield was of 13% obtained of extraction in a temperature of 78°C and pH 13. The sample showed characteristic signals of (1 $\rightarrow$ 6)-D-glucan and (1 $\rightarrow$ 4)-D-mannas.

Keywords: polysaccharides, optimization, response surface methodology, characterization.

1. Introduction

β -D-glucans are the most commonly used term for homopolysaccharides that has β -D-linkages in the backbone, and may also possess β -D-glucosidic linkages at position 6 in different, often repeating units (branches). In nature, β -D-glucans are widespread and are found in plants, algae, bacteria, yeast and mushrooms. β -D-glucans possess differences in molecular weights and degree of branching. Yeast β -D-glucan is a particulate carbohydrate that consists of glucose is a major constituent of the cell (Dalmo & Bogwald, 2008).

Yeast polysaccharides, especially, β -D-glucans are recognized as biological response modifier and used for the treatment of cancer and various infectious diseases both in modern medicine and traditional chemotherapeutic drug. They have drawn the attention of chemists and immunobiologists for their immunomodulatory, anti-tumor as well as antioxidant activities (Kozarski et al., 2011). Reactive oxygen species which damage lipids, proteins, carbohydrates, and nucleic acids are the roots of developing diseases like cancer, Alzheimer, and Parkinson (Zheng et al, 2005; Kubala et al, 2003; Takita et al, 2004). β -D-glucans from mushrooms are well-known antioxidant material and antinociceptive (Machova, et al, 2014; Baggio et al, 2010).

We report a sequential optimization strategy for β -D-glucan production by *Trichosporonoides* sp. CCMB 298, isolated in the Brazilian semi-arid region, through statistically designed experiments. Doehrllet design was used to describe the nature of the response surface in the experimental region, to search optimal medium composition for maximizing D-glucan yield. The D-glucan was characterized by FT-IR, ^1H and ^{13}C NMR spectroscopic techniques.

2. Materials and Methods

2.1 Microorganism

The *Trichosporonoides* sp. CCMB 298 was obtained from the Collection of Cultures of Microorganisms of Bahia (CCMB, Feira de Santana, Brazil). The microorganism was maintained on YM agar at 4 °C in an incubator (IGO 150 Cell Life – Jouan).

2.2 Culture conditions of *Trichosporonoides* sp. CCMB 298

The strain of *Trichosporonoides* sp. CCMB 298 was incubated in a YM culture medium for activation of 4 days at 30 °C. Cultivation in liquid media was carried out in 250 mL Erlenmeyer flasks containing 100 mL of (g/L): D-glucose, 15; $\text{KH}_2(\text{PO}_4) \cdot 3\text{H}_2\text{O}$, 2.0; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5; $(\text{NH}_4)_2\text{SO}_4 \cdot 7\text{H}_2\text{O}$, 2.0, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.1, pH 5.0. Flasks were inoculated with ten milliliters of cell suspension containing 10^9 UFC of Microorganism. The incubation was performed at 120 rpm for 96 hours in a rotary shaker. After the fermentation, the liquid culture medium, on which Microorganism was grown, was filtered and centrifuged (Centrifuge 5804R –Eppendorf, São Paulo, Brazil) at 8,000 g for 15 min at 4°C, and the precipitate, containing the biomass from *Trichosporonoides* sp. CCMB 298, was used to extract the biopolymers (Dong et al. 2008).

2.3 Extraction of yeast polysaccharides (poly298)

Several authors have extracted polysaccharides of fungi using time ranging from 30 min to 6 hours, and temperatures ranging from 60 to 100°C. In this case, the optimization of the parameters can ensure better performance and generate less costs (Bohn; BeMiller, 1995; Luo et al, 2008; Wu et. al, 2005).

The powder of dry biomass (1g) was extracted several times with 20 volumes of sodium hydroxide solutions at different pHs (9, 10, 11, 12 and 13), temperatures (50, 70 and 90°C). The suspension was centrifuged at $10.000\times g$ for 10 min. It was added to the supernatant three times of the volume of absolute ethanol, stirred vigorously and left overnight at 4°C. The precipitated polysaccharides were centrifuged at $8000\times g$ for 30 min and the supernatant discarded. The precipitate of crude polysaccharides was dried at 55 °C to a constant weight and the polymer was weighted by a scale (Sartorius ALC-110.4, Germany). The polysaccharides was determined by phenol–sulfuric acid method (Dubois, et.al, 1956). The product yield was measured at the (w/w) % of β -D-glucan per unit mass of biomass yeast.

2.4 Experimental design

The Doehlert experimental design contained two variables, Temperature and extraction time, and three replicates at the center of the domain lead to a total of 9 experiments (Table 1). Measurements were performed in triplicate to verify the best conditions to extraction of polysaccharide. The pH was studied at five levels, ranging from 9 to 13°C, and the temperature was studied at three time points, ranging from 50 to 90°C. The experimental errors were evaluated from replication of a central point. The experimental data was processed using Statistica software. All the experiments in this step were carried out in random order.

2.5 The number-average molecular weight (MW_n) and degree of polymerization (DP_n)

The MW_n and DP_n of the polysaccharides were determined by the measurement of the reducing value using 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959) and

the measurement of total carbohydrates using the phenol–sulfuric acid method (Dubois et al, 1956); $DP_n = [(total\ carbohydrates\ in\ \mu g\ of\ D-glucose)/(reducing\ value\ in\ \mu g\ of\ maltose)] \times 1.9$; $MW_n = [(DP_n) \times 162] + 18$ (Vettori, Franchetti, Contiero, 2012).

2.6. Determination of the protein concentration

Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin (BSA) as a standard.

2.7. Characterization of polysaccharides (poly298) by spectroscopic analysis

The polysaccharide was characterized using a Fourier Transform Infrared Spectrometer (PE IR Spectrum AsciiPeds 1.60, Spectrum One FT-IR Spectrometer). The dried polymer was grinded with potassium bromide (KBr) powder and pressed into pellets for FT-IR spectral measurement in the frequency range of $4000-500\ cm^{-1}$, with 20 scans. In NMR spectroscopic analysis, all spectra were recorded at 343 K on a Varian Inova 500 spectrometer operating at 11.7 T, observing 1H at 500 MHz and ^{13}C at 125 MHz, and using direct probe. The samples were dissolved in $D_2O/DMSO$ (6:1) and transferred to NMR tube. DMSO was used as a chemical shift reference. D_2O (99.9%) and DMSO (99.9%) were from Cambridge Isotope Laboratories, Inc. (USA).

2.8. Monosaccharide Composition of polysaccharide

The polysaccharide (poly298) (20 mg) was hydrolyzed with 2 M TFA at $100^\circ C$ for 8 h, followed by evaporation (Ruthes et al, 2013). Filtered liquids were analyzed by high-performance liquid chromatography (HPLC) using Amines® HPX-87H (300 mm x 7.8 mm i.d.) (9 μm) column (Merck, Darmstadt, Germany) and refractive-index

detection; the injection volume was 12 μL . The mobile phase was acetonitrile (MeCN): deionized H_2O (H_2O) (82:18) at a flow rate of 0.4 ml min^{-1} , and the column temperature was maintained at 25°C . For peak identification, an external standard of glucose was used. Determination of the monosaccharide composition was done in two independent experiments with consistent results.

3. Results

3.1 Extraction of polysaccharides (poly298)

A 9-run Doehlert design with three and five levels, including three replicates at the centre point, was used for fitting a second-order response surface. The three centre point runs were added to provide as a measure of process stability and inherent variability. The considerable variation in the polysaccharide yield from the *Trichosporonoides* sp. CCMB 298 under different conditions was shown in Table 1.

Table 1. Doehlert experimental design from optimization extraction of polysaccharides (poly298)

Experiment	pH	Temperature ($^\circ\text{C}$)	Observed Mass of poly298 (mg)	Predictd Mass of poly298 (mg)
1	10 (-0,5)	90 (+0,866)	44.1	41.75
2	12 (+0,5)	90 (+0,866)	45.0	47.35
3	9 (-1)	70 (0)	32.0	34.35
4C	11 (0)	70 (0)	44.0	45.00
5C	11 (0)	70 (0)	46.0	45.00
6C	11 (0)	70 (0)	45.0	45.00
7	13 (+1)	70 (0)	52.0	49.65
8	10 (-0.5)	50 (-0.866)	21.0	18.65
9	12 (+0.5)	50 (-0.866)	26.0	28.35

(C): central point; coded values are presented in the parentheses

The model adequacy was checked by an F-test and the determination coefficient R^2 . The analysis of variance (Table 2) showed that this regression model was highly significant ($P < 0.05$) with F-value of 14.72.

Table 2. Analysis of variances in the regression model for optimisation of polysaccharides (poly298) of *Trichosporonoides* sp. CCMB 298

Source of variations	SS	Df	MS	Fcal	Ftab	Rsqr
Regression	846,007222	5	169,2014	14,44	9.01	0.961
Residual	35,135000	3	11,71167			
lack of fit	33,1350	1	33,1350	33,13	18.51	
Pure error	2,0000	2	1,0000			
Total error	881,1422	8				

SS - Sum of square; Df - Degree of freedom; MS - Mean square; Fcal - calculated F value; Ftab - tabulated F value; Rsqr - R-square.

The fitness of the model was further confirmed by a satisfactory value of determination coefficient, which was calculated to be 0.9610, indicating that 96.10% of the variability in the response could be predicted by the model. Furthermore, the predicted poly298 mass by the final quadratic model, along with the corresponding values observed, were given in Table 1.

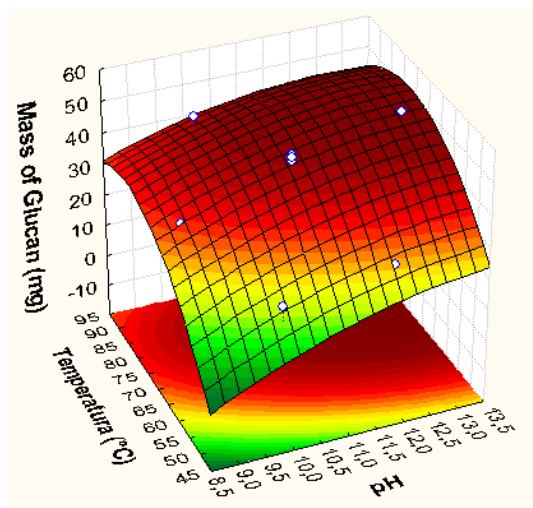
The polynomial model for poly298 extraction was regressed by considering only the significant term sand shown as below:

$$\text{poly298 (\%)} = +72.139 (\pm 9,015) + 5.915(\pm 1.340)(\text{pH}) - 0.1833(\pm 0.057)(\text{pH})^2 + 1.169(\pm 0.092)(T) - 0.006(T)^2 + 0.0132(\pm 0.006)(\text{pH})*(T)$$

(T): Temperature ($^{\circ}\text{C}$)

From the results of Table 1, we can construct three-dimensional graphics, relating the variables in question (Figure 1).

A



B

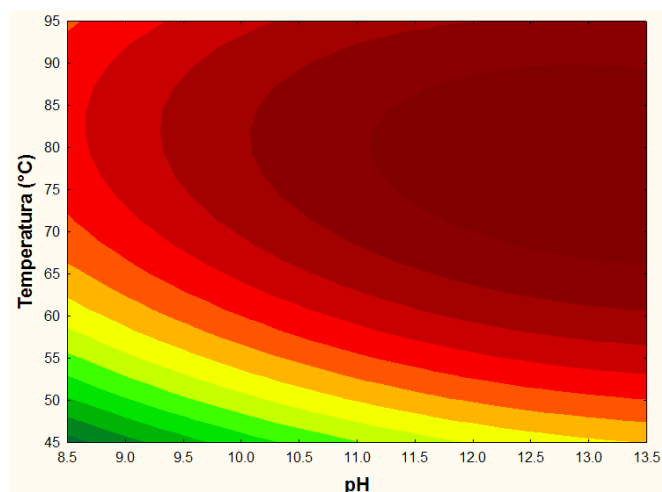


Figure 1. (A): Response surface plots for the extraction of polysaccharides (poly298).

(B): Isoresponse contour plot of temperature versus extraction time for poly298 production.

The optimum conditions for extraction of poly298 can be obtained from the optimal point of the model. The analysis of results suggests that temperatures below 60°C allow an inefficient extraction at any pH studied. Temperatures above 60°C provide a good extraction pH above 10, with optimum values of extraction equivalent to 78°C for temperature and pH for 13.

3.2 Characterization of polysaccharide by spectroscopic analysis and HPLC

The analysis of the infrared spectra of biomass obtained was performed to identify the main functional groups. Figure 2 shows a signal at 3263 cm^{-1} assigned to the hydroxyl stretching vibration of the polysaccharide. The FT-IR spectrum showed peaks at 1045 cm^{-1} , which is due to the stretching of anomeric C_1H group vibration (Yu, 2002) suggested for polysaccharides gum pellets (Figure 2).

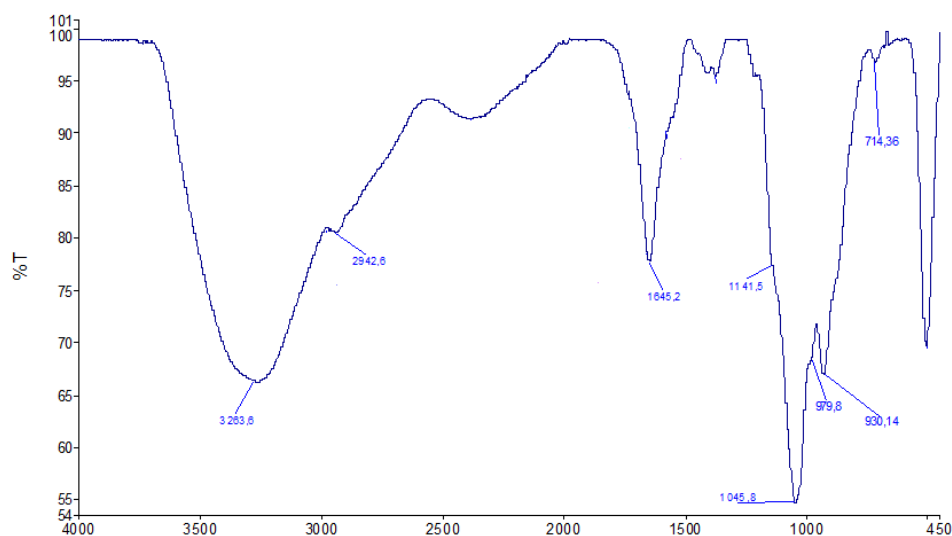


Figure 2: FT-IR spectrum of polysaccharides (poly298) produced from *Trichosporonoides* sp. CCMB 298. The dried polymer was grinded with potassium bromide (KBr) powder and pressed into pellets for FT-IR spectral measurement in the frequency range of $4000 - 500 \text{ cm}^{-1}$, with 20 scans.

It is also important to note that the spectra showed absorption peak at 930 cm^{-1} that is an indicative of β -linked glycosidic bonds. The FT-IR spectrum showed peaks at 1045 cm^{-1} , which is due to the stretching of anomeric C_1H group vibration (Yu, 2002) suggested for polysaccharides gum pellets. The band at about 1141 cm^{-1} was assigned to the valent vibrations of the C-O-C bond and glycosidic bridge (Shingel, 2002). The intense absorption in the region between 1645 cm^{-1} is related to the presence of functional groups corresponding to proteins such as amines, amides and aromatic compounds (Ahmad et al, 2010). The presence of proteins was confirmed by the technique described by Bradford (1956). The MW_n and DP_n of the poly298 were 48.716 and 300 respectively.

The ^{13}C NMR spectrum of sample and ^1H NMR spectrum (Figure 3) reveals signals characteristic of polysaccharide.

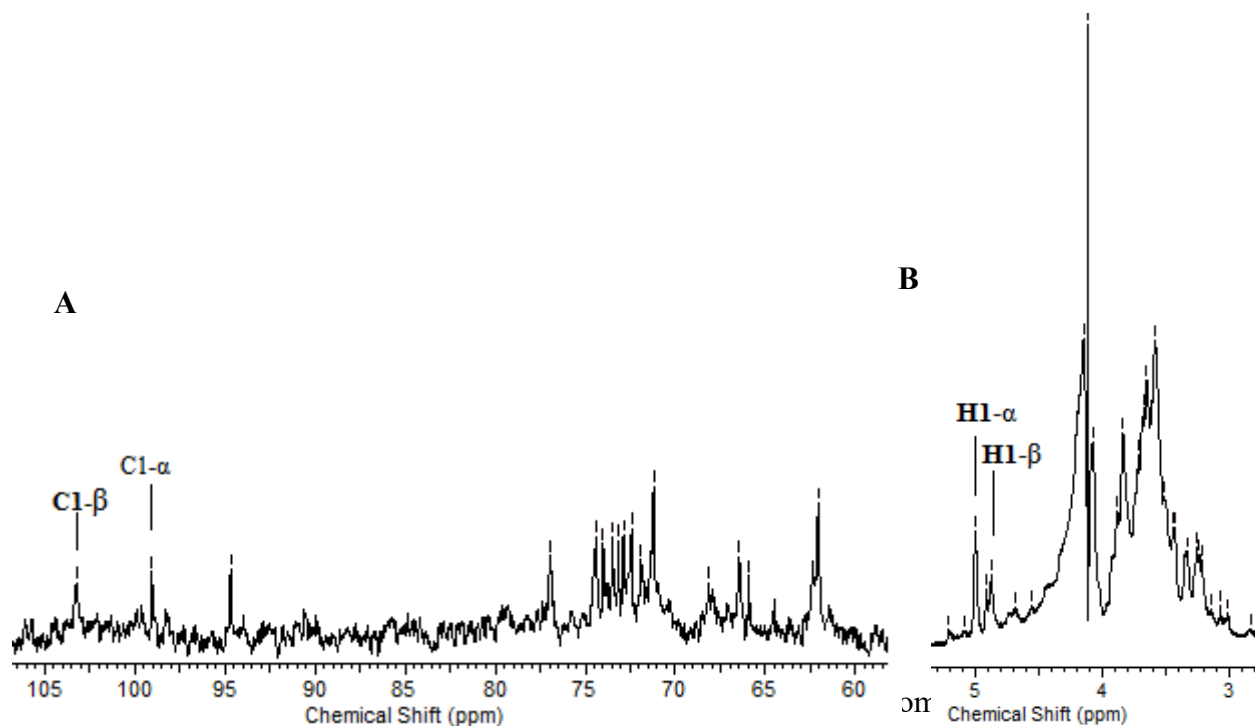


Figure 3. (A) ^{13}C NMR spectrum and (B) ^1H NMR spectrum. Experiments were performed in $\text{D}_2\text{O}/\text{DMSO}$ (6:1) at 343K, operating at 11.7 T, observing ^1H at 500 MHz and ^{13}C at 125 MHz (chemical shifts are expressed in δ ppm).

The signals $\delta^{13}\text{C}/\text{H}^1$ 103.2/4.55 and 99.0/5.0 were characteristic of a β -D-glucan and α -D-glucan respectively, by comparison with data from the literature. Signals between $\delta^{13}\text{C}$ 62.0 and 77.0 were characteristic of C-6, C-4, by comparison with data from the literature (Wu, 2005; Majumder et al, 2009). The sample showed characteristic signals of (1 $\rightarrow$ 6)-D-glucan and (1 $\rightarrow$ 4)-mannan.

The results of the HPLC analysis obtained after hydrolysis of polysaccharide is shown in Figure 4. The retention time standard of glucose and mannose were equivalent

to 13.39 and 14.30 min respectively. The monomer composition demonstrates the presence of mannose and glucose after hydrolysis with TFA. Quantitative analysis of individual peaks associated spectrometric analysis to suggest that the majority of poly298 consists of β -(1 $\rightarrow$ 6)-D-glucan (87%) followed by α -(1 $\rightarrow$ 4)-D-mannan (13%).

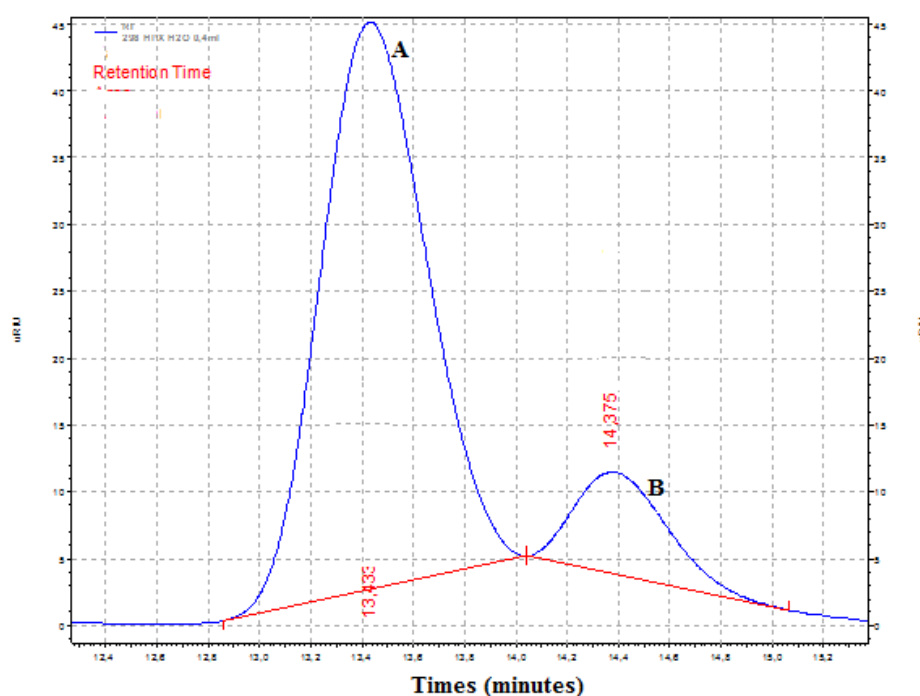


Figure 4. HPLC analysis of polysaccharides (poly298) after acid hydrolysis with TFA. (A): Glucose. (B): Mannose. The injection volume was 12 μ L. The mobile phase was acetonitrile (MeCN): deionized H₂O (82 : 18) at a flow rate of 0.4 mL min⁻¹, and the column temperature was maintained at 25°C.

4. Discussion

The application of RSM was able to predict the optimum conditions for extraction of polysaccharides (poly298). This type of study is able to meet the conditions applied by several authors (Yoshida et al, 2005; Ishibashi et al, 2004; Luo et al, 2008; Tada et al, 2007 and Oscar et al, 1999) and evaluate what would be the optimal condition of extraction of polysaccharide. From studies carried out by several authors, this study was able to identify an ideal condition. From the model it could be applied to

obtain the optimum conditions equivalent the 78°C and 13 to temperature and pH. The estimated by the model obtained, maximum yield was equivalent to 13.0%. These values of optimized extraction conditions were validated by flask culture study in triplicate sets of experiments and the maximum polysaccharides (poly298) production obtained was $12.8 \pm 2.2\%$ which was in good agreement with the predicted value. This is higher than the actual yield obtained by other authors (Yoshida et al, 2005; Ishibashi et al, 2004; Luo et al, 2008; Tada et al, 2007 and Li, et al, 2012);

The biological activities of polysaccharides are closely related to their dimensional structure. In addition to the improvement of extraction rate, the influence of extraction parameters on the structure and thus on the bioactivity of polysaccharides should also be taken into consideration in the process optimization. This difference may be the connection types, the size of the molecule or in association with proteins which may differ in pharmacological activities (Majumder et al, 2009).

Application of FT-IR spectroscopy has been shown to be a useful tool in monitoring structural changes in biopolymers. In spite of the fact that on the one hand the infrared characteristics of glucose are well known, on the other hand, information about glucans is rather insufficient. Therefore we applied this technique to glucans in the 1800–700 cm^{-1} region. The (C–O) and ring vibrations ranging at 1200–950 cm^{-1} as well as in the “anomeric region” at 950–700 cm^{-1} show differences in the α - and β -glucan (Majumder; Singh; Goyal, 2009)

After analysis of the infrared spectrum of the sample of D-glucan can be seen that the sample showed absorption regions characteristic of groups of α -D-glucan and β -D-glucan. The sample showed intense absorption in the region that suggests the presence of protein, which was confirmed by measurement made by the technique of Bradford (1956). Some techniques can be used to purify the sample, such as column

chromatography, precipitation with ammonium sulfate, among others, but it is unclear to what extent the lack of these proteins may interfere with pharmacological activity, since some studies attribute the pharmacological activity proteins involved in β -glucan and α -glucan (Chem; Serviou, 2007)

Analyses of NMR the anomeric carbons of β -D-glucan and α -D-mannan, 103.2 and 99.0 ppm had demonstrated presence of signals correspondents respectively. These results suggest the presence of D-glucans and D-mannans or presence of on ramifications to the main chain. The infrared ray analyses had respectively confirmed the presence of type linkings α and β , 930 cm^{-1} and 714 cm^{-1} , being that this finishes the signal if presented of Majunder, Singh and Goyal (2009) the isolated ones in this work had isolated D-glucan of *Leuconostoc dextranicum* with similar structural characteristics, then had attributed the signals (1 $\rightarrow$ 4)(1 $\rightarrow$ 6)-D-glucan. The (1 $\rightarrow$ 6)- β -glucan has demonstrated a biotechnological potential in the treatment of some illnesses, acting as immunomodulatory, being thus polysaccharides of *Trichosporonoides* sp. CCMB 298 “*in vitro*” and in can be applied in studies “*in vitro*” in order to test the capacity to stimulate macrophages to produce cytokines, and tests against carcinomas.

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CONCLUSÃO GERAL

O semi-árido baiano demonstrou ser uma fonte promissora de microrganismos capazes de produzir biomoléculas com potencial aplicação na área médico-farmacêutica. As leveduras, *Aureobasidium pullulans* (CCMB 324), *Pseudozyma* sp. (CCMB 306), *Trichosporonoides* sp. (CCMB 298) e *Rhodotorula mucilaginosa* (CCMB 33d1) isoladas no semi-árido foram estudadas a fim de otimizar as condições de produção dos polissacarídeos, e identificar o potencial biotecnológico, no tocante ao tratamento de doenças infecciosas, imunológicas e inflamatórias.

As condições ótimas de extração para as 4 leveduras estudadas foram semelhantes, girando em torno de 13 para pH, entre 80 e 90°C e 2 a 3 horas, sendo que o maior rendimento foi em torno de 26%, encontrados nas leveduras *Aureobasidium pullulans* (CCMB 324), *Pseudozyma* sp. (CCMB 306), podendo essas condições serem aplicadas em condições industriais, com intuito de se obter maiores rendimentos de polissacarídeos.

Todas as leveduras apresentaram em maior ou menor grau características de β -D-glicanos e α -D-mananos com ligações diversas (1 $\rightarrow$ 3, 1 $\rightarrow$ 4 e 1 $\rightarrow$ 6) além de tamanhos moleculares e graus de polimerização diferentes, entretanto, todas as características se encaixam na relação estrutura-atividade dos polissacarídeos, necessária para ter boa atividade farmacológica. Esse fato foi confirmado, já que todas apresentaram atividade analgésica “*in vivo*” nos modelos experimentais estudados.

Os estudos de atividade antinoceptiva, demonstraram que as leveduras *Aureobasidium pullulans* (CCMB 324), *Pseudozyma* sp. (CCMB 306), e *Rhodotorula mucilaginosa* (CCMB 33d1) produzem polissacarídeos com promissor potencial em aplicação como droga analgésica, apresentando potências (relação dose/efeito) variadas.

O mesmo estudo realizado demonstra que o mecanismo de ação dos polissacarídeos produzidos por essas leveduras, estão ligados à fase tardia da inflamação, provavelmente suprimindo a liberação de mediadores pró-inflamatórios.

Sendo assim, pode-se inferir que as leveduras isoladas do semi-árido baiano utilizadas nesse estudo, possuem potencial biotecnológico na aplicação como agente terapêutico com atividade analgésica. Entretanto, testes toxicológicos e farmacotécnicos devem ser realizados a fim de verificar sua segurança para propor uma possível utilização em humanos.

ANEXO A