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Changes in high and low molecular weight carbohydrates during *Rhizopus nigricans* cultivation on lemon peel

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Abstract

Rhizopus nigricans was cultivated in a liquid medium using lemon peel as the carbon source. During cultivation, changes were measured in high and low molecular weight carbohydrates from the growth medium, as well as changes in the uronic acid amount of the alcohol insoluble solids from the lemon peel before and after fermentation. The initial amount of carbohydrates in the cultivation medium originated from the solubilisation of small sugars and partial hydrolysis of lemon cell wall polysaccharides in the acidic medium during the autoclaving step of the growth medium preparation. A complex mixture of monosaccharides (fructose, glucose, xylose, inositol), cellobiose, unknown oligosaccharides, galacturonic acid oligomers (penta-, tri-, and monogalacturonic acid), and polysaccharides was solubilised in the cultivation medium. During fermentation the fungus grew, produced the pectic enzyme endopolygalacturonase and consumed free sugars (fructose and glucose) and galacturonic acid. A carbohydrate polymer fraction remained resistant to fermentation, while a fraction of lower molecular weight was consumed.

Keywords: Lemon peel; Galacturonic acid oligomers; *Rhizopus* cultivation

1. Introduction

Rhizopus species are industrially important micro-organisms, which produce enzymes through fermentation (Chitradon, Mahakhan, Poonpairoj, Kitpreechavanich, & Lotong, 1996; Ikasari & Mitchell, 1996; Lucio de Souza et al., 1996; Soccol, Stertz, Raimbault, & Pinheiro, 1995). *Rhizopus nigricans* is of special interest because of its virulence as well as its ability to produce pectic enzymes with thermoresistant properties (Ros, Saura, Salmerón, & Laencina, 1993). The citrus fruit transformation industry produces several waste materials and by-products which are potential growth substrates for the cultivation of micro-organisms. The growth conditions of these fungi are very important as the correct choice of agent for mycelium cultivation may intensify fungal metabolic processes, consequently contributing to a shortening of the cultivation period and an increase in the enzyme yield (Fros & Most, 1987). In this sense, it is important to fully understand the use of the growth substrate by the fungus during its cultivation. Following this approach, our work deals with the changes in high and low molecular weight carbohydrates during the cultivation of *Rhizopus nigricans* on a sample of lemon peel from the citrus fruit transformation industry.

2. Methods

2.1. Cultivation conditions

The composition of the culture medium used was that proposed by Spalding (1963) and the fresh peel of Spanish lemons obtained from the industrial process of essential oil and juice extraction was used as the carbon source. Subsequently, the pH of the fermenter was adjusted to 4.5 and then sterilised by autoclaving (120°C × 20 min), and the culture medium was inoculated with *Rhizopus nigricans* ATCC 24862. Inoculation was performed using one week old mycelium from a PDA plate, and cultivation was carried out in a 2000 ml fermenter with air agitation, pH control and sample extraction through a rubber septum under sterile conditions (Hellín, Ros, &, Laencina, 1998). The air feeding the fermenter was chemically sterilised. During cultivation a sample was taken from the culture bath at timed intervals and the endopolygalacturonase activity, uronic acids, and high and low molecular weight carbohydrates were determined.

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2.2. Preparation of alcohol insoluble solids from lemon peel

Lemon peel alcohol insoluble solids (AIS) were obtained before and after cultivation according to De Vries, Rombouts, Voragen, and Pilnik (1981), using a Soxhlet device and 70% (v/v) ethanol as extracting agent.

2.3. Analytical methods

Endopolygalacturonase activity was determined by the viscosimetric method (Pharr & Dickinson, 1973), using polygalacturonic acid as the substrate. Uronic acids were determined by the colorimetric 3,5-dimethylphenol assay (Scott, 1979). The neutral sugar content of the AIS was established by High Performance Anion Exchange Chromatography with Pulse Amperometric Detection (HPAEC-PAD) after pre-treatment (30°C, 1 h) with aqueous 72% sulphuric acid (Saeman hydrolysis) followed by hydrolysis with 1 M sulphuric acid (100°C, 3 h). HPAEC-PAD was carried out in a DX-500 Dionex Chromatography System (Dionex, Sunnyvale, CA, USA) using a CarboPac PA10 column with a CarboPac PA10 guardcolumn, both from Dionex. High quality water was used as the eluent at a constant flow of 1 ml/min and room temperature. In order to increase the response of PAD a post-column addition of 300 mM sodium hydroxide was made. Pure sugars from Sigma Chem. Co. (St. Louis, MO, USA) were used as standards. The molecular weight distribution of the polysaccharides was established by High Performance Size Exclusion Chromatography (HPSEC) using three Ultrahydrogel columns in series (500, 250, 120) with an Ultrahydrogel guardcolumn, all from Waters (Milford, MA, USA). 0.4 M sodium acetate (pH 3.0) was used as the eluent at a constant flow of 0.8 ml/min and room temperature. Detection was carried out using a L-7490 refractive index detector (RID) from Merck (Darmstadt, Germany). Calibration of the HPSEC columns was performed with standard pectins and galacturonic acid according to Schols, Posthumus, and Voragen (1990). Average molecular weight was established using Maxima SEC software from Waters. Soluble sugars were determined by HPLC using a CHO-682 column with a CHO-682 guardcolumn, both from Interaction (San Jose, CA, USA). High quality water was used as the eluent at a constant flow of 0.4 ml/min and 85°C. Detection was carried out by RID. Pure sugars from Sigma Chem. Co. were used as standards. Galacturonic acid oligomers were determined by HPLC using an ORH-801 column with an ORH-801 guardcolumn, both from Interaction. 5 mM sulphuric acid was used as the eluent at a constant flow of 0.6 ml/min and 30°C. Detection was carried out by RID. Pure galacturonic acid oligomers and short chain organic acids from Sigma Chem. Co. were used as standards.

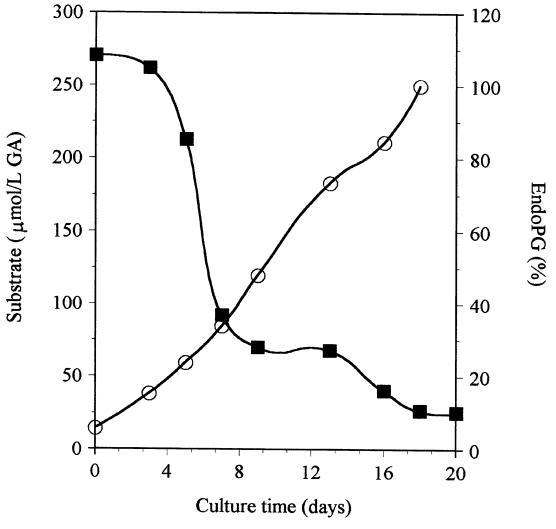

Fig. 1. Production of endopolygalacturonase by *Rhizopus nigricans* on lemon peel (○) and decrease of total amount of soluble galacturonic acid (■).

3. Results and discussion

3.1. Characterisation of the lemon peel used as the carbon source

Industrially processed Spanish lemon peel was used as the carbon source for the cultivation of *Rhizopus nigricans*. A characterisation of this material was carried out by determining the AIS content of the peel and the sugar composition of the AIS. AIS represent 7.7% (fresh weight basis) of the lemon peel used for cultivation. Lemon peel AIS had the following sugar composition (in % w/w): rhamnose 0.9, fucose 0.4, arabinose 5.3, xylose 2.8, mannose 1.4, galactose 4.1, glucose 28.3 and galacturonic acid 30.0.

3.2. Rhizopus nigricans cultivation on lemon peel

The culture medium was inoculated with *Rhizopus nigricans.* The increase of endopolygalacturonase activity in the cultivation medium during the growth period is shown in Fig. 1, and was followed by the consumption of the carbon source. This trend on microbial enzyme production has been reported by several authors (Nakamura et al., 1997; Ros, Núñez, Saura, Salmerón, & Laencina, 1991; Siedenberg et al., 1998). The galacturonic acid content of the AIS prepared from lemon peel decreased from 30% (w/w) before cultivation to 5% (w/w) after cultivation. Since the substrate line (Fig. 1) represents the total amount of soluble uronic acids in the liquid medium, a more detailed description of their composition and polymeric form was undertaken using high performance chromatographic techniques.

3.3. Changes in carbohydrates on molecular weight basis

Analysis by size exclusion chromatography of a sample just before inoculation indicated that carbohydrates solubilised by autoclaving in the cultivation medium contained a broad and heterogeneous molecular weight distribution

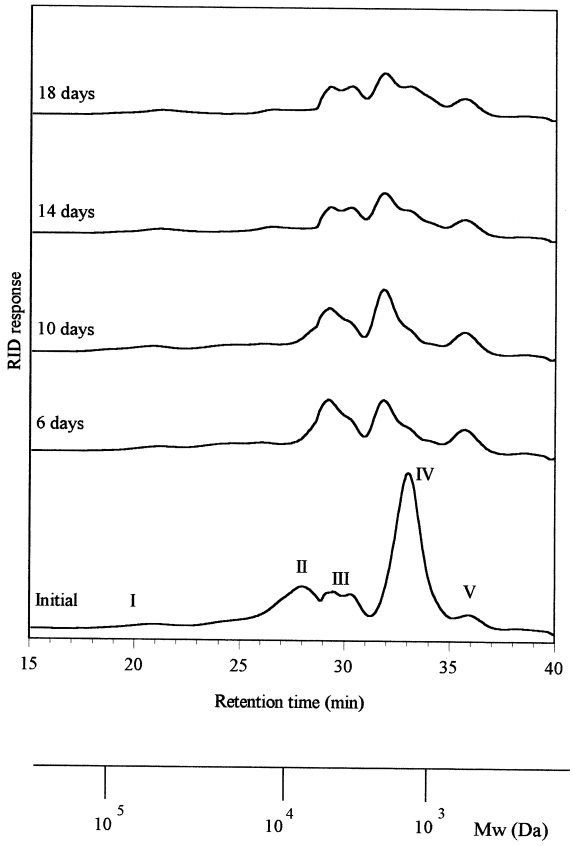

Fig. 2. High-performance size exclusion chromatography elution profile of a sample from *Rhizopus nigricans* cultivation on lemon peel at 0, 6, 10, 14 and 18 days of cultivation.

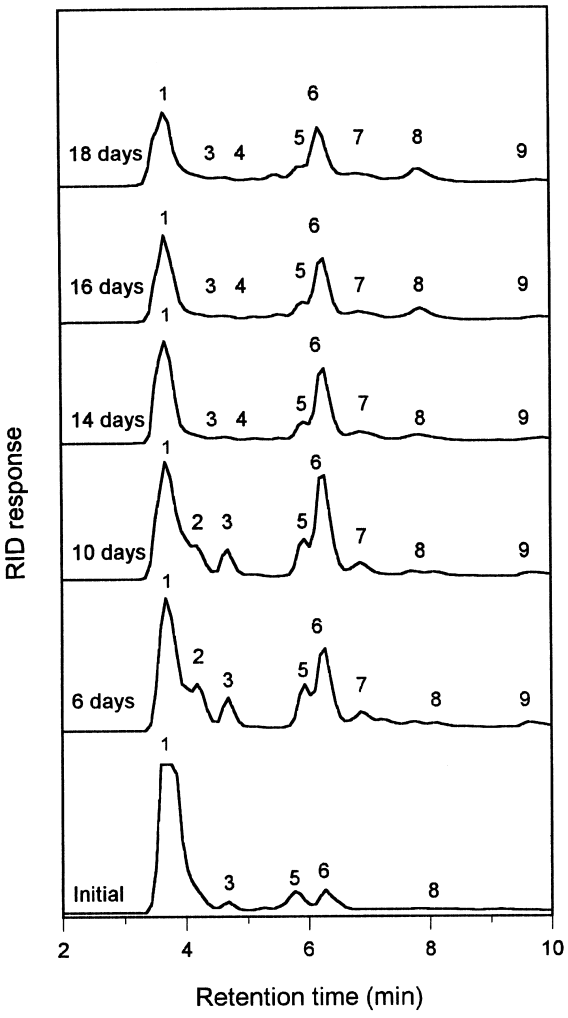

Fig. 3. High performance liquid chromatography elution profile of galacturonic acid oligomers and organic acids of a sample from *Rhizopus nigricans* cultivation on lemon peel at 0, 6, 10, 14, 16 and 18 days of cultivation: (1) pentagalacturonic acid; (2) tetragalacturonic acid; (3) trigalacturonic acid; (4) digalacturonic acid; (5) citric acid; (6) galacturonic acid; (7) pyruvic acid; (8) quinic acid; (9) succinic acid.

(Fig. 2). Up to five different populations (I–V) on molecular weight basis were found, including polymeric ($M_w >$ 5 kDa) and oligomeric (5–2 kDa) carbohydrates. Population I had higher molecular weight, appeared in lower amounts, and was unchanged during cultivation, indicating the inability of the fungus to degrade it. Populations II and III were quickly degraded within the first six days of cultivation, the degradation products being consumed by the fungus or added to the lower molecular weight populations resistant to the fungus. Population IV was quickly degraded and consumed within the first six days of cultivation, remaining finally as a resistant to consumption population. The speed of degradation and period of consumption (Fig. 2) coincide with the decrease in the available pectic substrate (Fig. 1). After eighteen days of cultivation there was still some pectic substrate (Fig. 1), but it was resistant to further degradation and consumption by the fungus (Fig. 2). In fact, pectic materials associated with arabinan and arabinogalactan and resistant to polygalacturonase activity, the so-called hairy regions of the pectins, have been found in various vegetable sources (Schols et al., 1990; Schols, Ros, Daas, Bakx, & Voragen, 1998; Schols & Voragen, 1994) and in lemon peel (Ros, Schols, & Voragen, 1996; Ros, Schols, & Voragen, 1998). Some bioactivities were proposed for such structures of pectic materials (Ros, Schols, Laencina, & Voragen, 2000; Samuelsen et al., 1996; Yamada, 1996; Yamada & Kiyohara, 1999), which may be of value as fermentation by-products.

3.4. Changes in sugars and uronic acids

Population IV had a low molecular weight (average M_w 1 kDa), which corresponds to a mixture of oligo- and monosaccharides. Analysis by HPLC of a sample taken just before inoculation indicated the presence of galacturonic acid oligomers (DP 1–5) and organic acids (Fig. 3). A sugar HPLC analysis of the same sample indicated the presence of monosaccharides (fructose, glucose, xylose, rhamnose, inositol), cellobiose and unknown oligosaccharides (Fig. 4). During fermentation galacturonic acid oligomers were consumed (Fig. 3) and some of them (tetra-, tri- and digalacturonic acid) appeared in the cultivation medium as

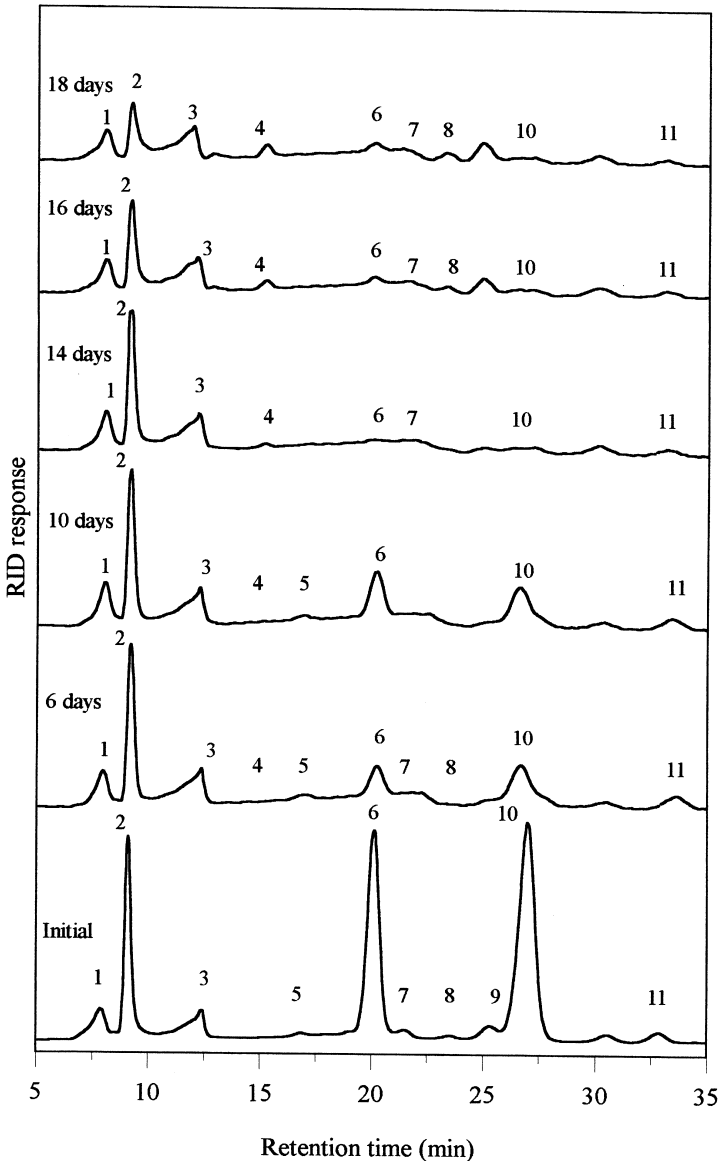

Fig. 4. High performance liquid chromatography elution profile of sugars of a sample from *Rhizopus nigricans* cultivation on lemon peel at 0, 6, 10, 14, 16 and 18 days of cultivation: (1, 2, 3) unknown oligosaccharides; (4) raffinose; (5) cellobiose; (6) glucose; (7) xylose; (8) rhamnose; (9) fucose; (10) fructose; (11) inositol.

intermediate products (Fig. 5). Penta- and monogalacturonic acid had initial concentrations of 8.5 and 1.6 mg/ml, respectively, which changed to 4.6 and 2.4 mg/ml after 18 days of cultivation. Among monosaccharides, the main sugars were glucose and fructose (initially 3.7 and 4.9 mg/ml, respectively), which were quickly consumed by the fungus during fermentation (Fig. 6). Other sugars, such as raffinose, xylose and inositol only changed in a narrow range of concentration (0–1 mg/ml). By comparing Figs. 5 and 6 it is possible to establish that the rates of consumption of oligosaccharides and monosaccharides were different. Initially, glucose and fructose were consumed at a higher rate than pentagalacturonic acid and the unknown oligosaccharides. Around the 14th cultivation day, when the amounts of glucose and fructose were fairly low, there was still undegraded pentagalacturonic acid (5.5 mg/ml) and unknown oligosaccharides (4.4 mg/ml). The slight increase observed for raffinose, glucose and xylose at the end of the cultivation could be related to the decrease of the unknown oligosaccharides, in that i.e. raffinose, glucose and xylose could come from an enzymatic degradation of the oligosaccharides. Some organic acids were found together with the galacturonic acid oligomers. Initially, these acids were citric acid (1.0 mg/ml) and quinic acid (0.3 mg/ml), while pyruvic and succinic acid were present due to the fermentation process. Pyruvic and succinic acid had almost constant concentrations during fermentation (1.0 and 0.4 mg/ml, respectively), while citric acid decreased from 1.0 to

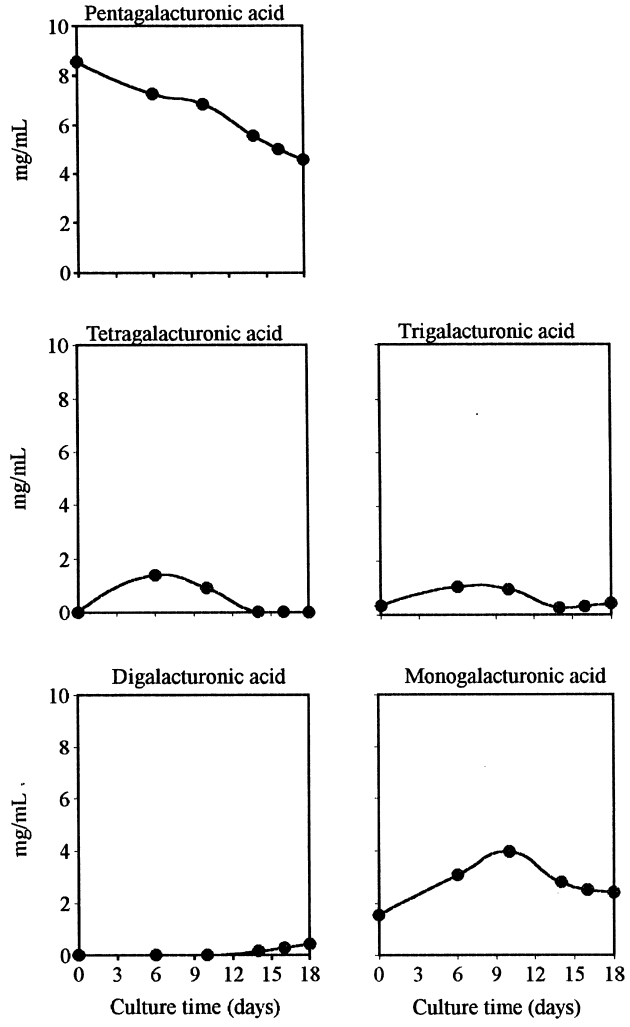

Fig. 5. The evolution of galacturonic acid oligomers during *Rhizopus nigricans* cultivation on lemon peel.

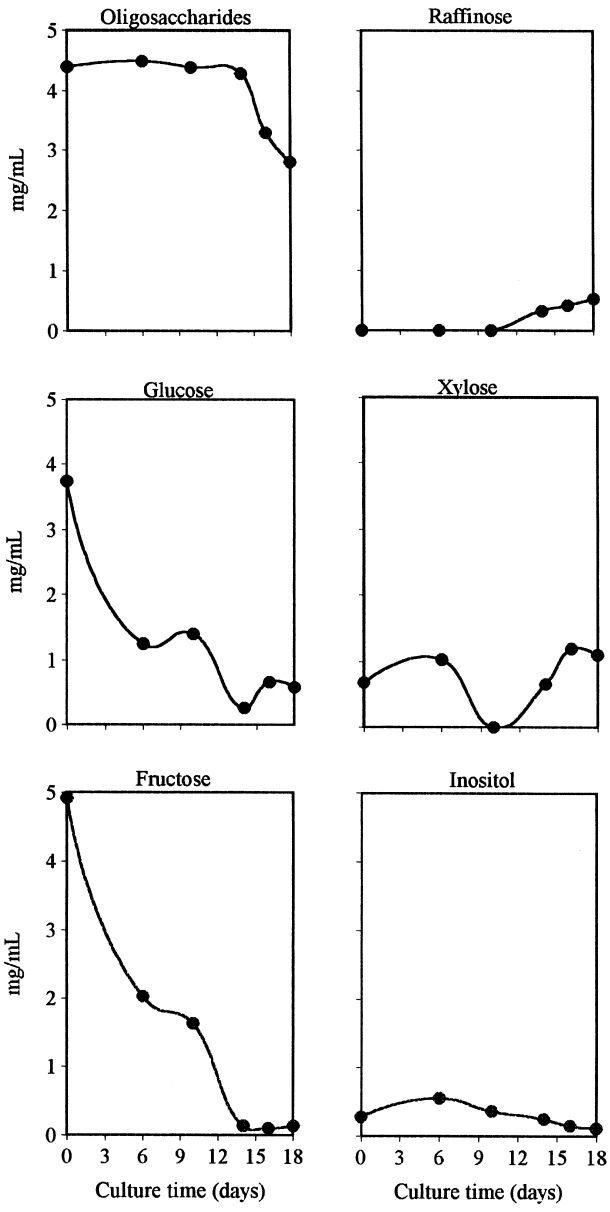

Fig. 6. The evolution of sugars during *Rhizopus nigricans* cultivation on lemon peel.

0.6 mg/ml and quinic acid increased from 0.3 to 1.0 mg/ml. This trend of galacturonic acid oligomer and oligo- and monosaccharide consumption during fermentation is similar to that reported for the production of endopolygalacturonase (Ros et al., 1991), xylanase (Siedenberg et al., 1997) and amylase (Goto et al., 1998).

4. Conclusions

The autoclaving step during preparation of the cultivation medium allows the solubilisation of a complex mixture of poly-, oligo- and monosaccharides from the lemon peel. Using these sugars *Rhizopus nigricans* grew and produced endopolygalacturonase, which degraded the polymeric pectins in the cell wall. These were then solubilised in the cultivation medium and consumed. The principal carbohydrates used by the fungus were glucose, fructose and galacturonic acid oligomers. A polymeric fraction was present which was resistant to enzymatic degradation, and, due to its potential bioactivity it could prove of interest, in the near future, to isolate it and establish its structure and composition.

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