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Detoxification of Patulin by Kombucha tea culture

La desintoxicación de patulina mediante cultivo de té de Kombucha

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Kombucha is a refreshing beverage, obtained by fermenting sugared tea with a symbiotic culture of acetic acid bacteria and yeast, consumed for its positive effects on human health. In this study, the potential of kombucha supernatant for reduction of patulin (PAT) of three toxigenic fungal strains (*Penicillium expansum* LC015096, *Talaromyces purpureogenus* LC015095, and *Acremonium implicatum* LC015097) in liquid medium and apple fruit was investigated. In liquid medium, kombucha up to 10% (v/v) significantly inhibited PAT production of *P. expansum* by 77.2% and that of *T. purpureogenus* and *A. implicatum* by 100%. In apple fruit, inhibition percent of PAT accumulation by the respective fungal strains was 49.8%, 100%, and 53%. In aqueous solution, kombucha cells showed a relative greater PAT uptake capacity than *Saccharomyces cerevisiae*. The maximum PAT uptake (64.67% and 60.69%) by viable and heat-treated kombucha cells was achieved at pH 3.0 throughout 48 h incubation, respectively.

Keywords: Kombucha tea; patulin (PAT); toxicity; apple; biosorption

La Kombucha es una bebida refrescante obtenida a partir de la fermentación del té endulzado con un cultivo simbiótico de acetobacterias y levadura, el cual es consumido por sus efectos positivos para la salud. En este estudio, se investigó el potencial de la Kombucha sobrenadante para la reducción de patulina (PAT) de tres cepas de hongos tóxicos (*Penicillium expansum* LC015096, *Talaromyces purpureogenus* LC015095 y *Acremonium implicatum* LC015097) en medio líquido y zumo de manzana. En medio líquido, la Kombucha con un porcentaje de hasta 10% (v/v) inhibió significativamente la producción de PAT de *P. expansum* en un 77,2% y la de *T. purpureogenus* y *A. implicatum* en un 100%. En el zumo de manzana, el porcentaje de inhibición de la acumulación de PAT mediante las cepas de hongos respectivas fue de 49,8%, 100% y 53%. En la solución acuosa, las células de Kombucha mostraron una mayor capacidad relativa de absorción de PAT que en *Saccharomyces cerevisiae*. La capacidad máxima de absorción de PAT (64,67% y 60,69%) mediante células de Kombucha viables y tratadas con calor se consiguió con un pH 3,0 mediante incubación de 48 h, respectivamente.

Palabras clave: Té de Kombucha; patulina (PAT); toxicidad; manzana; biosorción

Introduction

Kombucha is a traditional beverage of sugared black tea fermented with a symbiotic association of acetic acid bacteria and yeasts forming “tea fungus” for about 14 days (Jayabalan, Subathradevi, Marimuthu, Sathishkumar, & Swaminathan, 2008). Kombucha colony/mat represents a symbiotic relationship of *Acetobacter*, including *Acetobacter xylinum* as a characteristic species, and various yeasts, such as the genera of *Brettanomyces*, *Zygosaccharomyces*, *Saccharomyces*, and *Pichia* depending on the source (Mayer, Fromme, Leitzmann, & Gründer, 1995). Tea fungus broth is composed of two portions: a floating cellulosic pellicle layer and the sour liquid broth (Jayabalan, Malbaša, Lončar, Vitas, & Sathishkumar, 2014). The beverage has been claimed to be a prophylactic agent and to be effective against metabolic diseases, arthritis, indigestion, and various types of cancer (Sreeramulu, Zhu, & Knol, 2000). Beneficial effects and antioxidant activities of kombucha are attributed to a variety of active components and micronutrients produced during fermentation (Jayabalan et al., 2014).

Patulin (4-hydroxy-4H-furo[3,2-c]pyran-2(6H)-one) (PAT) is a secondary toxic metabolite produced by several species of filamentous fungi belonging to the genera of *Aspergillus*,

Byssoschlamys, *Gymnoascus*, *Paecilomyces*, and *Penicillium* (Frisvad & Thrane, 1996). PAT causes several human health effects including convulsions, nausea, ulceration, lung congestion, and epithelial cell degeneration in addition to its carcinogenic, genotoxic, immunotoxic, immunosuppressive, and teratogenic effects (Sant’Ana, Rosenthal, & Massaguer, 2008). Moreover, it has been reported to act as a phytotoxin (Ismaiel & Papenbrock, 2014). PAT has been detected in apples and apple products, and occasionally in other fruits such as pears, apricots, peaches, and grapes; being produced in the rotten parts of these fruits (Sanderson & Spotts, 1995; Sant’Ana et al., 2008). The European Union has set levels of PAT at 50 $\mu\text{g kg}^{-1}$ for fruit juices and apple juice ingredients, 25 $\mu\text{g kg}^{-1}$ for solid apple products (including apple compote, apple puree), and 10 $\mu\text{g kg}^{-1}$ for products intended for infants (European Commission, 2006).

Considerable attention has been paid to remove PAT from food and feed. Elimination of PAT by chemical methods (chemical addition of, for example, ascorbic acid) and physical approaches (clarification, filtration, and radiation) bears many drawbacks including expensive cost, low efficiency, significant changes to physical and chemical properties of food product, and impact on the environment (Assatarakul, Churey, Manns, & Worobo, 2012; Sant’Ana et al., 2008). In spite of reports on

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PAT removal from aqueous solution by lactic acid bacteria (LAB) and *Saccharomyces cerevisiae* (Fuchs et al., 2008; Guo, Yuan, Yue, Hatab, & Wang, 2012; Hatab, Yue, & Mohamad, 2012), no data have been published on biosorption of mycotoxins by kombucha mat. Nevertheless, due to active ingredients of this beverage that may exert beneficial effects on human health, the present study was undertaken to investigate the potential impact of kombucha on decreasing PAT level produced by three fungal species (*Penicillium expansum*, *Talaromyces purpureogenus*, and *Acremonium implicatum*) in liquid medium and decaying apples. Biosorption of PAT from aqueous solution by live and heat-treated biomass of kombucha tea was examined as influenced by incubation time and pH of the solution.

Materials and methods

Kombucha culture

The tea fungus used in this study was a traditional culture, kindly provided by Dr. Laila M. Abdel-Aty, Department of Agriculture Microbiology, Faculty of Agriculture, Ain Shams University, Cairo, Egypt. The starter used was a symbiotic culture between yeast and acetic acid bacteria mainly *A. xylinum*.

Fungal strains

The fungal strains used in this study were *P. expansum* B11 (LC015096), *T. purpureogenus* B8 (LC015095), and *A. implicatum* A5 (LC015097). They were all recovered from decaying apple samples obtained locally from several supermarkets (Giza, Egypt). The first and second strains were isolated from Red Spur Delicious samples and the third strain was isolated from Golden Delicious sample. The macro and microscopic features of the recovered fungal isolates were extensively studied on Czapek's and malt extract agar media according to Domsch, Gams, and Anderson (1980). These strains were molecularly identified based on the sequence of 18S rRNA–28S rRNA (flanking the sequence of ITS1, 5.8S rRNA, and ITS2). Sequences homologous to that of isolated fungi were identified in the NCBI GenBank database using BLASTP and BLASTX programs (Altschul et al., 1997). *S. cerevisiae* AX012, a commercial dry yeast strain, was obtained from Egyptian Starch, Yeast and Detergent Co. (El-Seiouf, Alexandria) and used as positive reference control in PAT biosorption assay. All fungal cultures were maintained on potato dextrose agar (PDA, Difco, Sparks, OK, USA) slants and stored at 4°C.

Inoculum preparation

Fungal spores from 7-day-old cultures of *P. expansum*, *T. purpureogenus*, and *A. implicatum* were harvested separately by flooding of slants with sterile water containing 0.1% Tween 20 and gently scrapping off spores with a sterile glass rod. Spore concentration was adjusted to $2 \times 10^6 \text{ ml}^{-1}$ using a hemocytometer.

Cultivation of kombucha and preparation of supernatant

Black tea (1.2%) was added to boiling water and allowed to infuse for about 5 min after which infusions were filtered through sterile sieve. Sucrose (10%) was dissolved in hot tea and the preparation (200 ml) was left to cool, poured into 500 ml sterilized glass jars. The prepared tea was inoculated with 3%

(w/v) of freshly grown tea fungus that had been cultured in the same medium for 14 days and 10% (v/v) of previously fermented liquid tea broth aseptically. The jar was carefully covered with a clean cloth and incubated in dark at 30°C for about 14 days (Jayabalan et al., 2008). Kombucha tea was filtered and the fermented tea was centrifuged at 10,000 rpm for 10 min and the supernatant was applied in the detoxification tests of PAT after determination of the physicochemical attributes of kombucha culture.

Physicochemical analyses of kombucha culture

Kombucha tea culture cultivated for 14 days was analyzed for the following physicochemical analyses:

Final pH

The final pH of the fermented broth was measured using a pH meter with a glass electrode (Hanna instrument).

Titrateable acidity (TA)

It was determined by titration of 10 ml fermented broth against 0.1N NaOH using phenolphthalein as a visual indicator (Roussin, 1996).

Organic acids

Acetic, citric, gluconic, and lactic acids were precipitated in the kombucha supernatant by adding excess of calcium hydroxide, after which 0.1N H₂SO₄ was added drop by drop to the precipitates of calcium salts till completely dissolved. Organic acids were then separated on thin layer chromatographic (TLC) plates and determined as per method reported (Harborne, 1984) after spotting with authentic organic acids (HPLC-grade) and developing in methanol: 5M NH₄OH (4:1). The TLC plates were then dried in air and sprayed with bromothymol blue indicator (0.04 g in 100 ml of 0.01M NaOH) as visualizer. Each organic acid spot was scrapped-off and eluted with 5 ml distilled water then titrated against 0.01N NaOH using phenolphthalein as indicator.

Wet weight

It was measured by draining kombucha mat on Whatman no. 1 filter paper under vacuum until no free water was drained out (Sreeramulu et al., 2000).

Protein content

It was determined in kombucha supernatant using bovine serum albumin as standard, according to Lowry, Rosebrough, Farr, and Randall (1951).

Cellulose

The bacterial cellulose pellicles were harvested by removing them from jar surface, weighing, boiling in 5N NaOH, and soaking in distilled water for 24 h (three exchanges to remove NaOH). Cellulose was then dried at 60°C for 2 days and weighed (Shezad, Khan, Khan, & Park, 2009).

Preparation of kombucha mat and *S. cerevisiae* cells for PAT biosorption experiments

Kombucha mat was prepared as described by Razmovski and Šćiban (2008). It was washed with an adequate amount of distilled water to remove media components. One part of fresh mat was cut by sterile scissors in pieces 4 mm × 5 mm and used in the biosorption experiments. The other part was heat-inactivated at 100°C until a constant weight. After which it was ground and the obtained dry mat was in the form of thin leaves.

S. cerevisiae used in the binding assay of PAT was prepared according to Guo et al. (2012) with some modifications. The yeast strain was reactivated in sterile water at 30°C for 5 h then cultivated on yeast extract peptone dextrose (YPD) agar (1% yeast extract, 2% peptone, 2% glucose, and 2% agar) at 30°C for 24 h. A loopful of yeast strain was transferred aseptically to YPD broth and incubated at 30°C for 24 h at 120 rpm, after which cells were harvested by centrifugation at 5000 rpm for 15 min and diluted with distilled water until reaching a cell concentration of 1.0×10^9 cells ml⁻¹. To obtain heat-inactivated yeast, cells were heated at 100°C for 1 h.

Effect of kombucha supernatant on PAT production by fungal strains grown in liquid medium

Czapek-Dox's supplemented with 0.5% yeast extract (CDYE broth, pH 5.5) was prepared and sterilized in conical flasks of 250 ml capacity, each containing 100 ml medium. Kombucha supernatant was filter-sterilized through a 0.45 µm cellulose membrane. Different volumes of sterilized supernatant were added separately to broth medium to give concentrations of 1.0–10% (v/v). To each flask, conidial spore suspension of *P. expansum*, *T. purpureogenus*, and *A. implicatum* (0.2 ml containing 2×10^6 spores per ml) was inoculated separately under aseptic conditions. Inoculated flasks treated with sterile distilled water instead of kombucha supernatant served as a control for each fungal strain. Flasks were then incubated at 30°C for 12 days, after which biomass yield and PAT concentration of fungal cultures were estimated. For determination of the dry cell weight, the fungal cultures were filtered through pre-weighed Whatman no. 1 filter papers, and weighed after drying to constant weight at 70°C. For determination of PAT concentration, the filtrate was extracted and undergone the necessary chromatographic analyses, as described later.

Effect of kombucha supernatant on PAT accumulation by fungal strains inoculated in apples

Mature and healthy apple fruits belonging to varieties of Golden Delicious and Red Spur Delicious were surface sterilized in a 2% sodium hypochlorite solution for 2 min, washed thrice with sterile distilled water, and dried under aseptic conditions. Thereafter, apples of each variety were cut aseptically into small slices (each slice was 5 g in weight, 0.5 cm wide) and 10 slices (50 g, total weight) were placed in a sterile Petri dish. Kombucha supernatant was diluted in different volumes of sterile distilled water to obtain the desired concentrations at 8.0%, 9.0%, and 10% (v/v). A quantity of 10 ml of each concentration was added to apple slices to give a moisture content of 20%. Slice inoculation of two apple varieties with the spore suspension of the three fungal strains was performed on the basis of the type of fungal strain recovered originally from each apple variety. The Red Spur Delicious slices were inoculated separately

with 1 ml of conidial spore suspension containing 2×10^6 spores of *P. expansum* and *T. purpureogenus*. The Golden Delicious slices were inoculated with spore suspension of *A. implicatum* at the same spore concentration. Inoculated apple slices treated with sterile distilled water instead of kombucha supernatant served as control. Incubation was carried out at 30°C till appearance of necrotic lesions on slices by *P. expansum* and *T. purpureogenus* after 14 days and by *A. implicatum* after 21 days. After which, PAT was extracted and quantified (see later).

PAT biosorption assay

Preparation of PAT working solution

The separated PAT from TLC plates and standard solid PAT (Sigma-Aldrich, Taufkirchen, Germany) were separately dissolved in ethyl acetate to obtain a stock solution of PAT at 10 mg ml⁻¹ and stored in the dark at -20°C. The ethyl acetate was evaporated by nitrogen steam and PAT was suspended in phosphate buffered saline (PBS, pH 4.0) to obtain 40 µg ml⁻¹ of working solution.

Biosorption experiments

Biosorption of PAT from PBS medium by both kombucha and *S. cerevisiae* cells was investigated under the influence of pH and incubation time. Kombucha (viable and heat-inactivated, both 0.25 g of dry matter) and yeast (viable and heat-inactivated, both 1.0×10^9 cells ml⁻¹) samples were suspended in 5 ml of PAT (40 µg ml⁻¹) working solution, vortexed shortly for 5 s then incubated for 5, 12, 24, and 48 h at 35°C. The effect of pH on reduction of PAT by kombucha and yeast samples was tested after incubation at 48 h. The pH of PBS medium containing PAT was adjusted to 3.0, 4.0, and 5.0 with 2N HCl. This pH range was tested because PAT has a high stability in this range (Topcu, Bulat, Wishah, & Boyaci, 2010). PAT solutions either without kombucha or yeast cells served as controls. At the end of incubation period, all samples were centrifuged at 6000 rpm for 10 min and the supernatant fluids for PAT residues were quantified spectrophotometrically (see later). The biosorption efficiency was expressed, as follows:

% Reduction = $[C_0 - C/C_0] \times 100$, where C_0 and C represented the initial and residual concentrations of PAT, respectively.

Extraction and determination of PAT from fungal cultures and apple samples

The pH of the culture filtrate was adjusted to 2.0 with 2N HCl and defatted with *n*-hexane, after which PAT was extracted with an equal volume of ethyl ether that was filtered over anhydrous sodium sulfate and then evaporated under vacuum to near dryness (Ismail & Tharwat, 2014). For extraction of PAT from decayed apple tissues, the samples were weighed and distilled water was added in 1:1 (w/w) ratio. Thereafter, PAT was extracted with an equal volume of ethyl ether as described earlier. By using chromatographic columns (Sydenham et al., 1995), the residue was dissolved in ethyl acetate (30 ml) to which toluene (70 ml) was added. The solutions were then passed through chromatographic columns packed with 15 g silica gel. PAT was eluted with ethyl acetate: toluene (30:70; 200 ml), which was collected and evaporated under vacuum to dryness.

By using precoated TLC plates with silica gel GF-254, the dried crude extract was dissolved in absolute ethanol and loaded with reference standard of PAT (Sigma-Aldrich) using toluene: ethyl acetate:formic acid (6:3:1, v:v:v) as a developing system. PAT spots ($R_f = 0.56$) were revealed as yellow color by spraying with phenylhydrazine hydrochloride solution (2 g in 100 ml water) and heating for 15 min at 130°C (supplementary Figure S1). Spots were scrapped off and eluted in 5 ml *n*-butanol. Color intensity was measured at 540 nm and the concentration was obtained by recording the optical density against a standard curve (Ismaiel & Tharwat, 2014).

Statistical analyses

Results were expressed as the mean \pm standard deviation (SD). Statistical significance was evaluated using analysis of variance (ANOVA, SPSS software version 22, IBM Corp., Armonk, NY, USA) test followed by the least significant difference (LSD) test at 0.05 level.

Results

Growth of kombucha starter culture

After 2 weeks of fermentation of sugared black tea with 3% of starter culture (tea fungus), growth of kombucha cultures was in the form of a flat macroscopic colony on the surface of the tea solution, taking on the shape of the receptacle. A thick jelly

membrane of tea fungus (Figure 1(a)) has been produced which was floating on the surface of tea broth. The original “mother” cultures produce a secondary layer of growth often called “baby” cultures (Figure 1(b)). Simultaneously, the tea ferments, producing a mildly acidic, usually carbonated, beverage that tastes rather like cider (Figure 1(c)). During fermentation of sugared tea, a few gas bubbles appear from the carbonic acid formed (Figure 1(a)) and a floating cellulose network that resembles a surface mold was apparent on the top of tea where cell mass of bacteria and yeast is attached (Figure 1(c)).

Physicochemical analyses of kombucha tea culture

The results given in Table 1 summarize the physicochemical attributes of kombucha black tea grown on sugared black tea solution (12 g Γ^{-1} black tea and 100 g Γ^{-1} sucrose, initial pH = 5.0). After 14 days of fermentation, the wet weight of kombucha mat was 12.04 ± 0.6 g and the pH decreased to 3.14. The TA was 3.16 ± 0.01 ml NaOH 100 ml $^{-1}$ fermented broth. Both protein and cellulose contents were satisfactory recording 1.81 ± 0.07 g Γ^{-1} and 1.22 ± 0.02 g Γ^{-1} , respectively. Analysis of kombucha organic acids indicated that citric acid had the highest concentration (2.77 ± 0.18 g Γ^{-1}), followed by gluconic acid (1.86 ± 0.12 g Γ^{-1}) and acetic acid (1.83 ± 0.04 g Γ^{-1}). L-lactic acid was being the lowest (0.702 ± 0.02 g Γ^{-1}) showing significant differences ($P \leq 0.05$) with the other concentrations of organic acids.

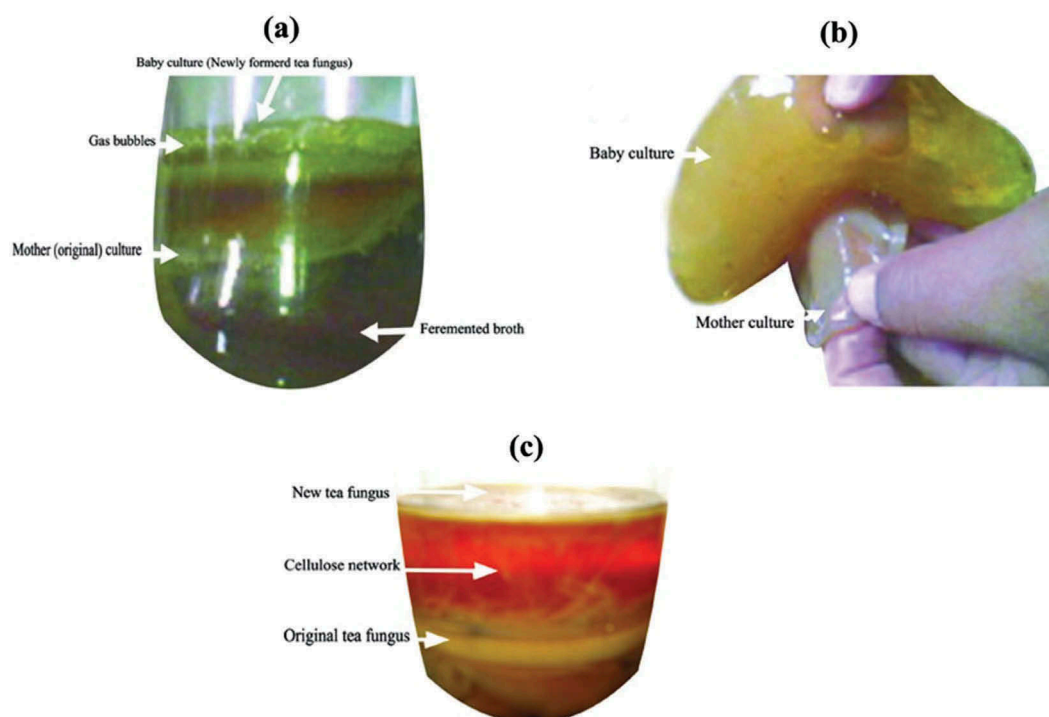


Figure 1. Photographs of kombucha culture grown on sugared black tea infusion after 14 days of incubation. (a) Kombucha black tea grown on 1.2% tea infusion sweetened with 10% sucrose showing a fermented broth and a floating daughter “baby” culture with a thick jelly membrane on the surface, a few gas bubbles develop from fermentation. (b) The mother and baby culture of kombucha after fermentation process on 1.2% tea infusion sweetened with 10% sucrose. (c) A cellulose network float between the original and new tea fungus after fermentation on 0.2% tea infusion sweetened with 10% sucrose.

Figura 1. Fotografías del crecimiento del cultivo de Kombucha en infusión de té negro endulzada después de 14 días de incubación. (a): El crecimiento de té negro de Kombucha en 1,2% de infusión de té endulzada con 10% de sacarosa mostrando un caldo de fermentación y un cultivo ‘bebé’ flotante con una gruesa membrana de gelatina en la superficie y unas pocas burbujas de gas provenientes de la fermentación; (b): Los cultivos madre y bebé de Kombucha después del proceso de fermentación con 1,2% de infusión de té endulzada con 10% de sacarosa; (c): Una red de celulosa flotaba entre los hongos originales y los nuevos de té después de la fermentación con 0,2% de infusión de té endulzada con un 10% de sacarosa.

Table 1. Physiochemical attributes of kombucha black tea culture after 14 days of fermentation at 30°C.

Tabla 1. Atributos físicoquímicos del cultivo de té negro de Kombucha después de 14 días de fermentación a 30°C.

Parameters	Level
Final pH	3.14 ± 0.01
TA (ml 100 ml ⁻¹)	3.16 ± 0.01
Wet weight of mat (g)	12.04 ± 0.6
Soluble protein (g l ⁻¹)	1.81 ± 0.07
Cellulose (g l ⁻¹)	1.22 ± 0.02
Organic acids (g l ⁻¹)	
Acetic acid	1.83 ± 0.04 ^b
Citric acid	2.77 ± 0.18 ^a
Gluconic acid	1.86 ± 0.12 ^b
L-lactic acid	0.702 ± 0.02 ^c

Notes: Calculated mean is for triplicate measurements from two independent experiments ± SD.

^{a-c}Means with different superscripts of organic acids in the same column are considered statistically different (LSD test, $P \leq 0.05$).

Notas: El promedio calculado es para las mediciones triplicadas de dos experimentos independientes ± SD.

^{a-c}Los promedios con diferentes superíndices de ácidos orgánicos en la misma columna se consideran estadísticamente distintos (test LSD, $P \leq 0,05$).

Influence of kombucha on production of PAT by fungal strains in growth medium

Results of the inhibitory activity of kombucha supernatant on dry cell weights and PAT concentrations of fungal strains grown in CDYE broth for 12 days are presented in Figure 2(a-c). In the

absence of kombucha supernatant (0%; positive control cultures), *P. expansum*, *T. purpureogenus*, and *A. implicatum* produced PAT in amounts of up to 7.41, 5.75, and 6.44 mg l⁻¹, respectively. The increase in kombucha supernatant concentrations (% v/v) caused a notable decrease in average values of both dry cell weights and PAT concentrations of treated fungal strains. At 5.0% kombucha supernatant, the dry cell weights of *P. expansum*, *T. purpureogenus*, and *A. implicatum* were significantly decreased by 36.0%, 21.7%, and 75.1%, respectively. At this concentration of kombucha, the PAT elaborated in culture filtrates of treated fungal strains was significantly reduced by 40.6%, 37.2%, and 70.2%, as compared with the respective control treatments. In the presence of 10% kombucha, PAT produced by *T. purpureogenus* and *A. implicatum* was completely inhibited, while PAT concentration produced by *P. expansum* was significantly reduced by 77.2%. At this higher concentration of kombucha, mycelial growth of *P. expansum* and *T. purpureogenus* was yet evident (0.92 and 1.45 g l⁻¹), while that of *A. implicatum* was completely inhibited.

Efficacy of kombucha in inhibition of PAT accumulation by fungal strains in apples

The effect of varying concentrations of kombucha supernatant (% v/v) on PAT-producing ability of the three fungal strains grown in apple samples is summarized in Table 2. Addition of kombucha supernatant to apple samples at concentrations 8.0–10% significantly reduced PAT accumulation ($P \leq 0.05$) in

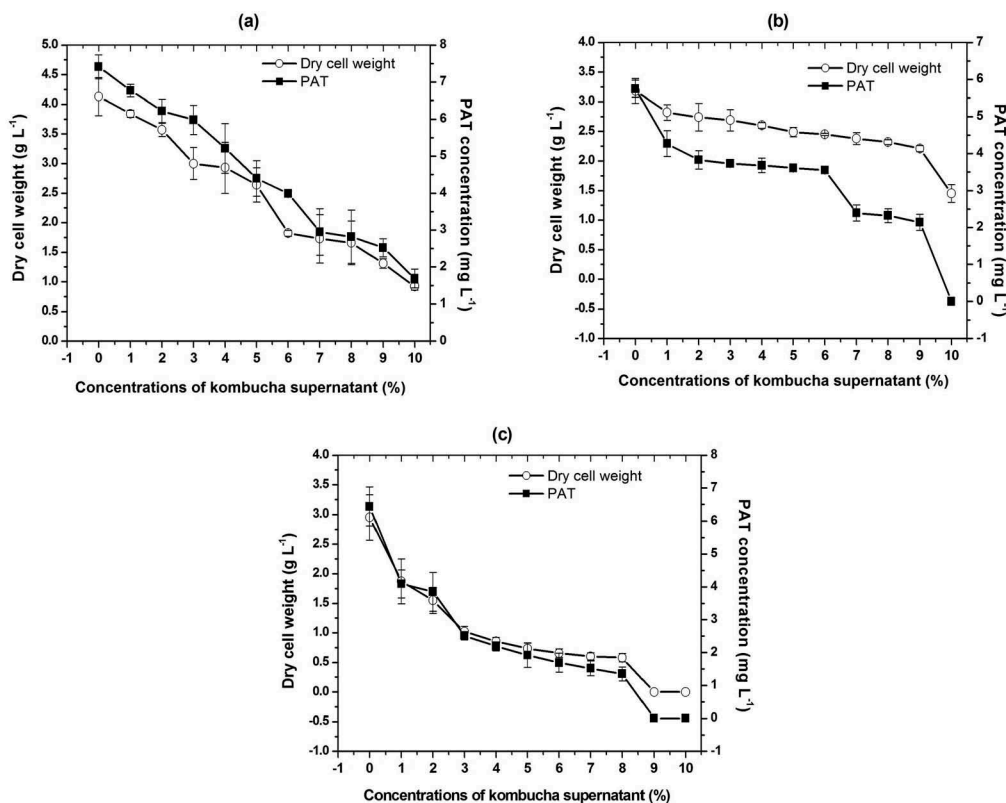


Figure 2. Effect of different concentrations of kombucha supernatant on growth and PAT production by (a) *P. expansum* LC015096, (b) *T. purpureogenus* LC015095, and (c) *A. implicatum* LC015097. Cultures were carried out at 30°C for 12 days in CDYE broth (pH 5.5). Data are shown as the mean ± SD of triplicate measurements from two independent experiments.

Figura 2. Efecto de las diferentes concentraciones de Kombucha sobrenadante en el crecimiento y producción de PAT mediante (a) *P. expansum* LC015096, (b) *T. purpureogenus* LC015095 y (c) *A. implicatum* LC015097. Los cultivos se realizaron a 30°C durante 12 días en el caldo CDYE (pH 5,5). Los datos se muestran como promedio ± SD de las mediciones triplicadas de dos experimentos independientes.

Table 2. Effect of fresh kombucha supernatant on PAT accumulation in local apples infected with *P. expansum*, *T. purpureogenus*, and *A. implicatum*.

Tabla 2. Efecto de el sobrenadante fresco de Kombuchaca en la acumulación de PAT en manzanas locales infectadas con *P. expansum*, *T. purpureogenus* y *A. implicatum*.

Local apples infected with	PAT production (mg kg ⁻¹) (% of reduction)			
	0.0%	8.0%	9.0%	10%
<i>P. expansum</i>	5.68 ^a	3.69 (35.0) ^b	3.49 (38.5) ^b	2.85 (49.8) ^b
<i>T. purpureogenus</i>	14.5 ^a	12.3 (15.1) ^b	5.93 (59.1) ^c	0.00 (100) ^d
<i>A. implicatum</i>	8.39 ^a	7.13 (15.0) ^b	4.36 (48.0) ^c	3.94 (53.0) ^c

Notes: Incubation was carried out at 30°C till appearance of the necrotic lesions on apple slices by *P. expansum* and *T. purpureogenus* after 14 days and by *A. implicatum* after 21 days, calculated mean is for triplicate measurements from two independent experiments ± SD.

^{a-d}Means with different superscripts in the same row are considered statistically different (LSD test, $P \leq 0.05$).

Notas: La incubación se realizó a 30°C hasta que aparecieron lesiones necróticas en los trozos de manzana mediante *P. expansum* y *T. purpureogenus* después de 14 días y mediante *A. implicatum* después de 21 días, el promedio calculado es para las mediciones triplicadas de dos experimentos independientes ± SD.

^{a-d}Los promedios con diferentes superíndices en la misma fila se consideran estadísticamente distintos (test LSD, $P \leq 0,05$).

the three fungal strains. The effect of kombucha on PAT accumulation in apples was dose-related, however concentrations lower than 8.0% showed no inhibitory effect on PAT accumulation. In the presence of 9.0% kombucha, there was 38.5%, 59.1%, and 48% inhibition of PAT production by *P. expansum*, *T. purpureogenus*, and *A. implicatum*, respectively. At 10%, the inhibition percent was increased to 49.8%, 100%, and 53%, respectively.

Biosorption of PAT from aqueous solution by kombucha compared with *S. cerevisiae* cells

The effects of incubation time on PAT biosorption by kombucha (viable and heat-treated cells) as compared with *S. cerevisiae* (viable and heat-treated cells) are given in Table 3. Removal percent of PAT from aqueous solution was significantly and continuously increased with incubation times ($P \leq 0.05$), which ranged between 33.33–44.77% and 7.46–11.94% after 5–12 h incubation for viable and heat-treated kombucha mat, respectively. After 24–48 h incubation, PAT removal rate increased between 48.25–57.22% and 25.87–40.76%, respectively. Comparable values of PAT removal percent were obtained by *S. cerevisiae* cells with respect to incubation time. After 5–12 h incubation, the removal percent ranged between 24.31–38.82% and 9.41–17.65% for viable and heat-treated yeast cells, respectively. After 24–48 h incubation of viable and heat-treated yeast cells in PAT solution, the removal percent was increased between 47.21–55.29% and 23.92–40.56%, respectively. Data further showed that at all incubation times tested, significant differences ($P \leq 0.05$) in the removal percent of PAT were obtained by viable cells, as compared with heat-treated cells of both kombucha and *S. cerevisiae*.

Results obtained in Table 4 showed the effect of pH at 3.0, 4.0, and 5.0 on PAT biosorption by viable and heat-treated cells of both kombucha and *S. cerevisiae*. The maximum PAT removal was achieved at pH 3.0 with statistically significant differences ($P \leq 0.05$). At this pH, the viable kombucha cells detoxified 64.67% and the heat-treated cells detoxified 60.69%, whereas the yeast cells were relatively less effective

Table 3. Effect of incubation time on the removal of PAT (40 mg l⁻¹) by kombucha and *S. cerevisiae* cells from aqueous solution at 35°C and pH 4.0.

Tabla 3. Efecto del tiempo de incubación en la extracción de PAT (40 mg l⁻¹) mediante células de Kombucha y *S. cerevisiae* de solución acuosa a 35°C y pH 4.0.

Incubation time (h)	Removal (%) by kombucha cells		Removal (%) by <i>S. cerevisiae</i> cells	
	Viable	Heat-treated*	Viable	Heat-treated†
5	33.33 ± 0.87 ^c	7.46 ± 1.49 ^c	24.31 ± 3.58 ^d	9.41 ± 3.11 ^d
12	44.77 ± 4.48 ^b	11.94 ± 1.50 ^c	38.82 ± 3.11 ^c	17.65 ± 2.30 ^c
24	48.25 ± 4.56 ^b	25.87 ± 2.28 ^b	47.21 ± 3.29 ^b	23.92 ± 2.96 ^b
48	57.22 ± 2.29 ^a	40.76 ± 2.23 ^a	55.29 ± 2.35 ^a	40.56 ± 2.55 ^a

Notes: Calculated mean is for triplicate measurements from two independent experiments ± SD.

*Significantly different from viable kombucha cells (LSD test, $P \leq 0.05$) at all different incubation times.

†Significantly different from viable *S. cerevisiae* cells (LSD test, $P \leq 0.05$) at all different incubation times.

^{a-d}Means with different superscripts in the same column are considered statistically different (LSD test, $P \leq 0.05$).

Notas: El promedio calculado es para las mediciones triplicadas de dos experimentos independientes ± SD.

*Significativamente diferente de las células viables de Kombucha (test LSD, $P \leq 0,05$) para todos los tiempos de incubación.

†Significativamente diferente de las células viables *S. cerevisiae* cells (LSD test, $P \leq 0,05$) para todos los tiempos de incubación.

^{a-d}Los promedios con diferentes superíndices en la misma columna se consideran estadísticamente diferentes (test LSD, $P \leq 0,05$).

Table 4. Effect of pH on the removal of PAT (40 mg l⁻¹) by kombucha and *S. cerevisiae* cells from aqueous solution at 35°C for 48 h.

Tabla 4. Efecto del pH en la extracción de PAT (40 mg l⁻¹) mediante células de Kombucha y *S. cerevisiae* en solución acuosa a 35°C durante 48 h.

pH	Removal (%) by kombucha cells		Removal (%) by <i>S. cerevisiae</i> cells	
	Viable	Heat-treated*	Viable	Heat-treated†
3.0	64.67 ± 2.28 ^a	60.69 ± 2.27 ^a	63.52 ± 1.17 ^a	52.54 ± 2.45 ^a
4.0	57.20 ± 2.30 ^b	41.29 ± 0.86 ^b	55.68 ± 4.45 ^b	40.78 ± 2.44 ^b
5.0	48.25 ± 3.75 ^c	36.31 ± 3.10 ^b	48.23 ± 4.23 ^c	31.37 ± 1.79 ^c

Notes: Calculated mean is for triplicate measurements from two independent experiments ± SD.

*Significantly different from viable kombucha cells (LSD test, $P \leq 0.05$) at pH 4.0 and 5.0.

†Significantly different from viable *S. cerevisiae* cells (LSD test, $P \leq 0.05$) at all pH values.

^{a-c}Means with different superscripts in the same column are considered statistically different (LSD test, $P \leq 0.05$).

Notas: El promedio calculado es para las mediciones triplicadas de dos experimentos independientes ± SD.

*Significativamente diferente de las células viables de Kombucha (test LSD, $P \leq 0,05$) a pH 4,0 y 5,0.

†Significativamente diferente de las células viables *S. cerevisiae* (test LSD, $P \leq 0,05$) para todos los valores de pH.

^{a-c}Los promedios con diferentes superíndices en la misma columna se consideran estadísticamente distintos (test LSD, $P \leq 0,05$).

in PAT uptake as the viable cells detoxified 63.52% and the heat-treated cells detoxified 52.54%. It was obvious that detoxification of PAT by both kombucha and yeast cells was decreased on increasing pH values. The PAT detoxifying potential of viable and heat-treated cells of both kombucha and yeast incubated at pH 5.0 was about 1.3 and 1.6 times, respectively; lower detoxifying potential than those incubated at pH 3.0. Data of PAT detoxification by kombucha and *S.*

cerevisiae cells also showed that significant differences ($P \leq 0.05$) in the detoxification capacity of viable cells were obtained at all pH values (except at pH 3.0 for kombucha) when compared with heat-treated cells.

Discussion

Tea fungus or kombucha is the common name given to a symbiotic growth of acetic acid bacteria and osmophilic yeast species in a zoogloeal mat which has to be cultured in sugared tea (Jayabalan et al., 2014). *A. xylinum* has been shown to be the primary bacterium in the colony and responsible for cellulose production (Mayser et al., 1995). Our observations on growth of 3% starter culture of kombucha in sugared black tea for 14 days showed that a flat macroscopic colony formed on the surface of the tea solution, taking on the shape of the receptacle and a newly formed daughter culture starts to float and forms a clear thin gel-like membrane across the available surface. The mother culture sinks later to the bottom of the tea broth and at this time, a few gas bubbles with a floating cellulose pellicle appear on the tea broth surface. Jayabalan et al. (2014) reviewed the same growth observations of kombucha in black tea sweetened with sucrose. This fungus-like mixture of microorganisms and cellulose is likely the reason behind why kombucha is also called “tea fungus” (Sreeramulu et al., 2000). The color of kombucha broth was lighter in comparison with the color of black tea and this suggested that polyphenols did undergo microbial change in acidic environment by enzymes liberated by bacteria and yeast (Jayabalan et al., 2008).

The physicochemical analyses of kombucha tea culture showed that the pH of filtrate decreased from 5.0 to 3.1 after 14 days of fermentation and the TA was 3.16 ± 0.01 ml NaOH 100 ml^{-1} filtrate. Additionally, a number of major organic acids such as acetic acid (1.83 g l^{-1}), citric acid (2.77 g l^{-1}), gluconic acid (1.86 g l^{-1}), and L-lactic acid (0.702 g l^{-1}) were detected. The decrease in pH is due to an increased concentration of these organic acids. This is in agreement with the findings of other studies (Jayabalan, Marimuthu, & Swaminathan, 2007; Sreeramulu et al., 2000). Kombucha mat wet weight (12.04 g) was comparable with that obtained by Sreeramulu et al. (2000) when they grew their kombucha tea starter culture on black tea sweetened with sucrose (10% w/v) and glucose (2.5% w/v). Cellulose and protein contents produced during fermentation in our experiment were 1.22 and 1.81 g l^{-1} . Cellulose is produced by *A. xylinum* and proteins likely represent extracellular proteins secreted by bacteria and yeasts during fermentation (Sreeramulu et al., 2000). Jayabalan et al. (2007) indicated that the protein content was in the range of 0.1–3.0 g l^{-1} , during 12 days of fermentation, afterward it continued to decrease because of yeast and bacterial extracellular protein decreases. The values of organic acids are varied in the literature. Malbaša, Lončar, and Djurić (2008) detected acetic acid and L-lactic acid in concentrations of 0.53 and 0.05 g l^{-1} , respectively, after 14 days of kombucha fermentation on 7% sucrose. Lončar, Malbaša, and Kolarov (2001) recorded a higher acetic acid value (1.3 g l^{-1}) in kombucha grown on 7% sucrose for 14 days, though L-lactic acid was not tested for the presence. Jayabalan et al. (2007) detected acetic acid and L-lactic acid in concentrations of 6.17 and 0.33 g l^{-1} , respectively, during fermentation on 10% sucrose for 15 days. These authors detected citric acid production (0.11 g l^{-1}) on third day of fermentation. Gluconic acid

concentration produced by our kombucha was satisfactory as that obtained previously (1.16 g l^{-1}) after kombucha cultivation on black tea sweetened with 1.51% glucose (Franco, Pefin, Mantovani, & Goicoechea, 2006).

In the present study, three local fungal strains identified as *P. expansum* LC015096, *T. purpureogenus* LC015095, and *A. implicatum* LC015097 were isolated from decaying apple samples. On using CDYE broth as cultivation medium, the three fungal strains produced PAT in amounts up to 7.41, 5.75, and 6.44 mg l^{-1} , respectively. In this regard, Steiman, Seigle-Murandi, Sage, and Krivobok (1989) found that PAT production is not a generic character, although *Aspergillus* and *Penicillium* are by far the most extensively represented. These authors reported the positive ability of 58 strains to produce PAT, among them are *Acremonium zeae* and *P. expansum*. Furthermore, Fang-chao, Chun-lin, Xiang-zheng, and Yi-lun (2011) found that *P. purpureogenum* F4 (*T. purpureogenus*) was PAT positive among 10 PAT-producing strains that were isolated from decayed pears. *P. expansum* is responsible for the blue mold rot being generally regarded as the main producer of PAT in apples (Sanderson & Spotts, 1995).

Treatment of the CDYE broth with kombucha supernatant caused a notable decrease in both growth and PAT production by toxigenic fungal strains and the effect was dose-related. Kombucha up to 10% was found to be effective in inhibiting PAT production by *T. purpureogenus* and *A. implicatum* and significantly reduced that by *P. expansum* by 77.2%. At this concentration, mycelial growth of *P. expansum* and *T. purpureogenus* was still evident, however that of *A. implicatum* was completely inhibited. Similarly, Sanzani, Schena, Nigro, De Girolamo, and Ippolito (2009) reported that quercetin and umbelliferone (two phenolic compounds) inhibited the *in vitro* production of PAT but did not affect *P. expansum* growth. However, Kumar and Prasad (1992) suggested that growth and aflatoxin production by *A. flavus* are proportionate processes. Reduction of both fungal growth and PAT production was possibly due to interference by organic acids, hydrolytic enzymes, ethanol, carbon dioxide, phenol, and antibiotics which are the main active components of kombucha broth (Jayabalan et al., 2014). Such interference may be at the biosynthetic levels. These products of fermented kombucha tea were able to inhibit proliferation of both spoilage and pathogenic microorganisms (Sreeramulu et al., 2000). Similarly, kefir filtrate (a fermented milk resulted from the metabolic activity of kefir grains which contain LAB, acetic acid bacteria, and yeast mixture coupled together in a slimy polysaccharide matrix) inhibited both growth and aflatoxin production of *Aspergillus flavus* at 10% (v/v) (Ismaiel, Ghaly, & El-Naggar, 2011).

In this study, addition of kombucha supernatant to sliced apple samples significantly reduced PAT accumulation by the three fungal strains. This may be explained on the basis of the acidity of organic acids in kombucha fermented beverages and other biological active compounds (proteins, antibiotics, enzymes, etc.) or metabolites other than acetic acid biosynthesized during the fermentation process. In this respect, Westby, Reilly, and Bainbridge (1997) showed that during food fermentations, the cyclopentanone moiety of aflatoxin B1 was reduced which results in aflatoxicol A and in the presence of organic acids, aflatoxicol A is irreversibly converted into the stereo-isomer aflatoxicol B which is about 18 times less toxic than aflatoxin B1. Under the conditions created in lactic acid fermentation ($\text{pH} \leq 4.0$), aflatoxin B1 is readily converted to

aflatoxin B2a which is also less toxic. Our results further revealed that the amount of PAT produced in untreated apple samples with kombucha (control treatments) revealed variation among the fungal strains tested. Sommer, Buchanan, and Fortlage (1974) have demonstrated that PAT amounts produced by different strains of *P. expansum* in Golden Delicious apples vary from 2 to 100 mg kg⁻¹. A clear variance was recorded among the isolates in their ability to attack fruits and to produce PAT; cultivars differ in their sensitivity to attack by the same strain and toxin production (Paster, Huppert, & Barkai-Golan, 1995).

In the present study, PAT detoxification from aqueous solution by viable and heat-treated kombucha increased with incubation time recording a significant removal percentage of 33.33%, 7.46% (at 5 h); and 57.22%, 40.76% (at 48 h), respectively. In this respect, Topcu et al. (2010) demonstrated PAT detoxification by viable and nonviable cells of *Enterococcus faecium* M74 and *E. faecium* EF031 strains, where PAT removal percentage by viable cells was 25.0%, 22.4% (at 5 h); and 41.6%, 45.3% (at 48 h), respectively. Meanwhile, removal percentage by nonviable cells was 23.5%, 19.7% (at 5 h) and 38.6%, 36.4% (at 48 h), respectively. Conversely, removal of aflatoxin B1 was a rapid process and its binding by *Lactobacillus amylovorus* CSCC 5160 increased from 52.6% (0 h) to 73.2% (72 h) (Peltonen, El-Nezami, Haskard, Ahokas, & Salminen, 2001). This was explained on the basis of binding of aflatoxin B1 and PAT by bacterial cells. Aflatoxin B1 was not bound strongly by viable cells of bacteria, and that some aflatoxin B1 could be released back into solution from bacteria–aflatoxin B1 complex after 24 h. However, PAT detoxification by viable and nonviable cells of bacteria increased with incubation time (Topcu et al., 2010).

The pH profile of PAT detoxification by viable and heat-treated kombucha mat showed that maximum removal of PAT was achieved at pH 3.0 and detoxification capacity was decreased on increasing pH. Accordingly, Topcu et al. (2010) and Hatab et al. (2012) found that the optimal removal of PAT from aqueous solution by bacterial cells was obtained at low pH values (pH 3.0 and 4.0). Kombucha tea mat has a high content of different functional groups on the cell walls and cellulose (i.e., hydroxyl, carboxyl, phosphate, and amino groups), which makes it very liable to the influence of pH (Razmovski & Šćiban, 2008).

In the binding assay of PAT, *S. cerevisiae* was used as a positive control to compare its uptake capacity with that of kombucha under the influence of pH and incubation time. Findings generally showed that comparable removal percents of PAT were obtained upon applying kombucha and *S. cerevisiae* as biosorbent samples (either viable or heat-treated) and in some cases the yeast cells were relatively less effective in PAT uptake. The detoxification of PAT from aqueous solution by kombucha tea biomass could be explained on the basis of the adsorbent ability of bacteria and yeast cells present in the cellulose biomass of kombucha. Bacterial cell wall peptidoglycans and polysaccharides have been suggested to be responsible components for the mycotoxin binding by bacteria (El-Nezami et al., 2004). Also, yeast cell wall carbohydrates and proteins were reported to be important components involved in PAT removal (Guo et al., 2012). Additionally, the hydrophobic interactions had a major role in adsorption of PAT (Guo et al., 2012).

In this study, viable kombucha biomass showed a greater ability to remove PAT than heat-treated biomass, which can

indicate that metabolic conversion of PAT by release of specific enzymes may occur. Reports regarding mycotoxins binding ability of viable and nonviable bacteria are contradictory. In agreement with our results, Fuchs et al. (2008) and Topcu et al. (2010) showed that viable bacteria had more detoxification capacity of PAT than nonviable cells and concluded that removal of mycotoxins from aqueous solution by viable or nonviable cells is strain dependent. Conversely, Oatley, Rarick, Ji, and Linz (2000) reported that heat-treated bacteria were often more efficient to remove aflatoxin B1 than viable cells. However, there was no significant difference between removal ability of viable and boiled cells of *L. lactis* ssp. *cremoris* ARH74 (Pierides, El-Nezami, Peltonen, Salminen, & Ahokas, 2000).

Conclusions

Overall, this study has been the first to demonstrate that kombucha tea has a significant ability to inhibit PAT production by three fungal species, *P. expansum*, *T. purpureogenus*, and *A. implicatum* in a liquid medium and in apple fruit. The inhibitory activity was found to be dose-related. Furthermore, kombucha tea biomass (viable and heat-treated) has been efficiently used as a biosorbent to remove PAT from aqueous solution. Its detoxification capacity was relatively greater than that of *S. cerevisiae* cells. Therefore, kombucha is not only being a prophylactic agent but also appears to be promising as safe alternative biopreservative offering a protection against intoxication from PAT.

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Disclosure statement

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Supplemental data

Supplemental data for this article can be accessed at [here].

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