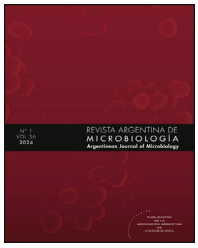
Revista Argentina de Microbiología xxx (xxxx) xxx–xxx

REVISTA ARGENTINA DE MICROBIOLOGÍA

www.elsevier.es/ram



ORIGINAL ARTICLE

Impact of *Kluyveromyces marxianus* VM004 culture conditions on the cell wall structure and its influence on aflatoxin B_1 binding

Carina Pereyra[a,b,*], **María del Pilar Monge**[c,d], **Silvestre Bongiovanni**[c,d], **Andrea Cristofolini**[d,e], **Sergio Campos**[f], **Lilia Cavaglieri**[a,d]

[a] *Departamento de Microbiología e Inmunología, Universidad Nacional de Río Cuarto, Ruta 36, Km 601, 5800 Río Cuarto, Córdoba, Argentina*
[b] *Instituto Para el Desarrollo Agroindustrial y de la Salud (IDAS), Member of Consejo Nacional de Investigaciones Científicas y Tecnológicas (CIC-CONICET), Argentina*
[c] *Departamento de Química, Universidad Nacional de Río Cuarto, Ruta 36, Km 601, 5800 Río Cuarto, Córdoba, Argentina*
[d] *Member of Consejo Nacional de Investigaciones Científicas y Tecnológicas (CIC-CONICET), Argentina*
[e] *Área de Microscopía Electrónica, Facultad de Agronomía y Veterinaria, Universidad Nacional de Río Cuarto, Ruta 36, Km 601, 5800 Río Cuarto, Córdoba, Argentina*
[f] *Departamento de Microbiologia e Imunologia Veterinária, Instituto de Veterinária, Universidade Federal Rural do Rio de Janeiro, Rod. BR 465, Km 7, Seropédica, 23890-000 Rio de Janeiro, Brazil*

Received 10 August 2023; accepted 1 July 2024

KEYWORDS
Aflatoxin B_1;
β-Glucans;
Cell wall;
Chitin;
Kluyveromyces marxianus

Abstract The aim of this study was to determine the impact of *Kluyveromyces marxianus* VM004 culture conditions on the cell wall (CW) structure and its influence on aflatoxin B_1 binding. The yeast was inoculated into two types of culture media: yeast extract-peptone-dextrose (YPD) broth and dried distiller's grains with solubles (DDG). The CW was extracted from the biomass produced in these media. AFB_1 (150 ng/ml) adsorption tests using the biomass (1×10^7 cells/ml) and the CW (0.001 g) were performed at pH 2 and pH 8. Transmission electron microscopy (TEM) evaluated the CW thickness, and infrared spectroscopy (IR) determined the CW composition. Biomass production in YPD was higher than that in DDG. Cell diameter (μm) and CW thickness (μm) increased in the DDG medium. The CW percentage obtained in DDG was higher than that in YPD. The absorbance of carbohydrates by IR was higher in YPD. pH influenced AFB_1 adsorption, which was lower at pH 8. The proportion of β-glucan and chitin present in CW was higher in the YPD medium. The IR method allowed to study the CW carbohydrate variation under the influence of these carbon sources. In conclusion, the culture media composition influenced the β-glucan and chitin composition and consequently, AFB_1 adsorption.

* Corresponding author.
E-mail addresses: cpereyra@exa.unrc.edu.ar, carinapereyra06@gmail.com (C. Pereyra).

https://doi.org/10.1016/j.ram.2024.07.004

Please cite this article as: C. Pereyra, M. del Pilar Monge, S. Bongiovanni et al., Impact of *Kluyveromyces marxianus* VM004 culture conditions on the cell wall structure and its influence on aflatoxin B_1 binding, Revista Argentina de Microbiología, https://doi.org/10.1016/j.ram.2024.07.004

PALABRAS CLAVE
Aflatoxina B_1;
β-Glucanos;
Pared celular;
Quitina;
Kluyveromyces marxianus

Impacto de las condiciones de cultivo sobre la estructura de la pared celular de *Kluyveromyces marxianus* VM004 y su influencia en la adsorción de la aflatoxina B_1

Resumen El objetivo de este estudio fue determinar el impacto de las condiciones de cultivo de *Kluyveromyces marxianus* VM004 en la estructura de la pared celular (PC) y su influencia en la adsorción de la aflatoxina B_1 (AFB_1). La levadura fue inoculada en dos medios de cultivo, extracto de levadura-peptona-dextrosa (YPD) y granos de destilería secos con solubles (DDG). De la biomasa obtenida en estos medios se extrajo la PC. Los ensayos de adsorción de la AFB_1 (150 ng ml^{-1}) utilizando la biomasa (1×10^7 cel ml^{-1}) y la PC (0.001 g) se realizaron con pH 2 y pH 8. El espesor y la composición de la PC se determinaron por microscopía electrónica de transmisión (TEM) y espectroscopía infrarroja (IR), respectivamente. La producción de biomasa en YPD fue superior a la lograda en DDG. El diámetro de las células (μm) y el espesor de la PC (μm) aumentaron en el medio DDG. El porcentaje de PC obtenido en DDG fue superior al obtenido en YPD. La absorbancia de carbohidratos por IR fue mayor en YPD. El pH influyó en la adsorción de la AFB_1, que fue menor al pH 8. La relación de β-glucano/quitina presente en la PC fue mayor en medio YPD. El método de espectroscopía IR permitió estudiar la variación de carbohidratos en la PC bajo la influencia de estas fuentes de carbono. En conclusión, la composición de los medios de cultivo incidió en la relación de β-glucano y quitina y, en consecuencia, en la adsorción de la AFB_1.

Introduction

In recent years, the so-called non-conventional yeast species have acquired relevance because of their positive contribution to food and beverages. *Kluyveromyces* species participate in the fermentation of beverages, dairy products, bakery products, sausages and various vegetables[11,29]. Among them, *Kluyveromyces lactis* and *Kluyveromyces marxianus* are considered generally recognized as safe (GRAS) microorganisms in the United States and have qualified presumption of safety (QPS) status in the European Union[16]. *K. marxianus* can grow on a variety of substrates and is the major producer of industrial enzymes for fermented foods and beverages, biofuels, and cell factory applications[30,32]. They are also a source of oligonucleotides, flavor enhancers in food products, prebiotics and immunostimulators[11]. In addition, they have been recognized as probiotic microorganisms[4,5]. Probiotics are defined as 'live microorganisms which, when administered in adequate amounts, exert a beneficial effect on the health of the consumer[7]. In the last years, functional foods that contain probiotic microbial strains responsible for health benefits in the host are being designed[6,11].

Moreover, mycotoxins are natural products, toxic secondary metabolites produced by filamentous fungi. The most important mycotoxins are aflatoxins (AFs), mainly represented by aflatoxin B_1 (AFB_1). Aflatoxins are produced by *Aspergillus* section *Flavi* group species. Economically and biologically the most important fungal species able to produce AFs are *Aspergillus flavus* and *Aspergillus parasiticus*[1,10]. Aflatoxins are among one of the most significant hazards to the feed supply chain and pose a threat to feed industries worldwide with a direct impact on feed safety, animal health and productivity. The occurrence of AFs is common in feed as natural contaminants in a variety of agricultural commodities of plant origin, especially in cereal grains, and are therefore often detected in animal feeds containing corn, soybean, and wheat, but can also be present in silage, haylage and pasture[33]. The ingestion of mycotoxin contaminated feeds can cause both acute and chronic diseases known as mycotoxicoses in most animal species such as pigs, dogs, cats, rainbow trouts, and ducklings[17].

Poor harvesting practices, improper drying, handling, packaging, storage, and transport conditions of cereals contribute to fungal growth and increase the risk of mycotoxin production. One of the strategies to prevent mycotoxicoses is the dietary supplementation with substances to make the toxin not available in the digestive tract and, therefore, to reduce its adverse effect. These strategies include the use of yeast cell wall (YCW) with potential to adsorb mycotoxins[23,34].

The YCW determines the shape of the cell and the integrity of the organism during cell growth and division. Its composition can vary under different growth conditions including type of culture, carbon source, temperature, pH, and aeration[19]. In particular, agroindustrial wastes, rich in carbohydrates and other nutrients, represent a good choice for microbial biomass biosynthesis[28]. *K. marxianus* was used in several biotechnological applications; however, no studies have shown the influence of the culture medium on the composition of the cell wall (CW) and its relationship with the adsorption of mycotoxins. Therefore, the aim of this study was to determine the impact of *K. marxianus* VM004 culture conditions on the structure of CW and its effect on AFB_1 binding.

Table 1 Dried distiller's grains (DDG) centesimal composition.

Dried distiller's grains (DDG)	
Components	Percentage
Dry matter	35.97
Water	64.03
Total protein	10.52
Total fats	4
Total fiber	3.08
Neutral detergent fiber (NDF)	3.76
Acid detergent fiber (ADF)	8.98
Starch	1.79
Sulphur	0.23
Phosphorus	0.32
Ashes	2.28

Materials and methods

Microorganism and culture media

K. marxianus VM004 was isolated from cheese whey, and characterized in our laboratory (and selected for its probiotic properties which were previously tested in *in vitro* and *in vivo* studies)[4,14].

K. marxianus VM004 was identified by molecular techniques through DNA extraction and sequencing of ITS regions: ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′), comparing sequences with the basic local alignment search tool (BLAST) within the National Center for Biotechnology Information (NCBI) database[4]. The strain was deposited in the National University of Rio Cuarto, Cordoba, Argentina (RC) collection center. The obtained sequences were deposited in GenBank under accession number KY421190 (see http://www.ncbi.nlm.nih.gov/nucleotide).

For the yeast growth, two culture media were used: yeast extract-peptone-dextrose broth (YPD) and dried distiller's grains with solubles (DDG). YPD medium was prepared according to the manufacturer's instructions. DDG was obtained from a local bioethanol producing plant. DDG medium was prepared by adding water (300 ml) to 75 g DDG, left at fluent steam for 20 min and then filtered. Then, yeast extract (1 g) and 0.1 g K_3PO_4 were added to 100 ml filtered DDG and autoclaved at 121 °C for 15 min. Its composition is shown in Table 1. The estimation of reducing sugars from the DDG medium was determined using the dinitrosalicylic acid (DNS) method. The amount of glucose from the DDG medium was 2.67 g/l while the amount of glucose in YPD was 20 g/l.

Biomass production

Yeast biomass production was performed following the methodology proposed by Nguyen et al. with some modifications[20]. The colony grown on malt extract agar (MEA) was inoculated into an *Erlenmeyer* flask (of 50 ml) containing YPD broth and incubated at 28 °C for 12 h to serve as a pre-inoculum. From this pre-inoculum, a cell suspension (1×10^7 cells/ml) was performed, and inoculated into five 500 ml *Erlenmeyer* flasks with 200 ml of medium each (total volume 1000 ml) and incubated at 28 °C for 12–14 h with aeration (150 rpm). Yeast cells were cultured to late exponential phase (about 12 h). They were harvested by centrifugation at 5000 rpm for 10 min, washed three times with water and twice with cold (4 °C) 0.1 M Na_2PO_4 buffer at pH 8.5. The biomass obtained from the YPD and DDG media was dried in forced air oven until constant weight and, the weight was expressed as g biomass/l culture medium. Means and standard error (SE) were compared using the Fisher's protected least significant test (LSD) ($p < 0.0001$). The analysis was conducted using Info Stat (version 2.03; University of Cordoba, Argentina) software.

Yeast cell wall preparation

Yeast cells used for the CW preparation were suspended in 0.1 M Na_2PO_4 buffer pH 8.5, and an equal volume of glass beads (0.45 mm diameter). They were cooled to 4 °C and broken by mechanical shaking for 30 s. The homogenate was cooled and disrupted for 30 s and this procedure was repeated five times. The glass beads were removed from the homogenate by decanting, and the CW was harvested by centrifugation at 5000 rpm for 15 min. The CW was washed five times with 0.1 M Na_2PO_4 buffer pH 8.5 and then, washed four times with distilled water. The temperature was kept below 4 °C during all operations. The CW was dried in forced air oven until constant weight.

Ultrastructural analysis of yeast cells

Yeast cell samples from the two different culture media were homogenized for 30 min, and centrifuged for 10 min at 10 000 rpm. The dry pellet was processed for TEM. The samples were processed following the methodology described in Pereyra et al.[25]. The sections were examined in an Elmiskop 101 transmission electron microscope (Siemens, Germany).

Infrared spectroscopy

The variation in the composition of the CW under the influence of the different culture media (YPD and DDG) was determined by the Fourier transformed infrared spectroscopy methodology. Dried CW (1 mg), mixed with potassium chloride (KCl) (200 mg), ground in an agate mortar and then, a tablet was made under pressure (≈15 ton cm^{-1}) applying dynamic vacuum for 15 min.

Measurements were performed in a Nicolet FTIR Impact 400 spectrometer. The spectrum includes an accumulation of 200 measurements to increase signal/noise ratio, and measured between 4000 and 400/cm with a resolution of 4/cm.

Aflatoxin B_1 determinations

Aflatoxin B_1 production, detection and quantification

Aflatoxins for *in vitro* assays were produced via the fermentation of rice by *A. parasiticus* NRRL 2999[13] (USDA, Agricultural Research Service, Peoria, IL). The fungus was grown on malt extract agar (MEA). AFs were extracted with chloroform following the procedure described in AOAC[3].

Table 2 Ultrastructural analysis of *Kluyveromyces marxianus* VM004: relationship between cell wall (CW) thickness and yeast cell diameter (μm).

Culture media	Ultrastructural analysis		
	Diameter of yeast cells (μm)	Thickness of yeast cell wall (μm)	Yeast cell wall thickness/cell diameter (μm)
YPD	2.83 ± 0.21^a	0.128 ± 0.017^a	0.045
DDG	4.58 ± 0.65^b	0.167 ± 0.031^b	0.036

YPD: yeast extract-peptone-dextrose broth; DDG: dried distiller's grains with solubles. The same letters do not indicate significant differences. Statistical analyses were performed for each column separately according to Fisher's least significant difference test (LSD) ($p < 0.0001$).

The sterile substrate (20 g) was placed in *Erlenmeyer* flasks and inoculated with 2 ml of an aqueous suspension containing 10^6 spores/ml. Cultures were allowed to grow for 7 days at 25 °C in the darkness and were shaken daily. On day seven, the *Erlenmeyer* flasks were autoclaved and the culture substrate was dried for 48 h at 40 °C in a forced air oven and then ground to a fine powder. The total AFs content in the culture extract was determined by high performance liquid chromatography (HPLC)[31]. The HPLC instrument was a Hewlett Packard chromatograph with a loop of 20 ml, equipped with a spectrofluorescence detector and a C18 column (Supelcosil LC-ABZ, Supelco; 150 mm, 4.6 mm, 5 mm particle size) connected to a precolumn (Supelguard LC-ABZ, Supelco; 20 mm, 4.6 mm, 5 mm particle size). AFB_1 was the only one produced in the highest concentration (60 μg/g). Levels of AFG_1, AFB_2 and AFG_2 were detectable but not quantifiable.

Aflatoxin B_1 adsorption

After the previously described incubation time, 1 ml 10^7 cells/ml or 0.001 g YCW was added to a microtube. The suspension was centrifuged to obtain a pellet and the supernatant was discarded. Cells or CW were washed with distilled water to remove traces of YPD or DDG broth. A pH 2 solution (1 ml) (containing 50 ml of potassium chloride 0.2 M and 13 ml of hydrochloric acid 0.2 M) and a pH 8 solution (containing 100 ml of 0.1 M KH_2PO_4 and 93.4 ml of 0.1 M NaOH) containing 150 ng/ml AFB_1 were added to each microtube containing the pellet. The mixture of yeast cells or CW and AFB_1 was homogenized and incubated on a rotary agitator at 37 °C for 40 min at 100 rpm. After the incubation period, the mixture suspensions were centrifuged and the supernatant containing the free toxin was transferred to another microtube. Controls only with AFB_1 and at each pH were obtained separately and performed in duplicates. The dose selected for AFB_1 was a proven one to reduce the productive performance in production animals[18].

Results

Biomass production

There were no significant differences in the biomass production of *K. marxianus* grown in YPD broth (4.98 g/l) and in DDG broth (4.24 g/l) ($p < 0.0001$).

Ultrastructural analysis of yeast cells after different cultural conditions by transmission electron microscopy

Table 2 shows the mean values of the yeast cell diameter and CW thickness for *K. marxianus* for each culture medium studied. The number of measurements for yeast cell diameter and thickness were 10 and 30, respectively. The yeast cell diameter and CW thickness of *K. marxianus* increased by 61.84% and 30%, respectively using DDG. The CW thickness/yeast cell diameter (μm) relationship was 25% higher using YPD.

Infrared spectroscopy of yeast cell wall

The spectra of the CW coming from the two culture media tested included three (3) regions: polysaccharides (950–1185/cm, proteins (1480–1700/cm) and lipids (2840–3000/cm) (Fig. 1).

β-Glucans and chitin present in the CW are the chemical structures implicated in the AFB_1 adsorption process. Figure 2 shows the IR spectra of β-glucans and amide bands from *K. marxianus* VM004 cultures in both culture media tested. The spectrum of YCW in YPD shows bands corresponding to —OH stretching and C—H stretching in the region between 2900 and 3500/cm. Furthermore, the infrared bands assigned to amide I and amide II (typical of chitin) were present at 1640 and 1562/cm, respectively. Vibration between C and O was identified in the region between 1000 and 1200/cm. β-Linked glycosidic bands were appreciable at 880/cm. In addition, in this case, (1-4)(1-6)-β-glucan absorption band was assigned at 765/cm. With regard to the spectrum of YCW taken in the DDG medium, similar peaks to those observed in YPD for —OH and C—H stretching were found at higher wavenumbers, being ~3400/cm and 2940; 2853/cm, respectively. Moreover, the infrared bands assigned to amide I and amide II (typical of chitin) were present at 1640 and 1560/cm, respectively. C—O—C and C—C stretching vibrations were also identified at 1200–990/cm. In addition, in-plane ring deformation of C—H and O—H bending modes was observed in the spectrum (1400–1200/cm). Vibrations related to (1-3)-α-D-glucan were observed at ~540/cm while the absorption corresponding to β-linked glycosidic bands was visible at 920–1060/cm. Table 3 summarizes the assignments of the different bands observed and

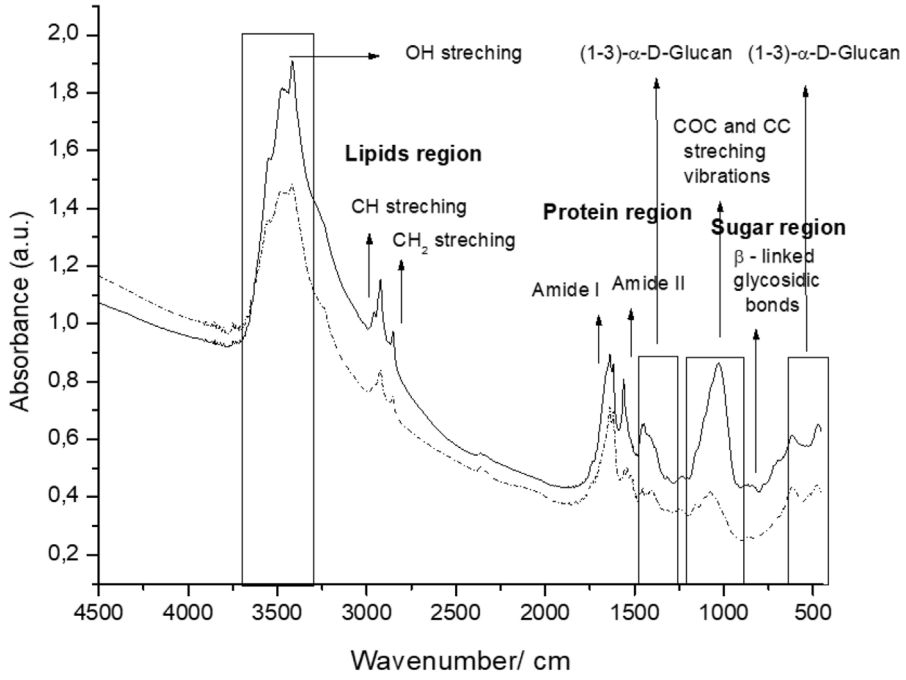


Figure 1 Infrared spectra of *Kluyveromyces marxianus* VM004 cell wall grown in YPD (-) and DDG (—) broth.

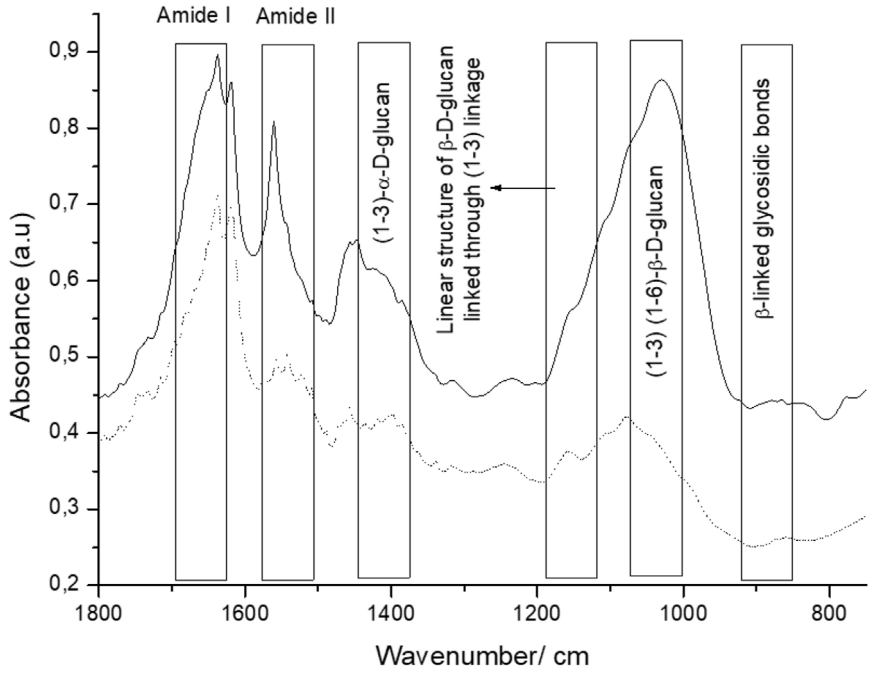


Figure 2 Infrared spectra of *Kluyveromyces marxianus* VM004 comparing β-glucans and amide bands of yeast cell wall from both culture media. YPD (-) and DDG (—) broth.

discussed in this work. Figure 2 shows a semi-quantitative comparison of the main infrared bands present in both culture media, with respect to β-glucans and amide bands.

Aflatoxin B_1 adsorption

The method was validated for linearity, accuracy, LOD and LOQ. Linearity of the method was tested by injecting three replicates (20 μl) of three levels of AFB_1 standard solutions (5–50 ng/ml). The accuracy of the method was determined by a recovery assay as described above and the average content of AFB_1 obtained was used to calculate the recovery percentage. The limit of detection (LOD) and limit of quantification (LOQ) for AFB_1 were calculated based on signal-to-noise (S/N) ratios of 3:1 and 15:1, respectively, but were experimentally obtained

Table 3 Functional groups resulting from curve fitting of the *Kluyveromyces marxianus* VM004 cell wall FITR spectra obtained using different culture media.

Functional groups	Wavenumber/(cm)	
	YPD	DDG
—OH stretching	3400	3400
C—H stretching	2940	2940
—OH_2 stretching (CH_2 of sugar)	2853	2853
Amide I (C═O) + (N—H) (indicative of chitine)	1640	1640
Amide II (N—H) + (C—N) (C═O) (chitine)	1562	1560
CH_2 in plane (CH_2OH of sugar)	–	1465
In-plane ring deformation including CH and OH bending modes	–	1400–1200
(1-3)-α-D-Glucan	–	1366
COC and CC stretching vibrations (characteristic of polysaccharides)	1200–990	1200–990
Linear structure of β-D-glucan linked through (1-3) linkage	–	1160 and 1074
(1-3)(1-6)-β-D-Glucan	1044	1060–1042
COC and CC stretching vibrations (characteristic of polysaccharides)	1200–990	–
(1-3)(1-6)-β-D-Glucan	1044	–
(1-3)(1-6)-β-D-Glucan	930	–
(1-3)(1-6)-β-D-Glucan	–	921
β-Linked glycoside bands	880	876
Referred to anomeric structure around glycosidic bonds	–	850
(1-3)-α-D-Glucan	–	850
(1-4)(1-6)-β-D-Glucan	765	770
(1-3)-α-D-Glucan characteristic	–	542
(1-3)-α-D-Glucan characteristic	–	454
(1-3)-α-D-Glucan characteristic	–	420

YPD: yeast extract-peptone-dextrose broth; DDG: dried distiller's grains with solubles.

Table 4 Aflatoxin B_1 adsorption by *Kluyveromyces marxianus* VM004 cell wall from different culture media and pHs.

Culture media	*K. marxianus* VM004 cell wall		
	(g)	Adsorption (%)	Media ± SD
		pH 2	pH 8
YPD	0.001	45.3 ± 7.5	29.6 ± 6.05
DDG		37.7 ± 0.7	6.5 ± 0

YPD: yeast extract-peptone-dextrose broth; DDG: dried distillers grains with solubles. The initial concentration was 159.3 ± 10.6 ng/ml.

injecting standard dilutions with the corresponding S/N ratio.

Table 4 shows the AFB_1 adsorption percentage using the yeast cell and the CW. In general, the adsorption percentage was influenced by the assayed pH. At pH 2 the adsorption was higher than that at pH 8 in the two culture media studied.

The adsorption percentage (mean ± SD) at pH 2 for *K. marxianus* VM004 grown in YPD broth was 8.40 ± 1.11% and at pH 8 the percentage decreased until 1.63%. Cells grown in DDG adsorbed 13.8 ± 2.3% at pH 2, while at pH 8 the adsorption was 5.2 ± 1.8% (data not shown). The extracted CW obtained from YPD adsorbed 45.3 ± 7.5% of AFB_1 at pH 2, and 29.6 ± 6.05% at pH 8. The extracted CW obtained from DDG adsorbed a lower percentage compared with YPD at both pHs studied. The CW adsorption from DDG at pH 2 was 37.7 ± 0.7% and 6.5 ± 0% at pH 8.

Discussion

The present study evaluated the influence of two carbon sources on the ultrastructure and composition of the cell wall of *K. marxianus* VM004 using transmission electron microscopy and IR spectroscopy, respectively, and its effect on the adsorption of AFB_1.

K. marxianus is a yeast widely used for different biotechnological applications, such as enzyme production, single cell protein, aromatic compounds, bioethanol, lactose reduction in foods, production of whey bioingredients, bioremediation, as anticholesterolemic agent, heterologous protein production, among others[32]. The *K. marxianus* strain used in this study for AFB_1 adsorption has demonstrated probiotic properties, such as resistance to gastrointestinal conditions, aggregation, coaggregation to pathogenic microorganisms, production of antimicrobial substances and as a substitute growth promoter for antibiotics, improving health and productive parameters of weaned piglets[4,14].

The intention of this study was to produce *K. marxianus* biomass in a reasonable way and compatible with commercial needs using an alternative medium such as DDG as a carbon source, which contained 2.67 g/l of glucose, while the YPD broth contained 20 g/l of glucose. Both culture media proved to be recommended for biomass and CW production. Yeast biomass was used for CW extraction in order to demonstrate AFB_1 adsorption. The percentage of CW from *K. marxianus* in DDG was similar to that extracted by Nguyen et al.[20] and higher than that extracted by Aguilar Uscanga et al.[2]. Nguyen et al.[20] used a medium based on yeast extract

(5 g/l) and glucose (50 g/l) and obtained values between 29.5 and 32.5% of CW and Aguilar Uscanga et al.[2] used different carbon sources (glucose, mannose, galactose, sucrose, maltose and ethanol) and found that the percentage of CW in dry weight was 10% for the culture performed with 25% ethanol in the sucrose culture.

Mycotoxin contamination represents a global problem in the animal feed industry, and several methods for preventing mycotoxicosis are being studied. Yeasts have great potential to reduce the economic damage caused by mycotoxin intake. Several studies have been reported on the biodegradation and adsorption of mycotoxins using different yeast species mainly *Saccharomyces cerevisiae*[8,15,21].

The use of *K. marxianus* as an adsorbent of nivalenol (NIV), zearalenone (ZEN) and deoxynivalenol (DON) has been demonstrated[27]. However, there are no reports of *K. marxianus* CW use as mycotoxin adsorbent. In this study, live cells and CW of *K. marxianus* VM004 were used to determine AFB_1 adsorption.

In this study, the adsorption was influenced by pH. The adsorption at acid pH was higher than at alkaline one. The influence of pH was observed in previous studies using commercial yeast CW, demonstrating that at pH 2 the AFB_1 adsorption was higher than at neutral pH[23]. Ye et al. showed that the adsorption of AFB_1 by humic acid polymers is pH dependent and that the transition to alkaline conditions can lead to desorption[35]. Pereyra et al. studied the influence of pH on the conidia wall of *Aspergillus niger* aggregate and its relationship with ZEN adsorption[24]. They showed that at pH 2 the ZEA adsorption was lower than at pH 6. pH could act by changing the composition of the yeast wall or the chemical structure of the toxin. The effect of pH is difficult to explain; future research should focus on the study of the influence of pH on the chemical groups of the CW and mycotoxin.

In the present work, the adsorption of AFB_1 using live cells and CW from *K. marxianus* VM004 in a simulated gastric and intestinal pH solution was studied. The percentage of AFB_1 adsorption using only CW was higher than using the living cell. However, it is important to note that the use of the living cell would have a probiotic effect in addition to the AFB_1 adsorbent. Yiannikouris et al. found that 6177 μg/ml of AFB_1 were adsorbed per 100 μg/ml CW[36]. Pereyra et al. studied the adsorption of AFB_1 with CW from commercial yeast (*S. cerevisisae*) applying mathematical models to explain the type of interaction of the toxin with the adsorbent[23]. They found values of 0.29 to 0.40 (g/g) at pH 2 and from 0.061 to 0.15 (g/g) at pH 6. These values are higher than those studied in our study at pH 2 and pH 8, but they are hypothetical values. Pereyra et al. studied the adsorption of AFB_1 using CW of probiotic strains of *S. cerevisiae* and *Saccharomyces boulardii*, obtaining similar values to those in this study[25].

Yeast CWs are composed mainly of polysaccharides, proteins and lipids that offer numerous functional groups for the interaction, such as carboxyl, hydroxyl, phosphate and amine groups, as well as hydrophobic adsorption sites, such as aliphatic chains and aromatic carbon rings[22]. For these reasons, the chemical structure of the toxin, the composition of the adsorbent and the pH of the medium where adsorption occurs must be taken into account to determine the adsorption efficiency.

It is known that the three-dimensional structure of the polysaccharides that make up the CW allows the adsorption of mycotoxins or their metabolic derivatives[37,38]. The interaction of mycotoxins with YCW is due to the helical conformation of (1-3)-β-glucans, which participate in Van der Waals bonds and hydrogen bonds, while (1-6)-β-glucans strengthen the Van der Waals bonds and stabilize the interaction[36].

In this study, the TEM technique was used to evaluate the relationship between CW thickness and cell diameter, to determine the proportion of CW in *K. marxianus* strain VM004. This relationship shows an accurate estimate of the CW content. The cell diameter was almost double in DDG. The use of DDG increased the CW thickness of the strain; however, the ratio of CW thickness and cell diameter ratio was better using YPD.

In this study the CW composition of *K. marxianus* VM004 was evaluated using IR spectroscopy. This is a fast, precise and accurate method, requiring little sample preparation, for the determination and quantification of the carbohydrates that make up the YCW[12,26]. The functional groups present in CW can be identified by this technique because each group has a unique energy absorption band[9]. With this technique it was possible to study the carbohydrate variation of CW under the influence of two carbon sources (YPD and DDG broth). Furthermore, the IR spectroscopy analysis indicated the presence of C—O, O—H and N—H groups, which are related to protein and carbohydrate components, mainly chitin and β-glucans involved in the adsorption of AFB_1.

Taking into account the results of the IR spectroscopy, a semi-quantitative comparison of the main infrared bands present in both cell cultures was done. In this sense, the bands referred to glucan and its derivatives (region between 1400 and 800/cm of the infrared spectra) were present in a higher proportion for the yeast grown in the YPD broth. Moreover, the amide bands of chitin were observed at 1640 and 1560/cm. In this case, a similar behavior than that observed for glucan could be noted. Based on these results, it is possible to confirm that the presence of both biomolecules is higher in the case of yeast that is evaluated in YPD broth than in DDG broth.

In particular, we were focused on the identification of β-D-glucan and chitin of the *K. marxianus* VM004 strain. In our studies, it was determined that the proportion of β-glucan and chitin was higher in the cells grown in YPD than those in DDG. However, the percentage of CW and thickness of the CW was higher in the cells grown in DDG. Taking these results into account, the amount of CW and its composition were relevant for the adsorption of AFB_1. Future studies should focus on the three-dimensional structure between β-glucan and chitin to determine how it affects AFB_1 adsorption.

Additional studies are required to better describe the mechanism of adsorption. It is necessary to identify all the chemical structures and composition not only of the CW but also of the toxin and the environment where they interact. Yianninkouris et al. demonstrated that β-D-glucans are the yeast components responsible for the complexation of zearalenone (ZEN), and that the reticular organization of β-D-glucans and the distribution between β-(1-3)-D-glucans and β-(1-6)-D-glucans play a major role in the efficacy[38]. On the other hand, Jouany et al. defined the molecular indices of the binding ability of a single helix of β-D-glucans for AFB_1[10]. The aromatic ring, the lactone and ketone groups of AFB_1 form polar or electron bonds with the glucose units

in the single helix of β-D-glucans, which maintain the toxin linked to the glucans. In our study, the presence of the different types of glucans responsible for adsorption and the presence of chitin was determined. Although the study is semi-quantitative, a ratio of 1 was observed between chitin (as indicated by the amide bands) and β glucans in the CW extract obtained from YPD. While in the DDG, the CW extract showed a higher presence of chitin than of beta glucans. Chitin is responsible for the rigidity of the CW. However, a high amount of chitin hinders the adequate three-dimensional conformation of β glucans for the effective adsorption of the mycotoxin. In another work, the adsorption of ZEN was studied using four strains of *S. cerevisiae* with different ratios of total β glucans and chitin where adsorption was a dependent strain and taking into account the percentage of insoluble β glucans[32].

In conclusion, this is the first time that the adsorption capacity of AFB_1 by a CW obtained from a probiotic strain of *K. marxianus* VM004 is reported. In addition, the use of DDG as a carbon source could replace a synthetic medium such as YPD for the production of biomass and CW intended to be used as an adsorbent of AFB_1. The use of *K. marxianus* VM004 CW as a mycotoxin adsorbent is a strategy to reduce animal exposure to mycotoxins.

Key contribution

There are no reports of *K. marxianus* cell wall use as aflatoxin B_1 adsorbent. In this study, live cells and cell wall of *K. marxianus* were used to determine the AFB_1 adsorption.

Conflict of interest

The authors declare that they have no conflicts of interest.

Acknowledgements

The authors are grateful to the Universidad Nacional de Rio Cuarto, Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina (CONICET), Agencia Nacional de Promoción Científica y Tecnológica (ANPCYT-FONCYT) which supported this study through grants.

References

1. Agriopoulou S, Stamatelopoulou E, Varzakas T. Advances in occurrence importance, and mycotoxin control strategies: prevention and detoxification in foods. Foods. 2020;137:1–48.
2. Aguilar Uscanga B, Solis Pacheco J, François J. Estudio de la variación de la composición de los polisacáridos contenidos en la pared celular de la levadura *Saccharomyces cerevisiae*. e-Gnosis. 2005;12:1–8.
3. Association of Official Agricultural Chemists (AOAC). Official methods of analysis 979.22 see column chromatographic procedure. Gaithersburg (MA): AOAC; 1994.
4. Díaz-Vergara L, Pereyra C, Montenegro M, Pena G, Aminahuel C, Cavaglieri L. Encapsulated whey-native yeast *Kluyveromyces marxianus* as a feed additive for animal production. Food Addit Contam. 2017;34:750–9.
5. Diosma G, Romanin DE, Rey-Burusco MF, Londero A, Garrote GL. Yeast from kefir grains: isolation, identification, and probiotic characterization. World J Microbiol Biotechnol. 2014;30:43–53.
6. Fadda ME, Mossa V, Deplano M, Pisano MB, Cosentino S. In vitro screening of *Kluyveromyces* strains isolated from Fiore Sardo cheese for potential use as probiotics. LWT Food Sci Technol. 2017;75:100–6.
7. FAO/OMS. Joint FAO/WHO expert consultation on evaluation of health and nutritional properties of probiotic in food including powder milk with live lactic acid bacteria; 2001.
8. Fiori S, Urgeghe P, Hammami W, Razzu S, Jaoua S, Migheli Q. Biocontrol activity of four non- and low-fermenting yeast strains against *Aspergillus carbonarius* and their ability to remove ochratoxin A from grape juice. Int J Food Microbiol. 2014;189:45–50.
9. Jin L, Bai R. Mechanisms of lead adsorption on chitosan/PVA hydrogel beads. Langmuir. 2002;18:9765–70.
10. Jouany JP, Yiannikouris A, Bertin G. The chemical bonds between mycotoxins and cell wall components of *Saccharomyces cerevisiae* have been identified. Arch Zoot. 2005;8:26–50.
11. Karim A, Gerliani N, Aïder M. *Kluyveromyces marxianus*: an emerging yeast cell factory for applications in food and biotechnology. Int J Food Microbiol. 2020;333:1–24.
12. Kuligowski J, Quintás G, Herwig C, Lendl B. A rapid method for the differentiation of yeast cells grown under carbon and nitrogen-limited conditions by means of partial least squares discriminant analysis employing infrared micro-spectroscopic data of entire yeast cells. Talanta. 2012;99:566–73.
13. Magnoli A, Monge M, Miazzo R, Cavaglieri L, Magnoli C, Merkis C, Cristofolini A, Dalcero A, Chiacchiera S. Effect of low levels of aflatoxin B_1 on performance, biochemical parameters, and aflatoxin B_1 in broiler liver tissues in the presence of monensin and sodium bentonite. Poult Sci. 2011;90:48–58.
14. Magnoli A, Parada J, de la Torre F, Watson S, Poloni V, Fochesato A, Martínez M, Coniglio M, Ortiz M, Cavaglieri L. Respiratory tract clinometry, fat thickness, haematology and productive parameters associated with direct-fed microbials used as growth promoter antibiotic alternative in weaned piglets. Vet Anim Sci. 2022;16, 100246.
15. Magnoli A, Rodriguez M, Poloni V, Peralta M, Nilson A, Miazzo R, Bagnis G, Chiacchiera S, Dalcero A, Cavaglieri L. Novel yeast isolated from broilers' feedstuff, gut and faeces as aflatoxin B_1 adsorbents. J Appl Microbiol. 2016;121:1766–76.
16. Marcišauskas S, Boyang Ji B, Nielsen J. Reconstruction and analysis of a *Kluyveromyces marxianus* genome-scale metabolic model. BMC Bioinformatics. 2019;20:1–9.
17. Martinez-Miranda M, Rosero-Moreano M, Taborda-Ocampo G. Occurrence, dietary exposure and risk assessment of aflatoxins in arepa, bread and rice. Food Control. 2019;98:359–66.
18. Murugesan G, Ledoux D, Naehrer K, Berthiller F, Applegate T, Grenier B, Phillips T, Schatzmayr G. Prevalence and effects of mycotoxins on poultry health and performance, and recent development in mycotoxin counteracting strategies. Poult Sci. 2015;94:1298–315.
19. Naruemon M, Romanee S, Cheunjit P, Xiao H, Mc Landsborough L, Pawadee M. Influence of additives on *Saccharomyces cerevisiae* β-glucan production. Int Food Res J. 2013;20:1953–9.
20. Nguyen TH, Fleet GH, Rogers PL. Composition of the cell walls of several yeast species. Appl Microbiol Biotechnol. 1998;50:206–12.
21. Papp LA, Horváth E, Peles F, Pócsi I, Miklós I. Insight into yeast–mycotoxin relations. Agriculture. 2021;1291:1–13.
22. Pascual A, Pauletto M, Giantin M, Radaelli G, Ballarin C, Birolo M, Zomeño C, Dacasto M, Bortoletti M, Vascellari M. Effect of dietary supplementation with yeast cell wall extracts on performance and gut response in broiler chickens. J Anim Sci Biotechnol. 2020;11:1–11.
23. Pereyra C, Cavaglieri L, Chiacchiera S, Dalcero A. The corn influence on the adsorption levels of aflatoxin B_1 and zearalenone by yeast cell wall. J Appl Microbiol. 2012;114:655–62.

24. Pereyra C, Cavaglieri L, Poloni V, Chiacchiera S, Cristofolini A, Merkis C, Dalcero AM. Effect of pH on ultra-structure of wall dead conidia of *Aspergillus niger* aggregate and their relation to the zearalenone adsorption capacity. World Mycotoxins J. 2016;9:389–95.
25. Pereyra C, Gil S, Cristofolini A, Bonci M, Makita M, Monge M, Montenegro M, Cavaglieri L. The production of yeast cell wall using an agroindustrial waste influences the wall thickness and is implicated on the aflatoxin B_1 adsorption process. Food Res Int. 2018;111:306–13.
26. Plata MR, Koch C, Wechselberger P, Herwig C, Lendl B. Determination of carbohydrates present in *Saccharomyces cerevisiae* using mid-infrared spectroscopy and partial least squares regression. Anal Bioanal Chem. 2013;405:8241–50.
27. Repečkienė J, Levinskaitė L, Paškevičius A, Raudonienė V. Toxin-producing fungi on feed grains and application of yeasts for their detoxification. Pol J Vet Sci. 2013;16:391–3.
28. Sharma NK, Beniwal V, Kumar N, Kumar S, Pathera AK, Ray A. Production of tannase under solid-state fermentation and its application in detannification of guava juice. Prep Biochem Biotechnol. 2014;44:281–90.
29. Tamang JP, Watanabe K, Holzapfel WH. Review: Diversity of microorganisms in global fermented foods and beverages. Front Microbiol. 2016;377:1–28.
30. Timmermans E, Langie I, Bautil A, Brijs K, Buvé C, Van Loey A, Scheirlinck I, Van der Meulen R, Courtin CM. Study of the fermentation characteristics of non-conventional yeast strains in sweet dough. Foods. 2023;830:1–16.
31. Trucksess M, Stack M, Nesheim S, Albert R, Romer T. Multifunctional column coupled with liquid chromatography for determination of aflatoxins B_1, B_2, G_1, G_2 in corn, almonds, Brazil nuts, peanuts and pistachionuts: collaborative study. J AOAC. 1994;6:1512–21.
32. Varela J, Gethins L, Ross P, Morrissey J. Applications of *Kluyveromyces marxianus* in biotechnology. In: Satyanarayana T, Kune G, editors. Yeast diversity in human welfare. Singapore: Springer Science+Business Media; 2017.
33. Xu R, Kiarie E, Yiannikouris A, Sun L, Karrow N. Nutritional impact of mycotoxins in food animal production and strategies for mitigation. J Anim Sci Biotechnol. 2022;13:69.
34. Xu R, Yiannikouris A, Shandilya UK, Karrow NA. Comparative assessment of different yeast cell wall-based mycotoxin adsorbents using a model- and bioassay-based in vitro approach. Toxins. 2023;15:104.
35. Ye SQ, Lv XZ, Zhou AG. In vitro evaluation of the efficacy of sodium humate as an aflatoxin B_1 adsorbent. Aust J Basic Appl Sci. 2009;3:1296–300.
36. Yiannikouris A, André G, Poughon L, François J, Dussap C, Jeminet G, Bertin G, Jouany J. Chemical and conformational study of the interactions involved in mycotoxin complexation with β-D-glucans. Biomacromolecules. 2006;7:1147–55.
37. Yiannikouris A, François J, Poughon L, Dussap C, Bertin G, Jeminet G, Jouany JP. Alkali extraction of β-D-glucans from *Saccharomyces cerevisiae* cell wall and study of their adsorptive properties towards Zearalenone. J Agric Food Chem. 2004;52:3666–73.
38. Yianninkouris A, Francois J, Poughon L, Dussap C, Jeminet G, Jouany JP. Adsortion of zearalenone by beta-D-glucans in the *Saccharomyces cerevisiae* cell wall. J Food Prot. 2004;67:1195–200.