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Removal of hexavalent chromium using chitosan prepared from shrimp shells
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Removal of hexavalent chromium using chitosan prepared from shrimp shells

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Contamination of the aqueous environment by heavy metals and due to the discharge of metal containing effluents into the water bodies is one of the environmental issues of the century. Thus, in this work, the main concern has been the preparation of chitin and chitosan from the raw materials of shrimp shells and the characterization of the prepared chitosan by field emission scanning electron microscopy (FESEM) and Fourier transform infrared spectroscopy (FTIR). The work was then shifted to investigate the potentiality of Cr⁶⁺ adsorption with the prepared chitosan. The controlled parameters of adsorption process were studied. The percentage of Cr⁶⁺ removal using the shrimp chitosan was 64.29%.

Key words: Shrimp shells, chitosan, adsorption, chitin.

INTRODUCTION

Today water pollution is a major problem. There are many industries, such as textiles, leather, paper, plastics, electroplating, cement, metal processing, wood preservatives, paints, pigments, and steel fabricating industries (Shanker et al., 2005; Dima et al., 2015; Kim et al., 2015) that pollute water. Industrial waste water is a major source of various kinds of metal pollutants in natural water, such as lead (Pb), cadmium (Cd), zinc (Zn), and copper (Cu); these metals find their way into the water bodies through wastewaters (Mahvi et al., 2005). The presence of a low concentration of these heavy metals in water prevents the light and oxygen to penetrate into it. As a result, the photosynthetic activities are reduced in the aquatic environments (Alluri et al., 2007). These

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nature, highly selective, environmentally friendly, and has great adsorption ability due to bulky surface area. Chitin is the most abundant renewable, natural resource after cellulose. Chitin and its end product are biomolecules which have excessive potential, along with flexible biological activities that demonstrate biocompatibility and biodegradability. Chitosan (Figure 1) is a low-cost biopolymer that can be used as an ideal adsorbent for removing pollutants from the wastewater (Jung et al., 2007). It is the main module of the cell walls of fungi, the exoskeletons of crustaceans (crabs, lobsters and shrimps) and insects, the radulae of molluscs, and the beaks and internal shells of cephalopods, including squid and octopuses. Chitin is a long-chain homopolymer of the residues of N-acetyl-d-glucosamine, which are linked to each other by osidic-1, 4 bonds with the molecular formula of \((\text{C}_9\text{H}_{13}\text{O}_5\text{N})_n\) (Dutta et al., 2004). Chitin and its end products like chitosan are broadly recognized to have huge applications in several fields (Benhabiles et al., 2013a, b; Vakili et al., 2014; Bouhenna et al., 2015). Chromium (Cr) is a transition element located in the group VI-B of the periodic table with a ground-state electronic configuration of \(\text{Ar} \ 3d^{4}4s^1\). The stable forms of Cr are the trivalent \(\text{Cr(III)}\) and the hexavalent \(\text{Cr(VI)}\) species, although there are various other valence states which are unstable and short lived in biological systems \(\text{Cr(VI)}\) is considered the most toxic form of \(\text{Cr}\), which usually occurs associated with oxygen as chromate \(\text{CrO}_4^{2-}\) or dichromate \(\text{Cr}_2\text{O}_7^{2-}\) (Shanker et al., 2005).

Objectives of the present work are preparation of chitin and chitosan from the raw samples of shrimp shells, calculating the yield of chitin and chitosan from the raw samples of shrimp shells, characterization of the prepared Chitosan by using field emission scanning electron microscopy (FESEM) and Fourier transform infrared spectroscopy (FTIR) analyses and studying the effect of amount of adsorbent dose and contact time on the adsorption process.

**MATERIALS AND METHODS**

Fresh samples of shrimp shells were obtained from the local market (Rourkela). The samples were washed thoroughly with water and then dried for 24 h. Hydrochloric acid (analytical reagents, Rankem) and sodium hydroxides pellets (Rankem) were purchased from Rankem chemicals.

### Deproteinization

Deproteinization of chitin is the process in which 2% NaOH was used along with heating at 100°C. A magnetic bead was put inside the solution, and the process was carried out on the electromagnetic stirrer for a time period of 30 min. The solution was continuously stirred and heated for 30 min. The resulting solution was then washed several times with distilled water till the neutrality of the solution was obtained (that is, pH 7) and then it was washed with ethanol.

### Demineralization

Demineralization is the process in which dilute HCl (2% HCl solution) solution was used without heating. Mineral content present in the shells of the crustaceans is not the same for each of the species, so all the chitin resources do not require the same type of treatments. Here, the sample of shrimp shells was treated with HCl solution at ambient temperature. A magnetic bead had been put inside the solution, and the process was carried out on the magnetic stirrer for a time period of 30 min. The solution was continuously stirred. The resulting solution was washed for several times with distilled water till the neutrality of the solution was attained (that is pH 7) and then it was washed with ethanol. The demineralized sample was then filtered and dried in an oven for a period of 5 h at 60°C. The chitin synthesized was then weighed.

### Deacetylation

Deacetylation is the process with which chitosan can be prepared from chitin; which has been prepared from the raw and ground samples of shrimp shells. In this process, the sample is treated with concentrated NaOH (40%) (Sagheer et al., 2009). Detailed procedure followed for deacetylation was reported in author's previous publications (Suneeta and Kumar, 2014; Kumari et al., 2015).

### Preparation of stock solution

A stock solution of a heavy be prepared for the different batch experiments conducted for the adsorption process. 100 ppm of stock solution was prepared, that is, 100 mg (0.1 g) of chromium (\(\text{Cr}^{6+}\)) powder was added to 1000 ml of distilled water and the sample is taken in a beaker. The solution was stirred for producing a homogeneous concentration.

### Batch experimental procedure

The adsorption of heavy metal (\(\text{Cr}^{6+}\)) was studied using the chitosan sample (shrimp shells) in the batch operation for a contact time of 60 min. 30 ml of heavy metal solution was taken in the 150 ml conical flask and then a known amount of the chitosan was added to the conical flask. The conical flask was put into the shaker at 100 rpm. Sample of liquid (1 ml) was pipetted out at regular time interval of 60 min. Collected liquid sample was subjected to centrifuge till clear liquid was separated from chitosan. Using UV-Spectrophotometer at \(\Lambda_{max}\) 370 nm the absorbance of clear liquid sample was estimated. In order to obtain the dye concentration, the calibration curve was plotted, and the absorbance of the unknown dye solution obtained from spectroscopic analysis was used to estimate the dye concentration.

**Figure 1.** Structure of chitosan and combine with Cr(VI) (Samiey et al., 2014).
Figure 2. Shrimp shell chitin and chitosan (2% HCl and 2% NaOH).

Study of the effect of contact time
For the study of the effect of contact time on the adsorption of Cr\(^{+6}\) over chitosan, 30 ml from 100 ppm Cr\(^{+6}\) solution was taken in a conical flask and 0.1 g of chitosan (shrimp) is added in the flask at solution pH. The flask was kept at 25°C in the shaker at 100 rpm shaking speed. Then, the samples were pipetted out at the interval of 60 min. The chromium concentration in the samples collected was analyzed by using UV-spectrophotometer.

Study the effect of adsorbent dosage
Effect of adsorbent dosage on the adsorption of Cr\(^{+6}\) was studied by taking 30 ml from 100 ppm Cr\(^{+6}\) solution in a conical flask, and then different amounts of chitosan were added in different conical flasks at certain pH. Conical flask was kept in a shaker at 100 rpm at a temperature of 25°C, the samples were collected at a time interval of 60 min to obtain the Cr\(^{+6}\) concentration remaining in the solution after adsorption.

Characterization of chitosan

**FTIR**

Infrared spectra were obtained using a Perkin-Elmer type FTIR 1000 spectrometer at room temperature and using KBr pellet scanning method. Pellets were scanned at room temperature (25°C) in the spectral range of 400 to 4000 cm\(^{-1}\).

**FESEM**

FESEM characterizes a wide range of samples with unique low vacuum capabilities and ultra-high resolution low voltage imaging; low voltage [1 kV] resolution is 1.4 nm in high vacuum mode, while for non-conductive materials, the Nova Nano SEM is unique in offering the highest resolution (1.8 nm) at low voltages (3 kV).

RESULTS AND DISCUSSION

**FTIR**

The FTIR spectra of chitin and chitosan are shown in Figure 2. FTIR pattern of shrimp chitin was presented for better comparison. Chitosan has exhibited FTIR peaks at 3260, 3100, 2917, 2877, 2342, 1615, 1551, 1375, 1295, 1080, 1000 and 873 cm\(^{-1}\). The characteristic bands at 1551 and 1615 cm\(^{-1}\) and in the vicinity of 3093 and 3244 cm\(^{-1}\) correspond to the stretching vibration of C=O and NH in (NHCOCH\(_3\)), respectively. Complete demineralization was confirmed by the absence of mineral associated bands. Chitin has exhibited FTIR peaks at 3244 cm\(^{-1}\), 3093 cm\(^{-1}\), 2917 cm\(^{-1}\), 2334 cm\(^{-1}\), 1615 cm\(^{-1}\), 1551 cm\(^{-1}\), 1368 cm\(^{-1}\), 1264 cm\(^{-1}\), 1016 cm\(^{-1}\) and 889 cm\(^{-1}\). In chitosan, the absorption feature observed around 1623 cm\(^{-1}\) (bending vibration of NH of R-NH\(_2\)) indicates the high degree of deacetylation (Sagheer et al., 2009; Kumari et al., 2015). The degree of deacetylation of chitosan was found to be 65 %. The degree of deacetylation was calculated by using FTIR.

**FESEM**

The extracted shrimp shell chitosan was observed to
have layers of flakes, and its porous nature could be seen in some areas. In some portions of chitosan, fibril structures can easily be distinguished. With the increased magnification, crumbling flakes were observed with fibril structures in some portions of chitosan, similar observation reported in the study of Yen et al. (2009) (Figure 3).

**Effect of adsorbent dose**

The effect of adsorbent dosage on the adsorption of Cr\(^{6+}\) has been studied by taking 30 ml of 100 ppm Cr\(^{6+}\) solution in a conical flask, and different amounts of chitosan (that is, from 0.1 to 0.4 g) were added in different conical flasks. Separate studies have been carried out for different samples of chitosan (that is, chitosan from shrimp at pH=4, which is acidic medium). The conical flask was kept at 25°C in the shaker at 100 rpm for the time period of 30 min to obtain the uniformity in the solution after adsorption. The graph obtained was shown in Figure 4. As the dose of the adsorbent increased, keeping the time and speed of the shaker (rpm) constant, the adsorption of Cr\(^{6+}\) was also found to be increased, that is, percentage of removal of Cr\(^{6+}\) followed an increasing trend with the increased amount of adsorbent taken. Removal of Cr\(^{6+}\) was found to be 73.89% at a chitosan dosage of 0.4 g, that is, the Cr\(^{6+}\) removal is 5.54 mg/g of shrimp chitosan.

**Effect of contact time**

For the study of contact time on the adsorption of chitosan, 30 ml of 100 ppm Cr\(^{6+}\) solution was taken in a conical flask and 0.1 g of chitosan (shrimp) was added in the flask at solution pH (pH= 4, which is acidic medium). The flask was kept at 25°C in the shaker at 100 rpm shaking speed for time period of 60 min. The sample (1 ml) was pipetted out at the interval of 60 min to obtain the concentration remaining in the solution after adsorption. From the graph shown in Figure 5, it was found out that by keeping the amount of adsorbent and speed of the shaker (rpm) constant as the time progresses, the concentration of the heavy metal solution decreased, but after 2 h of the time, there was no significant change in the percentage of removal. The percentage of removal of the heavy metal for the shrimp chitosan was found as 64.29% (The Cr\(^{6+}\) removal is 19.29 mg/g of shrimp chitosan).
Shrimp shell waste is a good source of chitin and chitosan synthesis. Deproteinization, demineralization of shrimp shell, followed by deacetylation resulted in the formation of chitosan and chitosan formation is confirmed from the FTIR patterns. The percentage of removal of Cr\(^{6+}\) is increased with increase in adsorbent dosage (keeping the time and shaker speed (rpm) constant). Removal of Cr\(^{6+}\) was obtained at the maximum of 73.89% for the case of 0.4 g shrimp chitosan dosage (Cr\(^{6+}\) removal is 5.54 mg/g of shrimp chitosan). It is concluded that keeping the amount of adsorbent (0.1 g) and speed of the shaker (rpm) constant, with increased time of contact, the percentage removal of Cr\(^{6+}\) increased but after 2 h, no significant change in Cr\(^{6+}\) concentration was observed. 64.29% removal of Cr\(^{6+}\) was removed with 0.1 g shrimp chitosan dosage as adsorbent (Cr\(^{6+}\) removal is 19.28 mg/g of shrimp chitosan).

**Conflict of interests**

The authors have not declare any conflict of interest.

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**REFERENCES**


Influence of enzymes and ascorbic acid on dough rheology and wheat bread quality

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The combined action of ascorbic acid and two commercial enzymatic complexes containing amylase and xylanase/amylase was analyzed to determine their effects on dough rheology and bread quality. Seven bread formulations containing different concentrations of these improvers were used in the analysis. The rheological properties of each dough formulation were determined by moisture, gluten and farinograph tests. The breads were also characterized in general aspect - especially shelf-life - based on the presence of fungi. The dough rheology results showed that the formulation developed in the presence of 0.01% xylanase/amylase and 200 ppm of ascorbic acid was more efficient. Improved shelf-life was obtained from the formulations containing xylanase. The results showed that some technological characteristics of dough rheology and gluten index produced from the combination of these improvers can indicate in order to obtain specific features of the bread.

Key words: Food biotechnology, xylanase, amylase, dough properties, bread improvers.

INTRODUCTION

The development of baking technology is a phenomenon that has had a great impact on the food industry, and has increased the acceptance of food by the consumers (Asghar et al., 2011; Eddy et al., 2007). The use of different additives such as emulsifiers, oxidants and enzymes to improve the quality of bread is nowadays common practice (Nanditha and Prabhasankar, 2009; Asghar et al., 2011; Barrera et al., 2015). In Brazil the consumption of bread reaches 30 kg per capita. Due to this high demand, the Brazilian market imports about 50% of the wheat for domestic consumption from Argentina and Canada. The Brazil is the second largest importer of wheat in the world, consuming 9.5 tons per year (Scheuer et al., 2011). Different imported wheat blended with the national cultivars make it difficult to maintain the rheological properties of flour, as a
Table 1. Additives amount utilized in the breads formulations.

<table>
<thead>
<tr>
<th>Additives</th>
<th>Form 1</th>
<th>Form 2</th>
<th>Form 3</th>
<th>Form 4</th>
<th>Form 5</th>
<th>Form 6</th>
<th>Form 7</th>
<th>Form 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Life spring B (α-amylase)</td>
<td>-</td>
<td>-</td>
<td>0.02%</td>
<td>0.02%</td>
<td>-</td>
<td>0.05%</td>
<td>-</td>
<td>0.02%</td>
</tr>
<tr>
<td>Spring 2002 (Xylanase/Amylase)</td>
<td>-</td>
<td>-</td>
<td>0.01%</td>
<td>-</td>
<td>0.01%</td>
<td>0.02%</td>
<td>0.01%</td>
<td>-</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>-</td>
<td>200 ppm</td>
<td>200 ppm</td>
<td>200 ppm</td>
<td>200 ppm</td>
<td>500 ppm</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Without additives (-); Form (formulation); Form1, standard formulation. Are indicated the amount of additives to 200g of flour.

MATERIALS AND METHODS

Materials

The wheat bread was prepared using wheat flour Specht (Food Products Ltd., Joaçaba, Brazil; moisture content 10%, protein 14%, fat 2.2%, ash 1.8%, carbohydrates 72%), dry yeast (Leuvaft - Lesaffre, Rio Janeiro, Brazil), edible salt, sugar (Alto Alegre, São Paulo, Brazil), vegetable fat (Qualy, Paraná Brazil) and ascorbic acid (L-AA; Sigma-Aldrich).

The commercial enzymes used in the experiments were suitable for baking and produced by Granotec SA - Nutrition and Biotechnology (Curitiba, Brazil) with the following specifications: xylanase, endo-1,4-beta-xylanase produced by Aspergillus oryzae (spring 2002 product brand; 4,260 U/g) and α-amylase, a maltogenic alpha-amylase produced by Bacillus stearothermophilus (life spring B; 5,200 U/g).

Determination of enzymatic activity

The xylanase activity (Endo-1,4-beta-xylanase; E.C. 3.2.1.8) was measured according to Shah et al. (2006) and the α-amylase activity (α-1,4-glucanohydrolase, EC 3.2.1.1) was used the method of determining the starch saccharification activity, according Ammar et al. (2002). One unit of enzymes was defined as the amount of enzymes that produced 1 µmol of reducing sugar per minute under standard conditions.

Bread making process

The dough of the wheat bread was prepared using the following formulation: wheat flour, 200 g; sugar, 10 g; edible salt, 4 g; dry yeast, 0.15 g; fat, 20 g and water, 100 mL (Standard – without bakery additives). The test formulations were elaborated containing seven different combinations of enzymes and ascorbic acid (Table 1). The breads were produced in a standardized manner by the straight dough method, in which the ingredients were added to a planetary mixer (Arno, CL 390, São Paulo, Brazil). The dough was kneaded for 2 min at medium speed and then shaped by the cylinder (G Paniz, CS 390, São Paulo, Brazil). Each bread dough, obtained with different formulation (8 formulations drawn up in triplicate) was fermented for 2 h in a fermenter (Imeca, CS 390, Bauru, Brazil) at 35°C with 75% of humidity and baked for 25 min at 180°C at electrical cooker oven (Progás, Turbo Light intelligent PRP, Caxias do Sul, Brazil). Then breads were cooled for 30 min at room temperature, sliced and packaged for shelf-life analysis.

Determination of dough rheological properties

The rheological properties of each dough formulation were determined by moisture, gluten and farinograph tests. Analyses of wet, dry and index gluten were performed according to the AACC International Approved Method (AACC, 2000) No. 38-12, using the

consequence, dough and bread quality are affected (Scheuer et al., 2011). Similarly, there are many wheat varieties in the world, some of which are useful for bread making. Differences in baking quality of flours are affected by phenotypes and genotypes of wheat, and these factors determine the particular rheological properties of the bread (Gholamin and Khayatnezhad, 2011; Mirsaeedghazi et al., 2008).

Therefore, due to the variable technological quality of flour, the use of additives has become important to standardize the flour in terms of gluten strength, color and fermentability (Pecicová et al., 2010; Nanditha and Prabhaksanker, 2009). Likewise, the formation of a gluten network is essential for the production of bread with organoleptic qualities especially dough formation and bread crumb texture. The gluten network is responsible also for dough elasticity, resistance and stability, while carbon dioxide production is due to the action of enzymes and yeast on sugar (Enriquez et al., 2003; Aamodt et al., 2003). Thus, to improve the gluten network formation, the baking industry has been using flour improvers, among the oxidizing agents, which act directly on the structure of the gluten proteins, reinforcing the gluten network by the formation of disulfide bonds (Elkassabany and Hoseney, 1980).

Among the oxidizing agent quite studied is ascorbic acid. It was stated that it has influence on the fermented dough behavior and the correlation with the flour composition, such as the elucidation of the mechanism as improver on bread (Hrušková and Novotná, 2003; Every et al., 1999). Another type of wheat flour improvers are enzymes (Asghar et al., 2011). The enzymes most commonly used in baking are amylases, protease, glucose-oxidases and xylanases (Barrera et al., 2015; Moayedallaie et al., 2010; Kara et al., 2005; Martinez-Anaya and Jimenez, 1997). Thus, several studies have been conducted to elucidate the action of bread improvers and the combined effect of these food additives on bakery products (Stojeska and Ainsworth, 2008; Katina et al., 2006), but few have aimed at determining the effects of the combined action of ascorbic acid with xylanase and amylase on process. This study aimed to analyze the effects of ascorbic acid and enzymes on dough rheology properties and consequently bread quality. These formulations combine ascorbic acid and two commercial enzymatic complexes containing amylase and xylanase/amylase.
RESULTS AND DISCUSSION

Firstly the enzymatic activities of the two commercial enzymes for the presence of xylanase (spring 2002) and amylase (spring life B) was determined. According to the results, the additive for baking spring life B presented an α-amylase activity of 5,851 U/g, slightly higher than specified on the package (5,200 U/g). Moreover, the xylanase activity of the product spring 2002 was 1,898 U/g, significantly lower than the specified by the enterprise (4,260 U/g), and also showed amylase activity of 4,991 U/g (Figure 1), not informed by the manufacturer. This data is important because the combined use of enzymes can lead to the over-amylase; this is an overdose of α-amylase and in consequence leading to deleterious effects on bread (Van der Maarel et al., 2002).

In preliminary analysis (data not shown), the best concentration of each commercial improver was determined separately. The enzyme additives containing α-amylase (life spring B) or xylanase/amylase (spring, 2002) showed better results of bread volume and properties of dough rheological in a concentration of 0.02 and 0.01%, respectively, an intermediate concentration according to manufacturer's indications. Based on these results the design of the formulations was elaborated. The results of the analysis of dough rheological properties (farinograph, moisture and gluten) for the seven formulations tested showed that, in comparison with the standard formulation (control without improvers) or with each other, some parameters improved while other parameters worsened as summarized in Tables 2 and 3.

An important test to check the action of improvers on dough rheology is the farinograph (Malomo et al., 2011). As regards the farinograph characteristics analyzed, water absorption was not significantly different (p > 0.05) in the standard and test formulations (Table 2). However, dough development time was significantly lower for the formulation containing α-amylase and ascorbic acid (formulation 4), and longer for the formulation containing xylanase/amylase and ascorbic acid (formulation 5). Although a shorter development time indicates that the gluten network formation takes less time, with less energy input on the dough, on the other hand this may reflect a weakened dough and low gluten quality (Dua et al., 2009). These data are evidenced by the tolerance index, breakdown time and stability parameters, in which

Figure 1. Enzymatic activity of commercial preparations life spring B and spring 2002.
only formulation 5 (xylanase/amylase and ascorbic acid) showed a significant improvement (Table 2). The behavior of these parameters indicate that the flour is considered stronger (Dua et al., 2009), its power is improved as compared with control (formulation 1, without the additives) or with the other formulations.

Farinograph quality indicates the general quality of the mixture and water absorption in the dough of wheat flour. As might be expected, formulation 5 (Table 2) was improved, while formulations 3, 4 and 6 had the worst performance. The other formulations did not change significantly compared with the standard. The deterioration in bread quality can be explained by an excess of improvers or combinations of these, as had been expected for formulation 6, which contained excess of xylanase and especially of amylase (Van der Maarel et al., 2002). Although that bread formulations with different concentrations of xylanase does not present significant differences on bread specific volumes (Jaeckel et al., 2012), and of ascorbic acid even in excessive levels are not deleterious in dough (up 200 ppm), perhaps because of the limited presence of oxygen necessary for its action (Hrušková and Novotná, 2003). Formulation 3 shows a possible excess of amylase too since the enzyme is present in both additives - life spring B (5,891 U/g) and spring 2002 (4,991 U/g). According to Van der Maarel et al. (2002), the overdose of amylase makes the dough stickier due to the production of maltodextrin, damaging dough quality. Formulations 4 and 5, on the other hand, showed the highest volume and best structure and color crumb, according to visual comparison of the experienced bakers. In formulation 4, despite the worsening of the farinograph parameters (with the exception of development time), the action of amylase together with ascorbic acid possibly determined the final quality of the bread (Figure 2). At appropriate concentrations, this enzyme acts on the starch to increase fermentable sugars, interfering directly on bread volume (Katina et al., 2006), once the sugar released is one of the factors that affect the yeast cell growth and consecutively the production of CO₂ gas.

The presence of proteins is another important factor determining the quality of the flour or mixtures (Dua et al., 2009; Enriquez et al., 2003). This is because there is a direct correlation between protein percentage and gluten formation (Malomo et al., 2011), which finally affects bread quality (Ranhotra et al., 1992; Mirsaeeedghazi et al., 2008). In the gluten analysis, formulation 5 (which showed better results in a previous analysis) showed no statistically significant difference compared with control, despite having a higher amount of wet gluten (Table 3). However, in the analysis of dry gluten, formulations 3 and 4 showed a decrease, whereas other formulations showed no significant differences. Finally, in the gluten index analysis, formulation 6 showed a value similar to control, while formulations 5, 7 and 8 showed a lower value and formulations 1, 2 and 3 showed a significant increase compared to control (highlighted in Table 3).

The results therefore indicate that there is no direct correlation between index and wet gluten, as suggested by Enriquez et al. (2003). Although the gluten index parameter is indicative of gluten quality and gluten...
strength, this is not always associated with a large bread volume (Ranhotra et al., 1992). This was evidenced by formulation 5, which produced a bread with higher volume and the results of gluten analysis showed a lower gluten index. Evidently, this occurs because the factors involved in the final quality of bread are diverse and quite complex. Therefore, rheological parameters such as gluten index are not always able to account for final quality. Rouillé et al. (2000) showed that, despite the interference of different formulations containing ascorbic acid and alpha-amylase with hemicellulase activity, the mixing conditions appeared to be the main factor affecting bread volume. Thus, in our results, among the tested formulations on manufacture condition the best overall results presented was the formulation 5 (Figure 2, Tables 2 and 3).

In general, the analysis of the structure of the breads obtained with different formulations indicated that amylase (life spring B) was found to have a good impact on bread characteristics such as volume and visual structure, when compared to bread produced with standard formulation. As suggested by Ammar et al. (2002), the use of amylase on bread formulation to the obtainment of bread with a lower weight, higher volume and greater retention of softness, even after several days of conservation. Similarly, the bread made with the addition of xylanase/amylase (spring 2002) presented a smaller alveolar structure and larger volume in visual analysis of professional bakers (Figure 2). According to Shah et al. (2006), the use of partially purified xylanase from Aspergillus foetidus on dough has a positive influence on bread attributes such as crumb structure and loaf volume and promotes a significant improvement in textural properties, in accordance with the sensorial evaluation and the rheological properties. This improvement in texture is probably due to the redistribution of

![Figure 2. Effects of ascorbic acid and enzymes addition on characteristics of wheat bread.](image-url)
water, which increases the volume of the gluten and increases extensibility, resulting in a better oven spring (Shah et al., 2006). Bread formulations made from whole grain wheat flour and added with xylanase also had specific volumes significantly higher than those of the control sample (Jaekel et al, 2012). Bread obtained from formulation containing only ascorbic acid as improver has the volume visually higher and a more homogeneous alveolar structure as compared with the standard. Ascorbic acid is one of the most commonly used baking improvers due to its properties, which lead to an increase in dough strength and consequently in bread volume (Aamodt et al., 2003), acting specifically on the final rise (Shaheen et al., 2002), (Nakamura and Kurata, 1997).

Regarding the results of microbiological analysis performed the breads obtained with different formulations was not detected, the occurrence were filamentous fungi, yeasts, coliforms at 45°C or Salmonella sp. This is expected because after baking bread, microbiological levels decrease dramatically (Marin et al., 2002).

Another study performed was of the shelf-life determination. According to the results (data not showed) the bread control without the addition of improvers had a shelf-life of 7 days on average, showing high concentration of filaments fungi in this period. All formulations containing xylanase showed a statistically significant higher shelf-life, especially formulation 6 containing excess α-amylase, xylanase and ascorbic acid, which in all tests had a shelf-life of 13 days on average. These data require further study, especially to evaluate the correlation between the use of xylanase with a longer shelf-life. This analysis suggests that some combinations of improvers can be recommended for specific bread characteristics or applications. Examples of these applications are to obtain bread with higher volume, retard the retrogradation of bread, bleach bread, extended shelf-life or improve other parameters to improve baking performance.

Conclusion

The findings in this study have shown the potentiality of the combined use of improvers in bread production. Specific formulations in different combinations of ascorbic acid and xylanase and/or amylase were shown to produce particular effects on the rheological properties of dough. These combinations can be used to produce quality bread with specific characteristics. The formulation that showed the best dough rheology results was 0.01% xylanase/amylase (spring 2002) and 200 ppm of ascorbic acid. It produced good bread characteristics such as a large volume, as did the formulation combining α-amylase (life spring B) and ascorbic acid. All formulations containing xylanases showed increased shelf-life, most particularly the formulation containing excessive improvers which, despite an impaired dough rheology, showed the most extended shelf-life. A further study of the impact of these formulations on the sensory properties of bread is required, as well as additional studies on other rheological parameters, particularly to elucidate related biochemical processes or the ratio of the interactions between these improvers and the properties of different flours.

Conflict of interests

The authors have not declare any conflict of interest.

REFERENCES


Conflict of interests

The authors have not declare any conflict of interest.


Full Length Research Paper

**In vitro antifungal activity of *Dorstenia mannii* leaf extracts (Moraceae)**

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The leaves of *Dorstenia mannii* are used in traditional medicine in Cameroon and other African countries for the treatment of infectious diseases like malaria, skin rashes and stomach disorders. To substantiate this folkloric claim, the crude methanol extract and fractions from the leaves of *D. mannii* were investigated for their antifungal activity. The crude methanol extract was prepared from powdered dried leaves of the *D. mannii*. A portion was subjected to flash liquid chromatography on silica gel to afford test fractions. All test samples were screened for major groups of phytochemicals. Test samples and nystatin (reference) were evaluated for antifungal activity on ten yeasts using agar disc diffusion and broth microdilution methods. The qualitative analysis of crude methanol extract and fractions of *D. mannii* leaves revealed the presence of flavonoids, phenols, steroids and cardiac glycosides. In agar disc diffusion assay, seven of the ten pathogenic fungal strains were sensitive to the crude methanol extract (7/10), *n*-hexane ethyl-acetate (*Hex-EA*) (75%) (8/10) and ethyl-acetate (100%) (8/10). The minimum inhibitory concentrations (MICs) for the test samples varied from 80 to 1280 µg/ml. The crude extract and ethyl-acetate (100%) were the most active plant samples with both fungistatic and fungicidal effects (MIC/MFC values from 80 to 640 µg/ml) though not as the reference drug. *Candida tropicalis* was the least sensitive to the test samples. Some fractions exerted no fungicidal actions on *Cryptococcus neoformans*, *Candida lusitaniae* and *Candida tropicalis*. The present work shows that the crude methanol extract and fractions (*n*-hexane, ethyl acetate and residue) from the leaves of *D. mannii* possess growth inhibitory effect on pathogenic yeast. The active ingredients of this plant could be an addition to the antifungal arsenal to opportunistic fungal yeast pathogens.

**Key words:** Antifungal activity, *Dorstenia mannii*, yeasts, opportunistic candidiasis.

**INTRODUCTION**

Nowadays, fungal diseases have emerged and are being increasingly recognized as important public health problems owing to an ever-expanding population of immuno-compromised patients (Miceli et al., 2011). Fungal infections are usually associated with *Candida*, *Aspergillus* and *Cryptococcus* species but those due to *Candida* species represent the main opportunistic fungal infections worldwide, leading to high morbidity and
mortality in the population (Low and Rotstein, 2011). These changes are linked to the growing population of immuno-compromised patients. During the last three decades, Candida albicans has been the most prevalent pathogen in systemic fungal infections (Pfaller and Diekema, 2004). Presently, non albicans species of Candida account for more than 50% of fungal infections. The opportunistic yeast mostly reported are C. albicans, Candida tropicalis, Candida krusei, Candida parapsilosis, Candida kefyr, Candida glabrata, Candida dublensis and Candida rugosa and Cryptococcus neoformans (Banerje, 2009). Antifungal active principles are diverse and numerous, but few classes of them are currently available against yeast infections because many are toxic (Spaminiato and Leonardi, 2013). The high morbidity and mortality rates associated with opportunistic yeast infections indicate that current antifungal therapy to combat candidiasis is still ineffective. The current arsenal of antifungal drugs is exceedingly short and no new antifungal drugs are expected to reach the market any time soon (Pierce and Lopez-Ribot, 2013). Therefore, the discovery of new antimicrobial agents is still relevant. Among the potential sources of new agents, plants have long been investigated because they contain many bioactive compounds that can be of interest in therapy.

Dorstenia mannii Hook f. (Moraceae) is a perennial herb growing in the tropical rain forest of West Africa (Hutchinson and Dalziel, 1954). A decoction of the leaves is used for the treatment of many diseases, but mainly for rheumatism and stomach disorders (Bouquet, 1969). There are few pharmacological studies reported on D. mannii. In the genus of Dorstenia, there are many plant species that have been proven scientifically to exert antimicrobial properties (Ngadjui et al., 2000). Studies have shown that many species from the Dorstenia genus are present in different degree, and have antimicrobial activities (Swain et al., 1991; Abegaz and Ngadjui, 1999). Amongst others, in Cameroon, twigs of Dorstenia angusticornis have been tested for their in vitro antimicrobial activity (Kuete, 2007). Prenylated flavonoids isolated from D. mannii such as 6,8-diprenyleriodictyol (5), dorsmanin C (3) and dorsmanin F (7) were found to be potent scavengers of the stable free radical 1,1-diphenyl-2-picrylhydrazyl (Duffall et al., 2003). Compounds 3, 5 and 7 also inhibited Cu2+-mediated oxidation of human low density lipoprotein (Duffall et al., 2003). The twigs of Dorstenia elliptica have been tested for their in vitro antimicrobial activity against bacteria and fungi (Kuete, 2007). Some compounds from the twigs of D. mannii presented growth inhibitory activity mostly on Gram-negative bacteria and Mycobacteria (Mbaveng et al., 2012).

Outside these activities observed from this genus, traditional pharmacopoeia reveals other particular activities of certain species, such as anti-diabetic, anti-cholesterol, anti-oxidant and anti-malarial properties (Kuete, 2007). The twigs of Dorstenia barteri are used in Cameroon for their antimalarial properties (Iwu et al., 1992). In many African countries, the roots of Dorstenia barnimiana are used in the treatment of skin diseases (Iwu et al., 1992). The juices of the leaves of D. elliptica are used as eye drops in Congo (Bouquet, 1969). The decoction of leaves of Dorstenia poineettifolia is used to treat infected wounds (Tsopmo et al., 1998). The roots of Dorstenia contrajervia is used in Bolivia like an antitoxin against snake and insect bites and also to remove worms like tenea (Okunjji, 1998). It is equally used in Guatemala to treat diarrhea, in Costa-Rica, Brazil and Mexico to provoke menstruation (Logan, 1973). This same species is used in Argentina like a tonic. The roots of Dorstenia lanei are used as infusion against stomach ache, in mouth washing against tooth ache or in fraction mixed with the stems of Pycnanthus angolensis, plus small portion of white clay, to calm down head ache and fights rheumatism (Walker and Sillans, 1961). The roots of Dorstenia drakena present an anti-secretory effect, a diuretic activity, lachrymal activity, mydratic and spasmyloptic activities (Kyerematien et al., 1985). The decoctions of the leaves and roots of Dorstenia psilurus are used in the treatment of rheumatism, snake bites, against head ache and stomach ache (Bouquet, 1969).

To the best of our knowledge no study has investigated the effects of D. mannii leaf extract on yeast pathogens. Therefore, the present work was designed to assess the in vitro antifungal activity of D. mannii leaf extracts on some yeast pathogens.

**MATERIALS AND METHODS**

**Plant material**

The harvesting of plants was done at the Nkoljobe hill in April 2011, in Yaounde Centre Region of Cameroon Africa; geographic coordinates: 3° 52’ 0” North, 11° 31’ 0” East. The identification of the plants was developed at the National Herbarium in Yaoundé, Centre Region according to standard procedures, and were conserved under the voucher specimen reference number (No. 2135) at the National Herbarium.

**Plant extract preparation**

The air dried and powdered leaves of the D. mannii (1 kg) were soaked in methanol for 48 h at room temperature with constant temperature.
stirring. Then the extract was filtered using a funnel and a filter paper, and then kept in a flask. Removal of the solvent from the obtained extract under reduced pressure yielded 45 g of a dark-green residue that constituted the crude extract. A mass of 40 g of this organic extract was subjected to flash liquid chromatography on silica gel 60 (220 g), and eluted with n-hexane, hexane-ethyl acetate gradients (3:1), (1:1), (1:3), ethyl-acetate and finally with methanol to rinse the column, constituting the residue. A total of six fractions were made. The extracts were then kept separately in small bottles and put in the fridge to be used in the antifungal assay.

Phytochemical screening

The crude methanol extract and fractions were subjected to phytochemical screening using standard procedures (Sofowara, 1993).

Fungal strains and growth condition

The microorganisms constituted 10 yeasts (C. albicans ATCC 24433, C. albicans ATCC 2091, C. albicans ATCC 9002, C. tropicalis ATCC 750 Candida lusitaniae ATCC 200950, C. parapsilosis ATCC 22019, C. krusei ATCC 6258, Candida guillermondii, C. glabrata IP 35 and C. neoformans IP 95026). The reference strains (ATCC) were obtained from American Type Culture Collection (Rockville, USA). The two IP fungal strains were obtained from “Institute Pasteur” (Paris, France). The fungal strains were grown at 35°C and maintained on Sabouraud Dextrose Agar (SDA, Conda).

Preparation of paper discs and test solutions

Sterile discs of 6 mm in diameter were prepared from Fisher filter paper P5 (Catalog No. 09-801C). The discs were impregnated with the crude extract or fraction (10 μL) prepared using dimethylsulfoxide solution (DMSO) at the concentration of 5 mg mL⁻¹ (5 μg μL⁻¹).

Antifungal assay (Agar disc diffusion test)

The disc diffusion method (Chattopadhyay et al., 2001) was employed for the determination of antifungal activities of the crude extract and fractions prepared from D. manii leaves. Briefly, 0.1 ml of suspension of yeast containing 1.5 × 10⁶ spores/ml was spread on Sabouraud dextrose agar medium in 90 mm Petri dishes. Filter paper discs, 6 mm in diameter, were impregnated with 10 μl of test solutions (50 μg) and placed on the seeded plates. A negative control was prepared using the solvent (10% DMSO) employed to dissolve the plant extracts. Nystatin trade name Mycostatin® (10 μg/disc) was used as positive reference drug for fungi. The Petri dishes were sealed with parafilm and allowed for 30 min to permit migration of test samples before incubation at 35°C for 48 h. Antifungal activity was evaluated by observing the presence of growth inhibition zones around the paper discs. Each assay in this experiment was repeated three times.

Microdilution assay

The minimum inhibitory concentration (MIC) of the crude methanol extract and fractions were determined through broth microdilution method in 96-well micro-titre plates (Zgoda and Porter, 2001). The 96-well plates were prepared by dispensing into each well 100 μl of Sabouraud Dextrose broth. The test substances were initially prepared in a volume of 100 μl and each test sample was added into the first wells of the micro-titre plate. Serial two-fold dilutions of these test samples were made and 100 μl of inoculum standardized at 2.5 × 10⁵ CFU mL⁻¹ for yeasts (at 600 nm, Jenway 6105 UV/Vis spectrophotometer- 50 Hz/60 Hz) (Tereschuk et al., 1997) was then added into each well. The last wells (N°12) served as sterility controls (contained broth only) or negative control (broth plus inoculum). This gave final concentration range of 1280 to 2.5 μg/ml and 128 to 0.25 μg/ml for the methanol extract or fractions and reference substance, respectively. The plates were sealed with parafilm, then agitated with a plate shaker to mix their contents and incubated at 35°C for 48 h. The minimum inhibitory concentration (MIC) of each test sample was determined by visualizing the turbidity of the wells. The MIC corresponded to the lowest well concentration where no turbidity change was observed, indicating no growth of yeast. The minimum fungicidal concentration (MFC) was determined by adding 50 μl aliquots of the clear wells to 150 μl of freshly prepared broth medium and incubating at 35°C for 48 h. The MFC was regarded as the lowest concentration of test sample which did not produce a turbidity change as above. All tests were performed in triplicates.

Statistical analysis

Where applicable, the data were subjected to one way analysis of variance, and differences between samples at P ≤ 0.05 were determined by Student-Newmann-Keuls multiple range test using the Statistical Package for the Social Sciences (SPSS) program.

RESULTS

Phytochemical analysis

The qualitative analysis of the crude methanol extract and fractions of D. manii revealed the presence of some classes of compounds with potential antifungal activities (Table 1). Alkaloids, flavonoids, tannins, phenols, steroids and cardiac glycosides were detected. Coumarins and terpenoids were not detected in all the plant test samples. The fractions Hex (100%) and Hex-EA (25%) were poor in major groups of chemical constituents.

Antifungal activity

The crude methanol extract and some fractions from the leaves of D. manii showed antifungal activity. The growth inhibitory effect of these test samples on yeasts is shown in Table 2. Some of the test samples presented fungistatic and fungicidal activities though they were selectively active. Seven of the ten pathogenic fungal strains were sensitive to the crude methanol extract (7/10), Hex-EA (75%) (8/10), EA (100%) (8/10) as indicated with a plus sign “+”. The test samples that were sparingly active included Hex-EA (25%), Hex-EA (50%) and the residual fractions. No activity was observed for n-hexane (100%) at 5 μg/ml on all the tested yeasts for the discs diffusion assay. The MIC (μg/ml) and MFC (μg/ml) of the crude methanol extract and fractions from the leaves of D. manii on the tested yeast are presented in
Table 1. Phytochemical screening of *D. mannii* methanol extract and fractions.

<table>
<thead>
<tr>
<th>Groups of chemical constituents</th>
<th>Test substances</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Crude extract n-Hexane (100%)</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>+</td>
</tr>
<tr>
<td>Alkaloids</td>
<td>+</td>
</tr>
<tr>
<td>Coumarins</td>
<td>-</td>
</tr>
<tr>
<td>Phenols</td>
<td>+</td>
</tr>
<tr>
<td>Steroids</td>
<td>+</td>
</tr>
<tr>
<td>Saponins</td>
<td>+</td>
</tr>
<tr>
<td>Tannins</td>
<td>+</td>
</tr>
<tr>
<td>Chalones</td>
<td>+</td>
</tr>
<tr>
<td>Terpenoids</td>
<td>-</td>
</tr>
<tr>
<td>Cardiac glycosides</td>
<td>+</td>
</tr>
</tbody>
</table>

+ = Present; - = absent; Hex-EA, n-hexane ethyl-acetate.

Table 2. Antifungal activity of *D. mannii* methanol extract and fractions.

<table>
<thead>
<tr>
<th>Yeast</th>
<th>Test substances</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Crude extract n-Hexane (100%)</td>
</tr>
<tr>
<td>C. albicans ATCC 2091</td>
<td>+</td>
</tr>
<tr>
<td>C. albicans ATCC 9002</td>
<td>+</td>
</tr>
<tr>
<td>C. albicans ATCC 24433</td>
<td>+</td>
</tr>
<tr>
<td>Candida parapsilosis</td>
<td>+</td>
</tr>
<tr>
<td>Candida tropicalis</td>
<td>+</td>
</tr>
<tr>
<td>Candida krusei</td>
<td>-</td>
</tr>
<tr>
<td>Candida glabrata</td>
<td>-</td>
</tr>
<tr>
<td>Candida lusitaniae</td>
<td>-</td>
</tr>
<tr>
<td>Cryptococcus neoformans</td>
<td>+</td>
</tr>
<tr>
<td>Candida guillermondii</td>
<td>+</td>
</tr>
</tbody>
</table>

+ = Active; - = not active.

Table 3. In this microwell dilution assay, all the test samples demonstrated antifungal activity including the n-hexane (100%) fraction was previously not active. The MICs recorded varied from 80 to 1280 µg/ml. The crude methanol extract and EA (100%) were the most active plant test substances as they exerted both fungistatic and fungicidal effects on all the yeasts under study (MIC/MFC values from 80 to 640 µg/ml). *C. tropicalis* was the least sensitive yeast to the test samples from *D. mannii* leaves, followed by *C. krusei* and *C. guillermondii*. The reference substance (nystatin) inhibited the growth of all the yeasts under study with a MIC range from 1 to 4 µg/ml. Although, n-hexane (100%), Hex-EA (25%) and residue fractions inhibited the growth of selected yeast, their activities were not fungicidal on *C. neoformans*, *C. lusitaniae* and *C. tropicalis* respectively.

**DISCUSSION**

Many studies have confirmed the role of medicinal plants in health maintenance and promotion, but the major challenge is to provide scientific evidence (Arora and Kaur, 1999). Therefore, the present work was designed to assess the *in vitro* antifungal activity of *D. mannii* leaf extract on some yeast pathogens. The crude methanol extract and fractions from the leaves of *D. mannii* presented variations in antifungal activities across the tested fungal strains. These variations in antifungal activity could be due to the differences in the chemical
Table 3. Minimal inhibitory concentration (MIC)/minimum fungicidal concentration (MFC) of test substances (µg/ml).

<table>
<thead>
<tr>
<th>Yeast</th>
<th>Crude extract</th>
<th>n-Hexane (100%)</th>
<th>Hex-EA (25%)</th>
<th>Hex-EA (50%)</th>
<th>Hex-EA (75%)</th>
<th>Ethyl-acetate (100%)</th>
<th>Methanol residue</th>
<th>Nystatin</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. albicans ATCC 24433</td>
<td>80/320</td>
<td>640/640</td>
<td>160/640</td>
<td>80/320</td>
<td>80/320</td>
<td>80/160</td>
<td>320/640</td>
<td>1/4</td>
</tr>
<tr>
<td>Candida tropicalis</td>
<td>80/320</td>
<td>na/na</td>
<td>na/na</td>
<td>na/na</td>
<td>na/na</td>
<td>80/320</td>
<td>640/na</td>
<td>2/4</td>
</tr>
<tr>
<td>Candida glabrata</td>
<td>320/320</td>
<td>na/na</td>
<td>80/320</td>
<td>160/320</td>
<td>160/320</td>
<td>80/320</td>
<td>80/320</td>
<td>1/8</td>
</tr>
</tbody>
</table>

na = Not active.

The overall results obtained revealed that the crude methanol extract and fractions (n-hexane, ethyl-acetate and methanol residue) from the leaves of D. mannii in this study possess inhibitory effect on the growth of pathogenic yeast. These findings emphasize the evidence that frequent use of D. mannii leaf extracts could be an alternative to prevent infections from opportunistic fungal yeast pathogens.
Conflict of interests

The authors have not declare any conflict of interest.

ACKNOWLEDGEMENTS

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